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Serum response factor is modulated by the SUMO-1 conjugation system

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Abstract

Serum stimulation leads to activation of the serum response factor (SRF)-mediated transcription of immediate-early genes such as *c-fos* via various signal transduction pathways. We have previously reported that promyelocytic leukemia protein (PML) is involved in the transcriptional regulation by SRF. PML is one of the well-known substrates for modification by small ubiquitin-related modifier-1 (SUMO-1) and several SUMO-1-modified proteins associate with PML. Here, we report that SRF is modified by SUMO-1 chiefly at lysine[147] within the DNA-binding domain. Substitution of this target lysine for alanine did not affect the translocation of SRF to PML-nuclear bodies. The SRF mutant augmented the transcriptional activity under Rho A-stimulated condition but not under serum-starved condition, suggesting that activated SRF is suppressed by its sumoylation. These data support the transcriptional role of SUMO-1 conjugating system in cellular serum response.

Keywords: SRF; SUMO-1; PML; Serum response; Transcription

Serum stimuli start various signal transduction cascades to make fundamental biological responses such as cell proliferation, differentiation, growth arrest, and apoptosis. In these cellular contexts, immediate-early genes including *c-fos* and *Egr-1* are transactivated through the serum response element (SRE) in their promoter regions. Serum response factor (SRF) is a MADS (MCM1, Agamous, Deficiens and SRF) box transcription factor required for the activation of these immediate-early genes [1]. SRF and members of the TCF (ternary complex factor) family of Ets domain proteins form transcription factor complexes on the *c-fos* SRE for its transactivation [2]. The activity of the TCF is regulated through MAP kinase cascades [3], while SRF is activated by serum or mitogens, principally through pathways involving Rho-family GTPase Rho A [4] and phosphatidylinositol 3-kinase (PI3K) [5]. Growth stimulation induces phosphorylation of the N-terminus of SRF [3], but the importance of this modification in SRF is not fully understood. Thus, the regulatory mechanism of the SRF activity remains to be elucidated.

Increasing numbers of cellular proteins have been reported to be covalently conjugated with the small ubiquitin-related modifier-1(SUMO-1). The SUMO-1 conjugation system is analogous to ubiquitination system mostly linked to proteasomal degradation. SUMO-1 is activated in an ATP-dependent manner by SUMO-1 activating enzyme, a heterodimer of Sua1 and UBA2, and then transferred to the SUMO-1 conjugating enzyme, Ubc9, and subsequently attached to target lysines of substrates [6,7]. Although SUMO-1 ligases have been

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identified [8–11], their absolute requirement for the SUMO-1 conjugation reactions remains unclear. In addition, SUMO-1 modification targets the consensus sequence ΨKXE, where Ψ and K represent a hydrophobic residue and the SUMO-1 acceptor lysine in substrate proteins, respectively [12–14]. This modification is thought to regulate the function of substrate proteins through affecting subcellular localization and interaction with cooperative molecules [15,16], or through antagonizing against ubiquitination of the same target lysine [17].

Promyelocytic leukemia protein (PML) is localized to a discrete subnuclear domain called PML-nuclear bodies (NBs), also known as nuclear domain 10 or PML-oncogenic domains. PML is required for formation of PML-NBs and the SUMO-1 conjugation of PML is indispensable for targeting it to PML-NBs [15,18]. In addition, PML interacts with some transcription factors and their coregulators [19,20]. Sp100, p53, and Daxx, which localize in PML-NBs, are also sumoylated, but their translocation into the subnuclear domains does not require the modification [14,21,22]. Thus, sumoylation of transcription regulatory factors [22–30] further suggests the role of SUMO-1 conjugation system in transcriptional control. Recently, we have reported that SRF interacts with PML and CBP acetyltransferase in the PML-NBs upon serum stimulation, leading to the efficient serum response transcription of *c-fos* gene [31]. During the investigation, we found that SRF retains two putative consensus sequence(s) for sumoylation in the MADS DNA-binding domain and carboxyl-terminal transactivation domain. These lines of evidence led us to examine whether SRF can be modified with SUMO-1. In the present study, we show that SRF is conjugated by SUMO-1 at lysine 147 within the MADS domain. The modification was not required for the recruitment of SRF into PML-NBs, but it was likely to negatively control SRF-mediated transcription. These data suggest that SUMO-1 conjugation system is involved in modulating serum response transcription.

Materials and methods

Antibodies. Rabbit anti-SRF G-20 polyclonal and mouse anti-PML PG-M3 monoclonal antibodies (Santa Cruz Biotechnology), mouse anti-Flag M5 (Sigma), and anti-Myc 9E-10 monoclonal antibodies (Roche Diagnostics) were used in this study.

Cell culture. HeLa cells were grown in Dulbecco's modified Eagle's minimum essential medium (Invitrogen) supplemented with 5% (vol/vol) heat-inactivated fetal calf serum (FCS). For serum induction, cells were serum-starved in the medium containing 0.5% FCS for 24 h and then stimulated by 20% FCS for the indicated periods.

Construction of expression plasmids. Plasmids expressing Myc-tagged SRF were generated as previously described [31]. To construct plasmids expressing SRF(K147A), SRF(K506A), and SRF(K147/506A), site-directed mutagenesis was performed using the following oligonucleotides: 5′-GGCCGCGTGAAGATCGCGATGGAGTTCATCGAC-3′ and 5′-GTCGATGAACTCCATCGCGATCTTCACGCGGCC-3′ for K147A, 5′-ACCGCCCACAGCACCGCTAGCGAATGATCCGCCCGC-3′ and 5′-GCGGGCGGATCATTCGCTAGCGGTGCTGTGGGCGGT-3′ for K506A. To generate pcDNA3 Myc-SRF(K147/506A), both pcDNA3 Myc-SRF(K147A) and pcDNA3 Myc-SRF(K506A) were digested with *Bgl*II, and the fragment containing Myc and region encoding amino acids 1–244 of SRF(K147A) was ligated into predigested pcDNA3 Myc-SRF(K506A). The cDNAs for wild-type SRF and SRF(K147A) were inserted into the *Xho*I site of pEGFP-C1 (Clontech) (termed pEGFP-SRF and pEGFP-SRF(K147A), respectively). For constructing the plamids expressing GST-fused SRF and SRF(K147A) (130–264 amino acids), cDNAs for SRF and SRF(K147A) were amplified by specific primers: forward (5′-GGCGCGGGATTCGCGGTGAGCGGGGCCAAGCCG-3′), containing a *Bam*HI (underlined) and reverse (5′-GACTGTAAGCTTCACTTCAGTGTGTCCTTGGTCTC-3′), containing *Hin*dIII (underlined). The amplified cDNAs were cloned into predigested pGEX-2TH with *Bam*HI and *Hin*dIII. The cDNA for human SUMO-1 (amino acids 1–97) was also amplified from HeLa cDNAs by specific primers: forward (5′-GCCACCCTCGAGATGTCTGACCAGGAGGCAAAA-3′), containing an *Xho*I (underlined) and reverse (5′-GCCACCCTCGAGAAGCTTCTAACCCCCCGTTTGTTCCTGATA-3′), containing an *Xho*I and *Hin*dIII (underlined), to construct pcDNA3 Flag-SUMO-1. To generate plasmids expressing (His)6-fused Flag-SUMO-1, the fragment encoding Flag and SUMO-1 was obtained by digesting with *Bam*HI and *Hin*dIII, and cloned into pRSET (pRSET Flag-SUMO-1).

In vivo SUMO-1 conjugation assay. HeLa cells were lysed at 36 h after transfection in a TBS buffer (10 mM Tris–HCl, pH 8.0, and 150 mM NaCl) containing 2% SDS. The lysates were boiled for 10 min to denature all proteins and inhibit SUMO-1 deconjugation, and four volumes of 1% Triton X-100 were added to them. Subsequently, the lysates were sonicated for 2 min and centrifuged (13,000 rpm) at 4 °C for 20 min. The supernatants were incubated with appropriate antibodies at 4 °C for 90 min and then with protein A/G beads for 2 h. The beads were washed with 0.5 % Triton X-100 in the TBS buffer five times. Samples were loaded onto 8% SDS–PAGE followed by Western blot analysis.

In vitro SUMO-1 conjugation assay. Sf-9 insect cells expressing either Sua1 or hUba2 were lysed by sonication in a buffer containing 10 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 200 mM NaCl, 0.1% NP40, and 100 μg/ml PMSF. The lysates were centrifuged at 13,000 rpm for 15 min. The two supernatants were mixed with each other and used as a source of Sua1/hUba2. His-tagged Ubc9 and His-Flag-SUMO-1(G) were expressed in *Escherichia coli* BL21(SI) and purified by affinity chromatography using Probond Resin (Invitrogen) [23]. GST-fused SRF(WT) and SRF(K147A) were expressed in *E. coli* BL21(star) and purified using glutathione–Sepharose (Amersham–Pharmacia). GST-SRF was immobilized on glutathione–agarose beads and then incubated with Sua1/hUba2 and Ubc9 together with or without Flag-SUMO-1(G) at 25 °C for 120 min in a buffer containing 50 mM Tris–HCl, pH 7.4, 10 mM ATP, 10 mM MgCl2, and 2 mM DTT. The beads were washed with 0.2% Triton X-100 in PBS five times and boiled in SDS-sample buffer, followed by Western blot analysis.

Confocal laser scanning microscopic analysis. For immunofluorescence analysis, HeLa cells were subjected to the analysis 24 h after transfection with indicated plasmids using Fugene (Roche). These cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min. After washing cells with PBS, the cells were incubated with the primary antibodies at room temperature for 60 min. The samples were incubated with Cy3 (Amersham)-conjugated secondary antibodies for 60 min and visualized with a confocal laser scanning microscope (Olympus). GFP-fused proteins were detected using intrinsic fluorescence of GFP. To avoid bleed-through effects in double staining experiments, each dye was independently excited and images were electronically merged.

Luciferase assay. HeLa cells were transfected with 1 μg of reporter plasmids and 0.2 μg pRL-TK, which was used for monitoring the transfection efficiency, together with expression plasmids for the indicated protein by Lipofectamine (Invitrogen). At 7 h after transfection, the transfected cells were serum-starved for 24 h. The cells were harvested and lysed in a lysis buffer offered by the manufacturer (Promega). The insertless pcDNA and pEF-BOS were used as a mock vector. The luciferase activities were determined with dual-luciferase reporter assay system and a luminometer. Values are means and standard deviations of results from three independent experiments.

Band shift assay. Band shift assay was carried out as described previously [32]. Bacterially expressed GST-SRF (amino acids 130–246) for wild- and mutant (K147A)-types was incubated with DNA fragments containing five copies of SRE sequence.

Results

SRF is modified with SUMO-1 at lysine 147 in vivo and in vitro

We have reported that SRF interacts with PML in PML-NBs [31]. Several molecules localizing at PML-NBs are modified by SUMO-1 and their translocation to PML-NBs did not need the modification [14,21,22]. The amino acid sequences 147–149 and 506–508 of SRF coexisted with the minimal consensus sequence for sumoylation (Fig. 1A). Lysine 147 and lysine 506 seemed to be putative SUMO-1 acceptor sites in SRF. Lys^{147} and Lys^{506} resided within MADS and transactivation domain, respectively (Fig. 1B). To test whether SRF can be conjugated with SUMO-1, and to determine the target site(s) of SUMO-1 modification in SRF, we generated three plasmids expressing mutant-types of SRF in which lysines 147 and/or 506 were substituted for alanine, termed SRF(K147A), SRF(K506A), and SRF (K147/506A). Each of the wild- (WT) and mutant-types SRF in a Myc-fusion was expressed in HeLa cells, together with Flag-SUMO-1 (Fig. 2A). Western blot analysis showed that Flag-SUMO-1 was efficiently utilized to modify substrate proteins in the cells (data not shown).

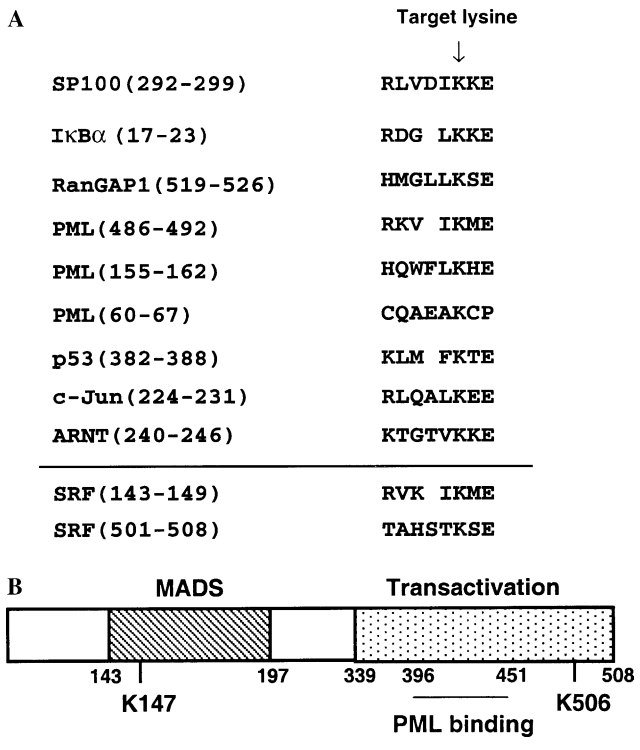

Fig. 1. Structure of serum response factor (SRF). (A) Consensus sequence for SUMO-1 conjugation. Alignment of SUMO-1 acceptor sites in known substrates is listed. An arrowhead indicates the lysine residue covalently modified by SUMO-1. (B) Structure of SRF. Lysine 147 and 506, which are putative target sites for SUMO-1 modification, reside in MADS box and transactivation domain, respectively.

Immunoprecipitation from the cell lysates was performed with anti-Flag, anti-Myc or control anti-HA antibodies and Western blot analysis was then carried out with anti-SRF antibodies. When the immunoprecipitates were probed with anti-Myc antibodies, some bands reacting with secondary antibodies against mouse IgG appeared near the position of the sumoylated SRF in all the lanes (data not shown). Therefore, we chose the use of the anti-SRF antibodies in the experiment. Wild- and mutant-types of SRF were equally expressed as shown by the presence of unconjugated SRF (~70-kDa) in Myc-precipitates (Fig. 2A; lanes 7–10). An approximate 95-kDa band of SUMO-1-conjugated SRF was significantly detected with anti-SRF antibodies in both Flag- and Myc-precipitates from the lysates of cells expressing wild-type SRF or SRF(K506A) (Fig. 2A; lanes 3, 5, 7, and 9), but not in the control precipitates (Fig. 2A; lanes 1 and 2). In contrast, SUMO-1-modification of both SRF(K147A) and SRF(K147/506A) was markedly reduced (Fig. 2A; lanes 4, 6, 8, and 10). The slightly faster migrating bands were faintly recognized in lanes 4 and 6, suggesting that endogenous protein probably dimerized with Myc-SRF was modified by Flag-SUMO-1. These data suggested that a fraction of SRF is modified by SUMO-1, chiefly at the lysine 147 which exists in the MADS domain with SRE-binding activity [33] (Fig. 1B).

To further clarify the SUMO-1 conjugation of SRF, we analyzed in vitro SUMO-1 transfer using recombinant proteins. Wild- and mutant (K147A)-types of SRF (amino acids 130–264) were prepared as GST fusion proteins. The reaction was performed in the presence of ATP using Sua1/UBA2, Ubc9, GST-SRF(WT) or GST-SRF(K147A), and Flag-SUMO-1. GST pull-down assays were then performed using glutathione–agarose beads (Fig. 2B). The use of anti-Flag antibodies showed that wild-type SRF formed an adduct with Flag-SUMO-1 in the presence of Sua1/hUba2 and Ubc9 (Fig. 2B; left panel). In addition, SUMO-1 conjugated form of wild-type SRF was similarly detected by anti-GST antibodies (right panel). SRF (K147A) mutant did not accept SUMO-1 molecule,

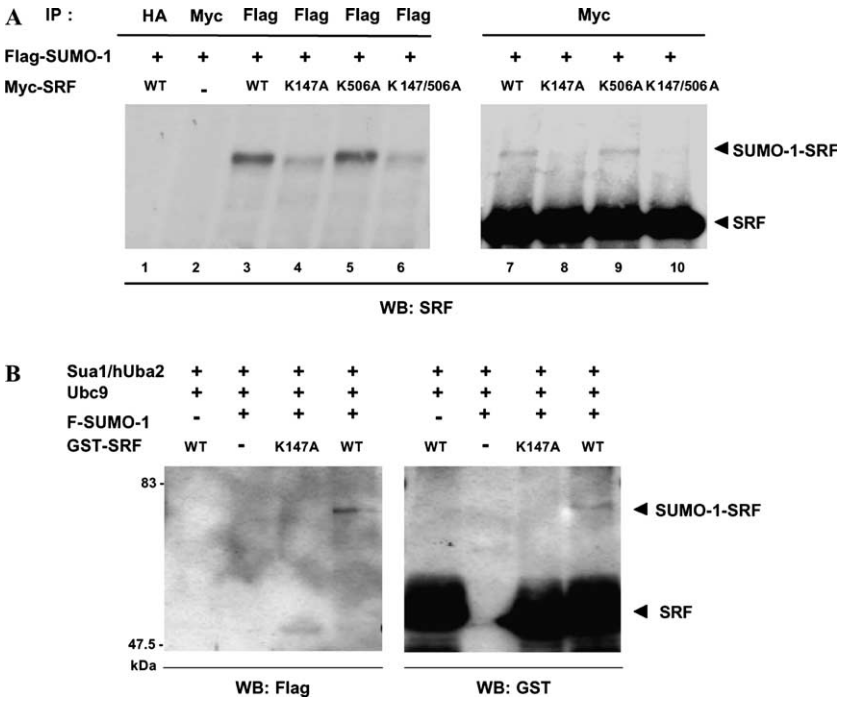

Fig. 2. SUMO-1 conjugation of SRF at lysine 147. (A) Conjugation of SUMO-1 to SRF in vivo. Lysines 147 and/or 506 of SRF were each substituted for alanine (K147A, K506A, and K147/506A). Lysates from transfected HeLa cells with indicated plasmids were immunoprecipitated with anti-Flag, anti-Myc or control anti-HA antibodies, and the immunoprecipitates were probed with anti-SRF antibodies. (B) Conjugation of SUMO-1 to SRF in vitro. GST-fused SRF(WT) and SRF(K147A) were immobilized on glutathione–agarose beads and subjected to SUMO-1 conjugation reaction containing recombinant Sua1/hUba2, Ubc9, and Flag-SUMO-1.

although unconjugated GST-SRF(WT) and SRF (K147A) were comparably present. Therefore, these data suggested the SUMO-1 modification of SRF at the lysine 147.

Effect of sumoylation on SRF-mediated transcription

SRF can localize to PML-NBs through binding PML [31]. To study the role of SUMO-1 modification of SRF, we tested whether sumoylation is required for translocation of SRF to the PML-NBs. Either green fluorescent protein (GFP)-fused SRF(WT) or GFP-SRF(K147A) was coexpressed together with PML in HeLa cells, and the cells were subjected to immunofluorescence analysis using a confocal laser scanning microscope (Fig. 3A). Both the wild- and mutant-types were similarly colocalized with PML in the bodies, indicating that SUMO-1 conjugation does not contribute to the translocation of SRF into PML-NBs.

The Rho family of small G-proteins affects SRF-dependent transcription, as is the case of serum stimulation [34]. Active forms of Rho A can potentiate SRF independently of TCF without the presence of extracellular stimuli [4,5]. The *SRE* consists of two distinct binding sites: the CArG box, which binds SRF, and the Ets box, which binds a TCF family of proteins. To examine whether sumoylation affects transcriptional activity of SRF, we used a pSRF-Luc reporter construct containing the CArG box, but not the Ets box, of the *c-fos* SRE promoter. This reporter enabled us to elucidate the transcriptional activity of SRF without any influence of TCF. Either Myc-tagged SRF(WT) or SRF(K147A) was expressed together with or without a constitutively active form of Rho A (Rho A.V14) (Fig. 3B). Western blot analysis using anti-Myc antibodies showed that both the wild- and mutant-types of SRF were comparably expressed. The luciferase activities under basal serum-starved condition were equally elevated in the cells expressing wild-type SRF and SRF(K147A). On the other hand, Rho A expression predominantly increased transcriptional activity of SRF(K147A), in comparison with that of wild-type. Similar results were obtained when the cells expressing wild-type SRF or SRF(K147A) were serum-stimulated (data not shown). Accordingly, SUMO-1 modification is likely to repress mitogen-stimulated SRF probably after transcriptional activation has been achieved.

We finally confirmed that SRF(K147A) does not have higher DNA-binding ability than wild-type, using a band shift assay (Fig. 3C). The five copies of *SRE*-containing DNA fragments were incubated with increasing amounts of bacterially expressed SRF proteins (amino acids 130–264) from wild-type and SRF (K147A). The appearance of high molecular SRF-bound SRE

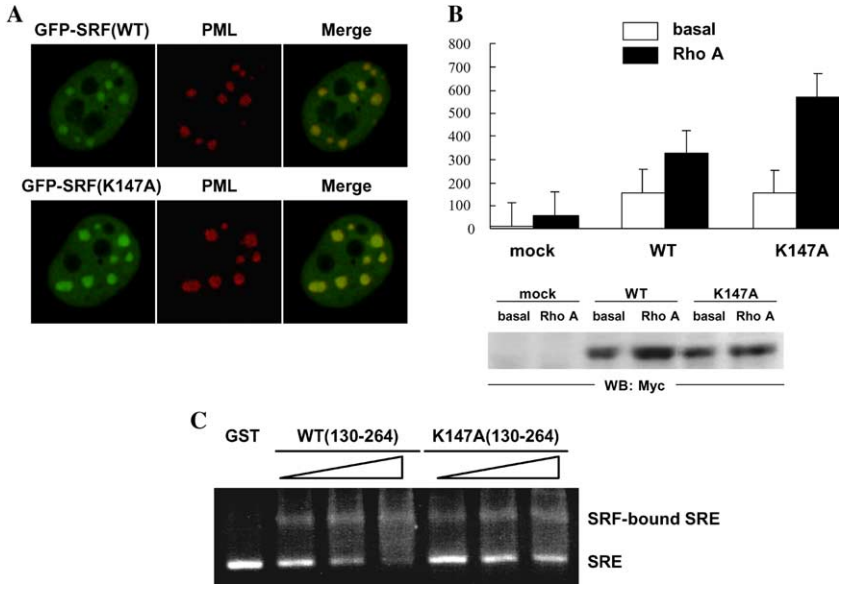

Fig. 3. Effect of SUMO-1 modification on SRF-mediated transcription. (A) SUMO-1 conjugation of SRF is not required for the translocation to PML-nuclear bodies. GFP-fused wild-type SRF or SRF(K147A) was coexpressed with PML in HeLa cells. (B) Effect of SUMO-1 modification on transcriptional activity of SRF. Plasmids (0.1 μg) expressing either SRF(WT) or SRF(K147A) were introduced in HeLa cells together with the 1 μg of pSRF-Luc, in combination with or without 0.4 μg of plasmids encoding Rho A.V14. The luciferase activity of the basal condition in combination with mock plasmid was normalized to 10. The expression level of the wild- and mutant-types of SRF was examined by Western blot analysis with anti-Myc antibodies. (C) Comparable DNA-binding ability of SRF(K147A) with that of wild-type SRF. SRF(K147A) or SRF(WT) was analyzed by electrophoretic mobility shift assay using DNA fragment containing *SRE*.

indicated that both SRF proteins complexed with the DNAs in a dose-dependent manner.In comparison with the case of wild-type, SRF(K147A) did not increase its DNA-binding ability. Taken together, these results suggested that SUMO-1 modification of activated SRF itself inhibits the transcriptional activity (see Discussion).

Discussion

In this study, we reported that SRF is post-translationally modified by SUMO-1 at the lysine 147 within the MADS domain. Protein modification by SUMO-1 is involved in inhibition of ubiquitination [17], control of subcellular localization [15,16], and functional regulation [22–30]. Sumoylation is thought to cause conformational changes of modified substrates, leading to alteration of interacting partner of the substrates [23]. Several transcription factors and coregulators have been recently shown to be functionally modulated by SUMO-1 conjugation [22–30]. Similarly, the sumoylation of SRF seems to suppress the transcriptional activity, because a mutant lacking SUMO-1 conjugation acquired higher transcriptional activity under Rho A- or serum-stimulated conditions. This SUMO-1 modification may affect the DNA-binding ability of SRF or create its interacting surface with a corepressor complex. Alternatively, the sumoylation may trigger the dissociation of SRF from coactivator complex. In response to mitogenic stimuli, SRF is rapidly activated and then repressed [31]. This combination of rapid activation and subsequent repression of immediate-early genes suggests a dynamic association of SRF with coactivator and corepressor complexes. We have recently reported that the interaction of SRF with PML in PML-NBs is crucial for both its transcriptional activation and suppression [31]. Silencing mediator of retinoic acid and thyroid hormone receptors (SMRT) is reported to associate with PML-mediated transcriptional repression [35]. Since SMRT is also shown to interact with SRF and inhibit its transactivation upon serum induction [36], SMRT might interact with SRF in PML-NBs. SUMO-1 modification did not affect the localization of SRF in PML-NBs, as were the cases of other sumoylated proteins including p53, Sp100, and Daxx [14,21,22]. SUMO-1 and SUMO-1-conjugating enzyme, Ubc9, coexist in the NBs, suggesting the possibility of SUMO-1 conjugation of substrates in the bodies [21]. Thus, it is possible that activated SRF could be sumoylated within PML-NBs. Sumoylation of SRF might alter the interacting partner from coactivator to corepressor in PML-NBs and contribute to temporal activation of SRF-mediated transcription.

SUMO-1 conjugation is found to occur in a small part of SRF and significantly affect the transcriptional activity of the protein under serum stimulation, as were the cases of other transcription factors [23–27]. These

suggested that SUMO-1 modification is transient and provided by a dynamic equilibrium between conjugation and deconjugation. In the same way, modification of c-Jun by SUMO-1 was reported to inhibit its transcriptional activity [27]. PML also modulates transactivation of AP-1 complex containing c-Fos and c-Jun [37], emphasizing the involvement of PML and SUMO-1 conjugating system in cellular serum response. Taken together, serum-induced transcription pathway via SRF and subsequent AP-1 complex exemplifies a series of functional links to PML-NBs and SUMO-1 conjugating system.

Acknowledgments

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