ARTICLE
Journal of Cellular Biochemistry 113:2721–2728 (2012)

Journal of Cellular Biochemistry

Protein Arginine Methylation of SERBP1 by Protein Arginine Methyltransferase 1 Affects Cytoplasmic/Nuclear Distribution

Yu-Jen Lee,[1] Wan-Yu Hsieh,[2] Ling-Yun Chen,[1*] and Chuan Li[2,3**]

[1]*Institute of Biochemistry and Biotechnology, Chung Shan Medical University, Taichung, Taiwan*

[2]*Department of Biomedical Sciences, Chung Shan Medical University, Taichung, Taiwan*

[3]*Department of Medical Research, Chung Shan Medical University Hospital, Taichung, Taiwan*

ABSTRACT

Protein arginine methylation regulates a broad array of cellular processes. SERBP1 implicated in tumor progression through its putative involvement in the plaminogen activator protease cascade, is an RNA-binding protein containing an RG-rich domain and an RGG box domain that might be methylated by protein arginine *N*-methyltransferases (PRMTs). Asymmetric dimethylarginine (aDMA) was detected in SERBP1 and an indirect methyltransferase inhibitor adenosine dialdehyde (AdOx) significantly reduced the methylation signals. Arginines in the middle RG and C-terminal RGG region of SERBP1 are methylated based on the analyses of different deletion constructs. The predominant type I protein arginine methyltransferase PRMT1 co-immunoprecipitated with SERBP1 and the level of bound PRMT1 decreased upon the addition of AdOx. Recombinant PRMT1 methylated SERBP1 and knockdown of PRMT1 significantly reduced the aDMA level of SERBP1, indicating that SERBP1 is specifically methylated by PRMT1. Immunofluorescent analyses of endogenous SERBP1 showed predominant cytoplasmic localization of SERBP1. Treatment of AdOx or PRMT1 siRNA increased the nuclear localization of SERBP1. Analyses of different deletions indicated that the middle RG region is important for the nuclear localization while both N- and C- terminus are required for nuclear export. Low methylation of the C-terminal RGG region also favors nuclear localization. In conclusion, the RG-rich and RGG box of SERBP1 is asymmetrically dimethylated by PRMT1 and the modification affects protein interaction and intracellular localization of the protein. These findings provide the basis for dissecting the roles of SERBP1. J. Cell. Biochem. 113: 2721–2728, 2012.

KEY WORDS: CYTOPLASMIC/NUCLEAR DISTRIBUTION; PRMT1; PROTEIN ARGININE METHYLATION; SERBP1

An RNA-binding protein (RBP) SERBP1 (SERPINE mRNA-binding protein 1) contains an arginine-rich segment in the N-terminus, an arginine- and glycine (RG)-rich segment in the middle and an RGG box in the C-terminus, but no typical RNA recognition motif (RRM) or K-homology (KH) region commonly found in most RBPs. SERBP1, first named as PAI-RBP1, was identified as a protein bound to the cyclic nucleotide-responsive sequence in the type I plasminogen activator inhibitor (PAI-1 or SERPINE 1) mRNA [Heaton et al., 2001]. Because plasminogen activators (PAs) are serine proteases important in thrombosis and

Abbreviations: SERBP1, SERPINE mRNA-binding protein 1; PAI, plasminogen activator inhibitor; PRMT, protein arginine methyltransferase; AdoMet, *S*-adenosylmethionine; aDMA, asymmetric N^G, N^G-dimethylarginine; AdOx, adenosine dialdehyde; RBP, RNA binding protein; GST, glutathion-*S*-transferase; hnRNP, heterogeneous nuclear ribonucleoproteins; RGG box, arginine- and glycine-rich motif; NLS, nuclear localization signal.The authors have no conflict of interest.

Additional supporting information may be found in the online version of this article.

Grant sponsor: National Science Council of Republic of China; Grant numbers: 93-3112-B-040-001, 96-2320-B-040-022-MY2, 98-2320-B-040-011-MY3; Grant sponsor: Chung Shan Medical University; Grant numbers: 93-OM-A-026, 93-OM-A-147, 94-OM-A-024, 99-OM-A-128.

*Correspondence to: Ling-Yun Chen, Institute of Biochemistry and Biotechnology, Chung Shan Medical University, No.110, Sec.1, Jian-guo N.Rd., Taichung 40201, Taiwan. E-mail: chenly@csmu.edu.tw

**Correspondence to: Chuan Li, Department of Biomedical Sciences, Chung Shan Medical University, No.110, Sec.1, Jian-guo N.Rd., Taichung 40201, Taiwan. E-mail: cli@csmu.edu.tw

Manuscript Received: 11 January 2012; Manuscript Accepted: 14 March 2012

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 22 March 2012

DOI 10.1002/jcb.24151 • © 2012 Wiley Periodicals, Inc.

fibrinolysis, tissue remodeling, as well as tumor invasion, the involvement of SERBP1 in cancer development was suggested [Koensgen et al., 2007]. However, the physiological cellular function of SERBP1 has not been elucidated.

A splicing isoform of SERBP1, CGI55, was identified as an evolutionarily conserved human gene by comparative genomics of human and *C. elegans* [Lai et al., 2000]. Both SERBP1 (all four possible splicing products including CGI-55 thereinafter) and its paralog Ki-1/57 interact with a chromatin-remodeling factor CHD-3 [Lemos et al., 2003]. Other SERBP1-interacting proteins are associated with promyelocytic leukemia nuclear bodies (PML-NBs) or are involved in sumoylation and transcriptional regulation [Lemos and Kobarg, 2006]. SERBP1 was also identified in the supraspliceosomes interacting with pre-mRNA, mRNA and spliceosomes [Chen et al., 2007], and in the interactomes of the 65 kDa subunit of U2 small nuclear ribonucleoprotein auxiliary factor U2AF65 (U2AF2) [Prigge et al., 2009]. SERBP1 was identified as an interacting partner of a Tudor domain containing protein TDRD3, and can localize to cytoplasmic stress granules (SGs) [Goulet et al., 2008] where the localization, stability and translation of mRNA are regulated in response to various cellular stresses.

Protein arginine methylation of Ki-1/57 by protein arginine methyltransferase 1 (PRMT1) was reported [Passos et al., 2006a], with similar modification of SERBP1 studied for comparison. Interaction of SERBP1 with PRMT1 was shown in another study isolating PRMT1-interacting proteins by affinity chromatography [van Dijk et al., 2010]. Protein arginine methylation is involved in various cellular functions such as signal transduction, protein subcellular localization, RNA processing and export, transcriptional regulation, and DNA repair [Bedford and Clarke, 2009]. The modification is catalyzed by a group of protein arginine methyltransferases (PRMTs) that can be divided into type I and type II, depending on the catalyses of forming asymmetric di-ω-N^G, N^G-methylarginines (aDMA) or symmetric di-ω-N^G, N'^G-methylarginine (sDMA) residues, respectively [Bedford and Clarke, 2009; Wolf, 2009].

Many RNA-binding proteins are modified in the RGG or glycine- and arginine-rich (GAR) motifs by protein arginine methylation. These motifs are frequently found in many RBPs and have been suggested for RNA as well as protein interactions [Pahlich et al., 2006; Bedford and Clarke, 2009]. The modification is important for the subcellular localization or functions of these proteins. For example, the RGG domain of hnRNPA2 can be arginine methylated, and RGG deletion as well as the inhibition of methyltransferases increase cytoplasmic distribution of hnRNPA2 [Nichols et al., 2000]. Methylation of hnRNPK by PRMT1 reduces its interaction with tyrosine kinase c-Src, inhibits its phosphorylation [Ostareck-Lederer et al., 2006], and influences the subcellular localization [Chang et al., 2011]. Methylation of hnRNPQ (NSAP1) by PRMT1 is important for its nuclear localization [Passos et al., 2006b].

Even though arginine methylation in SERBP1 has been reported [Passos et al., 2006a], there was no detailed analysis of the modification and it is not known whether the modification might be involved in modulating its intracellular localization. Cytoplasmic as well as nuclear subcellular localization of the protein have been reported with some inconsistencies [Lemos et al., 2003; Lemos and Kobarg, 2006; Passos et al., 2006a]. Based on the interaction network of SERBP1 revealed by now, SERBP1 might play different roles in different subcellular localization. Nuclear SERBP1 might be involved in transcriptional regulation, whereas cytoplasmic SERBP1 might be related to (PAI-1) mRNA stability. Thus, in the present report, we pinpointed the motifs modified by protein arginine methylation, investigated the subcellular localization of SERBP1, and correlated the protein methylation with its distribution.

MATERIALS AND METHODS

CONSTRUCTION OF PLASMIDS

The human SERBP1 cDNA clone was obtained from Source BioScience LifeSciences (Berlin, Germany). The full-length cDNA and deletions were amplified by polymerase chain reaction with primers listed in Supplementary Table 1. The PCR products were inserted into TA vector (Yeastern Biotech, Taiwan), and then cloned into pEYFP (Clontech, Takara Bio Inc., Shiga, Japan) or pFLAG-CMV2 (Sigma–Aldrich, St. Louis, MO) via *Eco*RI-*Bam*HI sites and into pGEX4T1 (GE Healthcare, Piscataway) via *Bam*HI-*Eco*RI sites.

PREPARATION OF RECOMBINANT PROTEINS AND IN VITRO METHYLATION ASSAYS

Plasmids derived from pGEX4T1 expressing recombinant glutathione *S*-transferase (GST)-SERBP1 (full-length or fragments) fusion proteins were transformed into *Escherichia coli* BL21 (DE3) cells. The protein expression was induced by 0.1 mM IPTG for 3 h at 37°C. The GST-fusion proteins were purified using Glutathione Sepharose affinity chromatography (GE Healthcare) according to the manufacturer's instructions.

Recombinant GST-fused PRMT1 and recombinant His-tagged fibrillarin protein were prepared as described [Ai et al., 1999]. Recombinant GST-fused SERBP1 protein (1 μg) was incubated with 1 μCi of [*methyl*-^{3}H] *S*-adenosylmethionine (60 Ci/mmol; Amersham, GE Healthcare) and GST-PRMT1 (2.7 μg) in the reaction buffer (50 mM sodium phosphate, pH 7.5) to the final volume of 15 μl. The in vitro methylation reaction and the subsequent analyses by fluorography were carried out as described [Chen et al., 2004].

CELL CULTURE AND TRANSFECTION

HeLa cells (ATCC CCL-2) were grown as described [Chen et al., 2004]. For transfection, cells grown to about 50% confluency were transfected with plasmids by Lipofectamine 2000 (Invitrogen, Camarillo, CA) according to the manufacturer's instructions. The cells were recovered after 24 hr of transfection and incubated with normal MEM medium containing 20 μM of adenosine dialdehyde (AdOx; Sigma–Aldrich). The cells were harvested after 48 hr of transfection (24 hr AdOx treatment) and cell extracts were prepared following the methods in [Chen et al., 2004]. Transient RNAi-mediated PRMT1 knock-down was performed using 250 pmol of *prmt1* siRNA that targets nucleotides 797-815 (5′-CGT TCT GCC TGC AAG TGA A-3′) and 1037-1055 (5′-CCA TCG ACC TGG ACT TCA A-3′) or control siRNA (5′-UUC UCC GAA CGU GUC ACU U-3′) synthesized by GenePharma Co., Ltd (Shanghai, China).

IMMUNOPRECIPITATION AND IMMUNOBLOTTING

HeLa cell extracts prepared from pFLAG-SERBP1-transfected cells were incubated with anti-FLAG-M2 affinity gel (Sigma–Aldrich) at 4°C overnight. Supernatant was removed after centrifugation and the beads were washed with TBS (50 mM Tris–HCl, pH = 7.5; 150 mM NaCl, 1 mM EDTA). The FLAG-tagged proteins were eluted by elution buffer containing 3× FLAG peptide (Sigma–Aldrich). Immunoblotting analyses were conducted basically as described in [Chen et al., 2004] with the following primary antibodies [1:2,000 for anti-SERBP1 from Abnova (Taiwan); 1:5,000 dilution of anti-FLAG from Sigma–Aldrich; 1:900 for ASYM24, 1: 2,500 for anti-PRMT1, 1:500 for anti-phosphoserine, 1:5,000 for anti-β-tubulin from Millipore (Billerica, MA)]. Chemiluminescent detection was performed using the VisGlow substrate for HRP (Visual Protein, Taiwan) according to the manufacturer's instructions.

IMMUNOFLUORESCENT ANALYSIS

HeLa cells grown on glass coverslips were incubated with 100 μM AdOx or not for 24 h and fixed with 2% paraformaldehyde (Sigma–Aldrich) in phosphate-buffered saline (PBS). The cells were washed with PBS, permeabilized with PBS containing 0.5% Triton X-100 and blocked with PBS containing 2% fetal bovine serum (FBS/PBS). The cells were incubated with FBS/PBS containing the primary antibody (1:200 for SERBP1) at 4°C overnight, washed with PBS containing 0.01% TritonX-100 (PBS-T), incubated with PBS-T containing FITC-conjugated anti-mouse antibodies (Jackson ImmunoResearch, West Grova, PA), and followed by DAPI (0.5 μg/ml, Sigma–Aldrich). The cells were washed with PBS-T and observed with a fluorescent microscope (ZEISS AXioskop2, Carl Zeiss, Oberkochen, Germany). Alternatively, for the detection of GFP-tagged SERBP1 in cells, transfected HeLa cells grown on coverslips were fixed, permeabilized, incubated with DAPI and visualized by fluorescent microscopy 48 h after transfection.

RESULTS

The amino acid sequence of SERBP1 (isoform 4) is shown in Figure 1A. This isoform containing no alternative exons is homologous to the only encoded SERBP1 we detected in zebrafish (Chang et al., unpublished data). An RG repeat in the middle and an RGG box at the C-terminus are both typical sequences modified by protein arginine methylation. Another putative methylarginine containing region is the arginine-rich sequence with multiple arginines in a short stretch close to the N-terminus. These regions were marked in Figure 1A and further analyzed in this study. A predicted nuclear localization signal in the middle RG region matching the (R[GA]x{0,2}[GA]R[GA]x[GA]R[GA]) signal (http://www.predictprotein.org/) was indicated. Serine or threonine phosphorylation sites in SERBP1 (summarized in http://www.uniprot.org/uniprot/Q8NC51) identified by phosphoproteomic studies

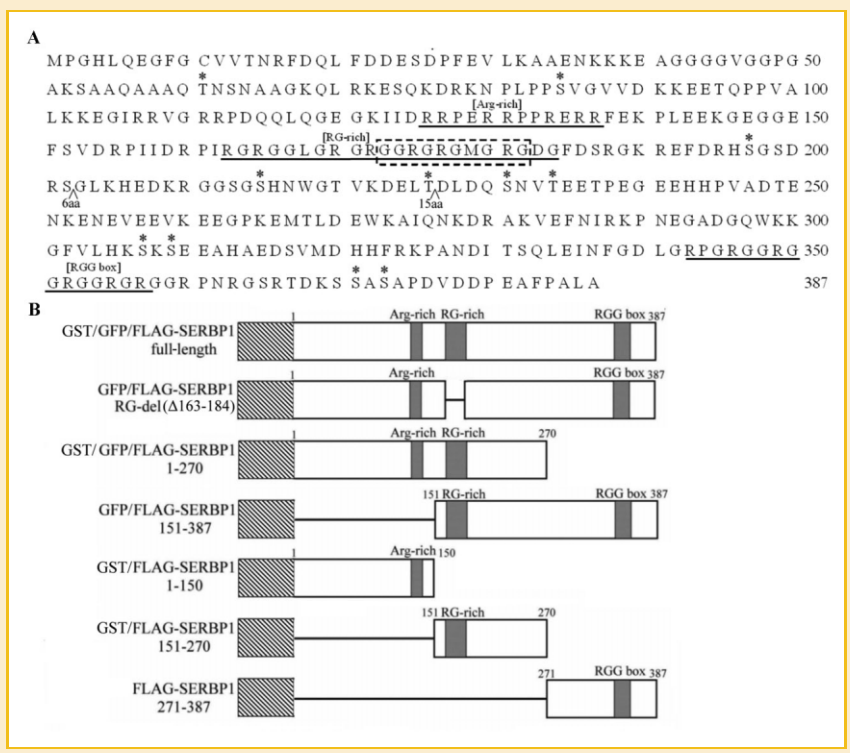

Fig. 1. The amino acid sequence of SERBP1 and schematic representation of recombinant SERBP1 proteins. A: The amino acid sequence of human SERBP1 isoform 4. The two insertion sites due to alternative splicing are indicated. Putative arginine methylation sites: Arg-rich (126–137), RG-rich (163–184), and RGG box (343–359) are underlined. Phosphorylated serine or threonine identified by phosphoproteomic studies are marked with asterisk. Putative nuclear localization signal (NLS, 173–182) is boxed. B: Schematic representation of full-length, and deletions or fragments of N-terminal, RG-rich and C-terminal domain of human SERBP1 fused in frame in plasmid pGEX4T1, pEYFP, or pFLAG-CMV-2. Fusion protein tags are indicated by striped boxes and the putative arginine methylation sites by gray boxes.

were also indicated (Fig. 1A). The constructs containing full-length or truncated SERBP1 proteins used in this study were illustrated in Figure 1B.

ASYMMETRIC DIMETHYLARGININE METHYLATION OF SERBP1

We examined arginine methylation of SERBP1 in vivo. FLAG-tagged SERBP1 purified from HeLa cell extracts were detected by Western blot analyses with ASYM24, an aDMA-specific antibody (Fig. 2A, lane 1). AdOx acts as an indirect methyltransferase inhibitor via blocking the activity of *S*-adenosylhomocysteine (SAH) hydrolase and accumulating SAH, the product inhibitor of methyltransferases. After AdOx treatment, arginine methylation level of the full-length FLAG-SERBP1 reduced significantly (Fig. 2A, lanes 1,2). The FLAG-tagged SERBP1 proteins in both samples were of similar level (Fig. 2A, lower panel), indicating that the reduced methylation signal of SERBP1 was not due to reduced expression of FLAG-SERBP1 in AdOx-treated cells. Co-transfection of PRMT1 siRNA reduced the arginine methylation signal of FLAG-SERBP1, suggesting that at least part of the SERBP1 methylation was catalyzed by PRMT1 (Supplementary Fig. S1).

To further map the arginine-methylation domain, FLAG-tagged SERBP1 proteins with deletions of the middle RG region (RG-del; deletion of residue 163-184), the N-terminus (containing residue 151-387), and the C-terminus (residues 1-270) were expressed in HeLa cells. Arginine methylation was detected in all three partially deleted SERBP1 and specific methylarginine signals were reduced upon AdOx treatment (Fig. 2A, lanes 3,4; Fig. 2B). All three constructs showed similar results, indicating that arginine methylation should be present in more than one domain.

We then prepared SERBP1 fragments containing only one putative arginine methylation motif. The C-terminal RGG box (271–387) was modified and the methylation level decreased upon AdOx treatment (Fig. 2C, lanes 5,6). However, no or very faint methylarginine signals were detected for either the N-terminal arginine-rich (1–150) or the middle RG-rich (151–270) fragment (Fig. 2C, lanes 1–4). Equivalent loading of FLAG-fusion proteins was confirmed by anti-FLAG detection. Since the C-terminal deletion

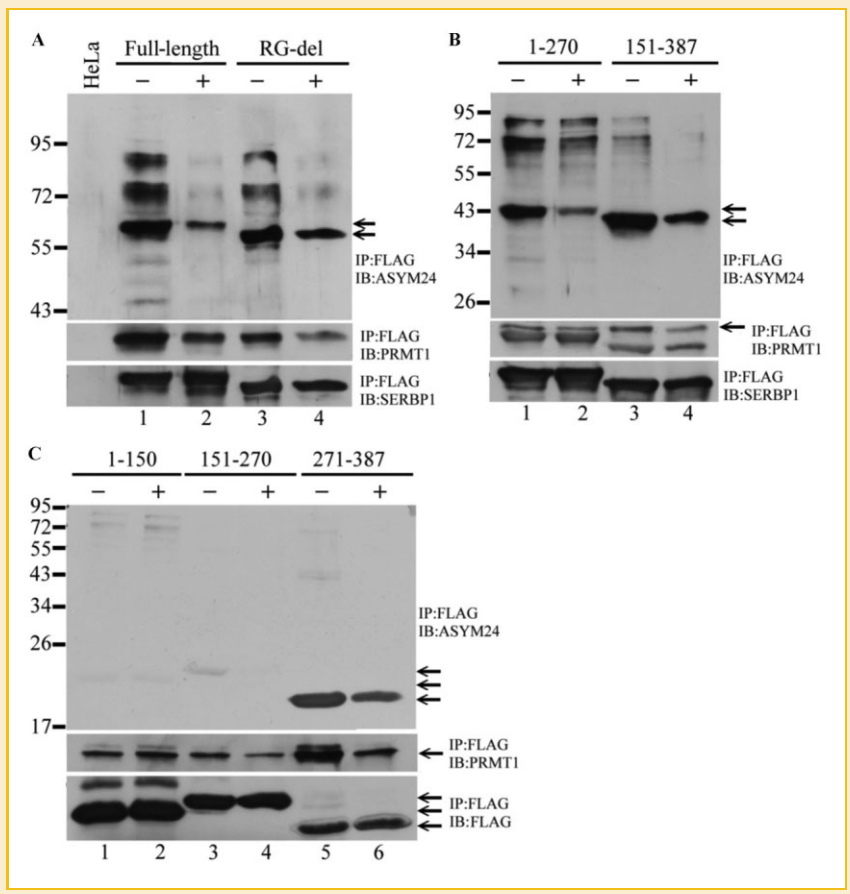

Fig. 2. Arginine methylation of truncated SERBP1 proteins in HeLa cells. Plasmids containing cDNAs encoding the FLAG-tagged SERBP1 proteins were transfected into HeLa cells. The FLAG-tagged proteins were analyzed by western blot with an aDMA-specific antibody ASYM24. The blot was stripped of the interacted antibodies and then re-probed with anti-PRMT1 (middle panel), and anti-SERBP1 or anti-FLAG antibodies (lower panel). A: Full-length FLAG-SERBP1 and RG-rich deletion (RG-del) separated by 8% acrylamide gel; (B) C- and N-terminal deletions (1–270 and 151–387) separated by 10% acrylamide gel. C: N-terminal (1–150), middle RG-rich (151–270) and C-terminal (271–387) fragments separated by 12.5% acrylamide gel. The arrows indicate the migration of FLAG-tagged SERBP1 protein in the upper and lower panels, or PRMT1 in the middle panel. "HeLa" indicates non-transfected HeLa cell extract. "+" or "−" indicates the addition of 20 μM AdOx to HeLa cell cultures or not.

containing residues 1–270 was methylated, it is likely that at least one single motif (1–150 or 151–270) contains the methylation sites. We then use in vitro methylation reaction to examine these fragments. GST-SERBP1 (full-length), GST-SERBP1 (1–270), and SERBP1 (151–270) could be methylated by PRMT1, but GST-SERBP1 (1–150) could not (Fig. 3). It is thus likely that the RG region but not the N-terminal arginine-rich region can be methylated by PRMT1. The modification of a single small RG fragment might be hindered in vivo by inefficient PRMT1 interaction.

INTERACTION OF PRMT1 AND SERBP1

The interaction between PRMT1 and SERBP1 has been reported through yeast-two hybrid analyses [Passos et al., 2006a] and affinity pull-down experiments [van Dijk et al., 2010]. We further evaluated the interaction of PRMT1 with full-length or truncated SERBP1 by co-immunoprecipitation in the present study. As shown in Figure 2 (middle panel), PRMT1 interacted with full-length and most of the SERBP1 fragments. Basically, PRMT1 interacted best with full-length SERBP1 (Fig. 2A, lanes 1,2), and fairly with the middle RG-deletion (Fig. 2A, lanes 3,4) or the C-terminal RGG region (271–387; Fig. 2C, lane 5,6). AdOx treatment not only reduced the aDMA level in these SERBP1 proteins, but also reduced the interactions with PRMT1 significantly. Low levels of interacted PRMT1 were detected for SERBP1 fragments containing the C-terminal deletion (1–270) or the N-terminal deletion (151–387; Fig. 2B), as well as only the N-terminal (1–150) or the middle RG domain (151–270; Fig. 2C, lanes 1–4). The weak interactions of PRMT1 with these constructs were not or only slightly affected by AdOx treatment.

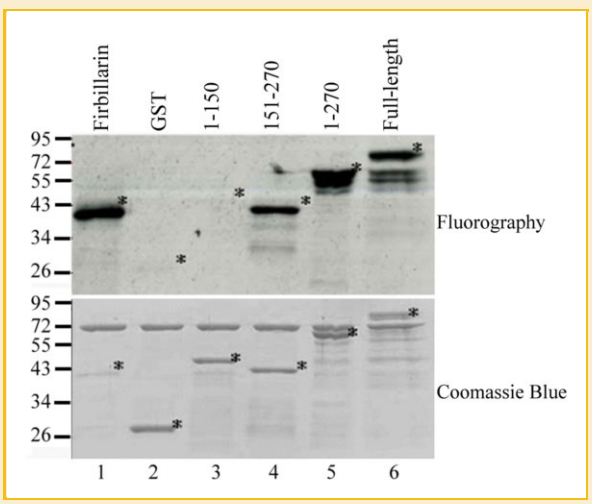

Fig. 3. In vitro methylation of recombinant SERBP1 proteins. Full-length or fragments (1–150, 151–270, 1–270) of the SERBP1 protein were expressed as GST fusions in *E. coli* and purified. The proteins were submitted to in vitro methylation using purified recombinant GST–PRMT1 as the enzyme source. Methylated proteins were separated by SDS–PAGE (12.5% acrylamide), stained with Coomassie Blue (lower) and detected by fluorography (upper). Asterisks (*) indicate the positions of putative substrates. Fibrillarin, known to be arginine methylated, was used as the positive control. GST was included as a negative control.

The co-immunoprecipitation experiment also revealed the interactions of SERBP1 with other aDMA-containing proteins. For example, polypeptides of molecular masses about 72 and 90 kDa appeared to interact with most SERBP1 constructs and the interacted proteins decreased significantly upon AdOx treatment (Fig. 2A,B). It might be resulted from reduced aDMA modification in these proteins upon AdOx treatment, or reduced interactions of these proteins with the hypomethylated FLAG-SERBP1, or both.

GENERAL SERINE PHOSPHORYLATION OF SERBP1 NOT AFFECTED UPON REDUCED ARGININE METHYLATION

Different posttranslational modifications of the same protein might crosstalk and affect each other. Specific phosphorylation at a threonine residue in the C-terminal RGG motif of Ki-1/57 but not CGI55 was reported [Passos et al., 2006a]. However, as multiple phosphorylation sites in SERBP1 had been determined (Fig. 1A), we analyzed whether serine phosphorylation of SERBP1 might be affected by arginine methylation. As shown in Supplementary Figure S2, the phosphorylation level of FLAG-SERBP1 at normal or hypo-methylation conditions was about the same. Equal loading (Supplementary Fig. S2, right panel) and reduced arginine methylation in the AdOx-treated samples were confirmed (data not shown). A phosphoprotein signal of the molecular mass about 100 kDa was reduced upon AdOx treatment, suggesting that the interaction of the phosphopolypeptide decreased with reduced SERBP1 methylation.

SUBCELLULAR LOCALIZATION OF SERBP1 AND PROTEIN ARGININE METHYLATION

The subcellular localization of SERBP1 has been reported previously, however, with contradictions as nuclear or cytoplasmic predominant [Lemos et al., 2003; Lemos and Kobarg, 2006; Passos et al., 2006a]. We thus re-examined the subcellular localization of the endogenous as well as transfected SERBP1 proteins in HeLa cells.

Immunofluorescent staining of endogenous SERBP1 showed that most of the SERBP1 proteins distributed in the cytoplasm. Nuclear granules and faint nuclear staining were observed in some cells (Fig. 4A). Intense nuclear staining of SERBP1 was observed with increased AdOx concentration (Fig. 4A). The nuclear staining was more intense than the cytoplasmic staining in some cells upon AdOx treatment. Localization of SERBP1 to cytoplasmic stress granules under specific cellular stresses was reported [Goulet et al., 2008]. Another general methylation inhibitor 5′-deoxy-5′ methylthioadenosine alone would not induce stress granules [Goulet et al., 2008], and we also did not observe SERBP1 in cytoplasmic granule-like foci after AdOx treatment.

Similar localization effects were observed for the treatment with PRMT1 siRNA (Fig. 4B). Knockdown of PRMT1 was confirmed by western blot analyses (Fig. 4C). As siRNA might not be evenly distributed to all of the treated cells, we counted the cells with different staining patterns (Fig. 4D). We showed the increase of cells that are cytoplasmic-dominant with nuclear staining, equal nuclear/cytoplasmic distributed, and nuclear-dominant. However, the cytoplasmic-dominant cells decreased.

We further expressed GFP-tagged full-length or partial deletions of SERBP1 in HeLa cells (Fig. 5). Transiently expressed GFP-SERBP1

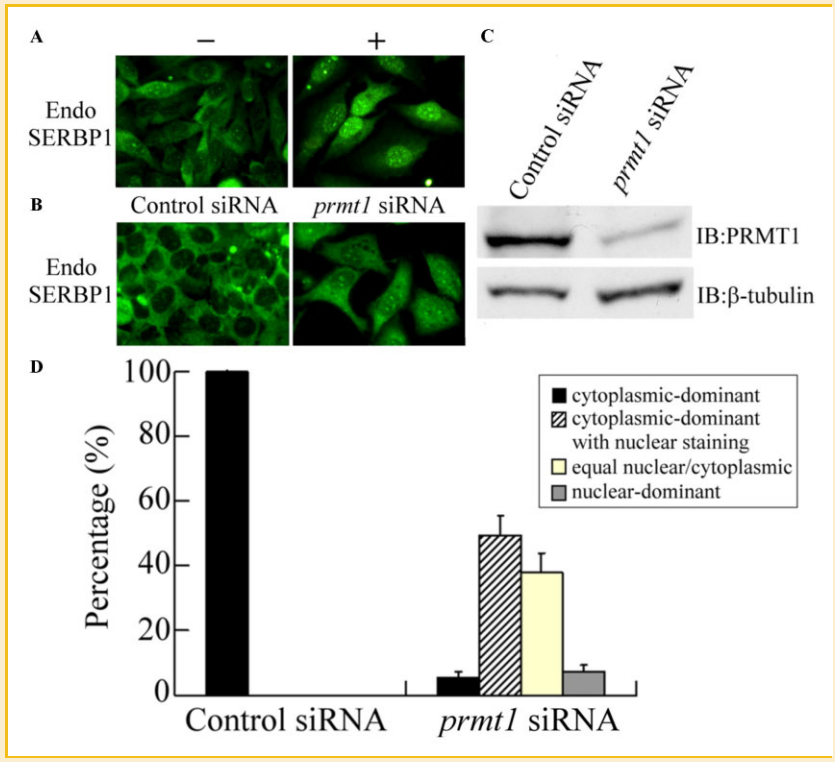

Fig. 4. Subcellular distribution of endogenous SERBP1 is affected by methylation and the presence of PRMT1. The cells were fixed and immunostained with anti-SERBP1 and visualized with FITC-conjugated secondary antibodies by fluorescent microscopy. (A) "+" or "−" indicate the treatment of 100 μM of AdOx for 24 h or not. B: HeLa cells were transfected with *prmt1* siRNA and control siRNA. C: Cell extracts from the siRNA-treated cells were immunoblotted with anti-PRMT1. Detection by anti-β-tubulin was used as a loading control. D: The quantification of siRNA-treated cells is represented as the percentage of the cells with different SERBP1 staining patterns (cytoplasmic-dominant, cytoplasmic-dominant with nuclear staining, equal nuclear/cytoplasmic, and nuclear-dominant). Data are the means ± SD of three independent experiments. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

(full-length) showed similar localization pattern as the endogenous SERBP1. The cytoplasmic localization of GFP-SERBP1(RG-deletion) was similar to that of the full-length SERBP1. The N-terminal-deleted (151–387) or C-terminal deleted (1–270) GFP-SERBP1 appeared to localize predominantly in the nucleus. Subsequent to AdOx treatment, the RG deletion, as the full-length SERBP1, increased its nuclear distribution. The N-terminal or C-terminal deletions did not change their localization upon AdOx treatment.

DISCUSSION

In this study, we showed evidences for asymmetric dimethylation of SERBP1 by PRMT1. Recombinant PRMT1 can catalyze SERBP1 methylation in vitro. A general methyltransferase inhibitor AdOx, as well as knockdown of PRMT1 by siRNA, reduced the aDMA level in FLAG- SERBP1 purified from HeLa cells. Furthermore, interactions of PRMT1 with various constructs of SERBP1 were illustrated by co-immunoprecipitation. AdOx treatment reduced the level of interacting PRMT1. The results suggested that when PRMT1 was inhibited, it interacted less with SERBP1 and resulted in the reduced aDMA level of SERBP1.

We constructed different deletions and fragments of SERBP1 fused with the FLAG-tag and analyzed the putative modification sites. The C-terminus with the RGG box appears to be the major site for asymmetric dimethylation. This segment can interact well with PRMT1 by itself compared with other single motif fragments that are barely modified. The interaction of its putative paralog Ki-1/57 with PRMT1 mapped by yeast two-hybrid analyses showed that fragments containing the N-terminus or the central region interacted stronger than the full-length protein. The C-terminal fragment interacted to about the same level as the full-length protein [Passos et al., 2006a]. The reasons that their results are different from that of our co-immunoprecipitation studies might be due to the differences of protein sequences or different techniques to evaluate the interactions.

Phosphorylation is the most studied posttranslational modification that can transduce signals and regulate protein activities. We showed that AdOx treatment that specifically reduced arginine methylation of SERBP1 did not affect overall serine phosphorylation level of SERBP1. No crosstalk of arginine methylation and phosphorylation in SERBP1 could be detected this way. As the phosphorylation sites are scattered in SERBP1 (http://www.uniprot.org/uniprot/Q8NC51) and are likely to be modified by different protein kinases, whether arginine methylation might influence specific phosphorylation or vice versa requires further investigation. However, a phosphorylated protein (about 100 kDa) interacted differentially with methylated or hypomethylated SERBP1 (Supplementary Fig. S2).

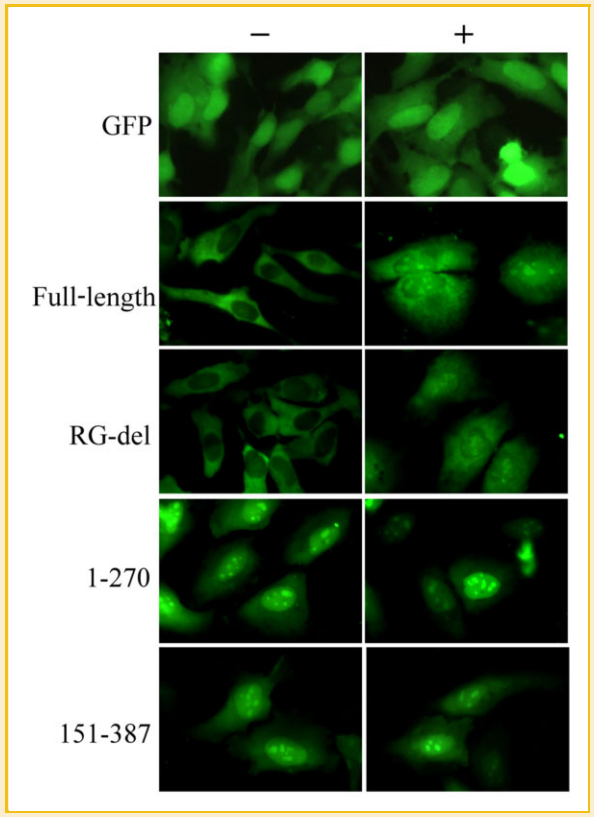

Fig. 5. Methylation status and deletions of specific domains can affect subcellular localizations of SERBP1. HeLa cells were transfected with plasmids expressing GFP or GFP-fused SERBP1 deletions. AdOx (100 μM) was included in the medium 24 h after transfection, and the cells were fixed after another 24 h. Distribution of GFP-tagged proteins was observed by fluorescent microscopy. "+" or "−" indicate the addition of AdOx or not. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Arginine methylation does not change the positive charge but increases the hydrophobic steric hindrance on the guanidinium nitrogens of arginine residues. The modification thus might lead to changes in protein–RNA or protein–protein interactions through the interference of RNA or protein bindings by reduced hydrogen bondings or increased hydrophobic interactions due to the methyl groups [Pahlich et al., 2006; Bedford and Clarke, 2009]. Moreover, the interactions of some arginine methylated polypeptides (at molecular mass about 90 and 72 kDa) with SERBP1 might also decrease with the reduced aDMA level of SERBP1 (Fig. 2). Besides the interaction of SERBP1 with chromatin-remodeling factor CHD-3 [Lemos et al., 2003] and proteins in PML nuclear bodies [Lemos and Kobarg, 2006] revealed by yeast-two hybrid analyses, SERBP1 was identified by proteomic studies to be present in the supraspliceosomes [Chen et al., 2007] and the interactomes of U2AF65 [Prigge et al., 2009]. It is thus possible that arginine methylation of SERBP1 can affect its interacting partners and associating complexes.

We determined the subcellular localization of SERBP1 and its putative relation with arginine methylation. We showed predominant cytoplasmic localization of endogenous SERBP1 with large nuclear granules and diffused nuclear staining in some cells. Similar results were obtained by immunofluorescent analyses of FLAG-tagged SERBP1 (data not shown), as well as by direct fluorescent microscopy of GFP-tagged SERBP1 in HeLa cells. Since the full-length and RG-deleted SERBP1 showed cytoplasmic localization while the N-terminal or C-terminal deleted SERBP1 appeared to localize mainly in the nucleus, it is likely that the middle RG region contains an NLS as predicted, and both the N- and C-termini are required for nuclear export.

High AdOx concentration as well as PRMT1 siRNA treatment increased nuclear distribution of SERBP1. RG-deleted SERBP1 was redistributed from cytoplasm to nucleus by AdOx treatment. On the contrary, nuclear-predominant distribution pattern of N-terminal or C-terminal deletion was not affected. Since arginine methylation of SERBP1 in the middle RG and C-terminal RGG regions has been demonstrated, we suspect that beside the putative NLS predicted in the RG region, hypomethylation of the C-terminal RGG region might facilitate nuclear distribution/retention of RG-deleted SERBP1. Alternatively, hypomethylation condition might activate factors involved in SERBP1 nuclear import/retention. The results showed that there are a few determinants for SERBP1 cytoplasmic/nuclear distribution. Differential association of SERBP1with proteins/complexes of specific subcellular localizations under different methylation conditions might also contribute to altered nuclear-cytoplamic shuttling of SERBP1.

PRMT1 mRNA levels increased and the balance of PRMT1 isoforms was altered in a number of breast cancer cells and tumors [Goulet et al., 2007]. Anomalous PRMT1 expression/distribution might lead to altered arginine methylation, protein interaction network, and subcellular distribution of SERBP1 in these cancer cells. Abnormal SERBP1 expression/distribution can have further pathological implications. SERBP1was reported to interact specifically with, and to be involved in the stability of PAI-1 (SERPINE 1) mRNA [Heaton et al., 2001]. Cytoplasmic SERBP1 is more likely to be related to PAI-1 mRNA stability whereas nuclear SERBP1might have other functions. Overexpression of SERBP1 in ovarian cancer was first identified by in silico data mining [Schmitt et al., 1999] and then determined experimentally. In epithelial ovarian cancer, SERBP1 as well as PAI-1 were overexpressed in tumors but not benign and normal tissues, and the expression level of SERBP1 correlates with advanced disease stage [Koensgen et al., 2007]. The involvement of SERBP1 in tumor invasion was demonstrated in the metastases of prostate cancer to different organs. SERBP1 was expressed at higher levels in prostate cancer lymph node and liver metastases versus bone metastases [Morrissey et al., 2008]. Since PAI-1 not only is a major regulator of plasminogen activator for cell migration and tumor invasion, but also plays signaling roles by interacting with a few matrix and cell surface proteins [Czekay et al., 2011], the alterations of SERBP1 arginine methylation can be involved in further tumor progression.

In conclusion, our study carefully dissected the arginine methylation sites of SERBP1 protein in the middle RG and C-terminal RGG regions. The aDMA level of SERBP1 appears to correlate with the degree of interaction between SERBP1 and PRMT1. The modification is important for cytoplasmic-dominant distribution of SERBP1. The information obtained in this study can

be the basis for further understanding in the physiological and pathophysiological roles of SERBP1.

ACKNOWLEDGMENTS

Fluorescence microscope (ZEISS AXioskop2) was performed in the Instrument Center of Chung Shan Medical University, which is supported by National Science Council, Ministry of Education and Chung Shan Medical University.

REFERENCES

Ai LS, Lin CH, Hsieh M, Li C. 1999. Arginine methylation of a glycine and arginine rich peptide derived from sequences of human FMRP and fibrillarin. Proc Natl Sci Counc Repub China B 23:175–180.

Bedford MT, Clarke SG. 2009. Protein arginine methylation in mammals: Who, what, and why. Mol Cell 33:1–13.

Chang YI, Hsu SC, Chau GY, Huang CY, Sung JS, Hua WK, Lin WJ. 2011. Identification of the methylation preference region in heterogeneous nuclear ribonucleoprotein K by protein arginine methyltransferase 1 and its implication in regulating nuclear/cytoplasmic distribution. Biochem Biophys Res Commun 404:865–869.

Chen DH, Wu KT, Hung CJ, Hsieh M, Li C. 2004. Effects of adenosine dialdehyde treatment on in vitro and in vivo stable protein methylation in HeLa cells. J Biochem 136:371–376.

Chen YI, Moore RE, Ge HY, Young MK, Lee TD, Stevens SW. 2007. Proteomic analysis of in vivo-assembled pre-mRNA splicing complexes expands the catalog of participating factors. Nucleic Acids Res 35:3928–3944.

Czekay RP, Wilkins-Port CE, Higgins SP, Freytag J, Overstreet JM, Klein RM, Higgins CE, Samarakoon R, Higgins PJ. 2011. PAI-1: An integrator of cell signaling and migration. Int J Cell Biol 2011:562481.

Goulet I, Gauvin G, Boisvenue S, Cote J. 2007. Alternative splicing yields protein arginine methyltransferase 1 isoforms with distinct activity, substrate specificity, and subcellular localization. J Biol Chem 282:33009–33021.

Goulet I, Boisvenue S, Mokas S, Mazroui R, Cote J. 2008. TDRD3, a novel Tudor domain-containing protein, localizes to cytoplasmic stress granules. Hum Mol Genet 17:3055–3074.

Heaton JH, Dlakic WM, Dlakic M, Gelehrter TD. 2001. Identification and cDNA cloning of a novel RNA-binding protein that interacts with the cyclic nucleotide-responsive sequence in the Type-1 plasminogen activator inhibitor mRNA. J Biol Chem 276:3341–3347.

Koensgen D, Mustea A, Klaman I, Sun P, Zafrakas M, Lichtenegger W, Denkert C, Dahl E, Sehouli J. 2007. Expression analysis and RNA localization of PAI-RBP1 (SERBP1) in epithelial ovarian cancer: Association with tumor progression. Gynecol Oncol 107:266–273.

Lai CH, Chou CY, Ch'ang LY, Liu CS, Lin W. 2000. Identification of novel human genes evolutionarily conserved in *Caenorhabditis elegans* by comparative proteomics. Genome Res 10:703–713.

Lemos TA, Kobarg J. 2006. CGI-55 interacts with nuclear proteins and co-localizes to p80-coilin positive-coiled bodies in the nucleus. Cell Biochem Biophys 44:463–474.

Lemos TA, Passos DO, Nery FC, Kobarg J. 2003. Characterization of a new family of proteins that interact with the C-terminal region of the chromatin-remodeling factor CHD-3. FEBS Lett 533:14–20.

Morrissey C, True LD, Roudier MP, Coleman IM, Hawley S, Nelson PS, Coleman R, Wang YC, Corey E, Lange PH, Higano CS, Vessella RL. 2008. Differential expression of angiogenesis associated genes in prostate cancer bone, liver and lymph node metastases. Clin Exp Metastasis 25:377–388.

Nichols RC, Wang XW, Tang J, Hamilton BJ, High FA, Herschman HR, Rigby WF. 2000. The RGG domain in hnRNP A2 affects subcellular localization. Exp Cell Res 256:522–532.

Ostareck-Lederer A, Ostareck DH, Rucknagel KP, Schierhorn A, Moritz B, Huttelmaier S, Flach N, Handoko L, Wahle E. 2006. Asymmetric arginine dimethylation of heterogeneous nuclear ribonucleoprotein K by protein-arginine methyltransferase 1 inhibits its interaction with c-Src. J Biol Chem 281:11115–11125.

Pahlich S, Zakaryan RP, Gehring H. 2006. Protein arginine methylation: Cellular functions and methods of analysis. Biochim Biophys Acta 1764: 1890–1903.

Passos DO, Bressan GC, Nery FC, Kobarg J. 2006a. Ki-1/57 interacts with PRMT1 and is a substrate for arginine methylation. FEBS J 273:3946–3961.

Passos DO, Quaresma AJ, Kobarg J. 2006b. The methylation of the C-terminal region of hnRNPQ (NSAP1) is important for its nuclear localization. Biochem Biophys Res Commun 346:517–525.

Prigge JR, Iverson SV, Siders AM, Schmidt EE. 2009. Interactome for auxiliary splicing factor U2AF(65) suggests diverse roles. Biochim Biophys Acta 1789:487–492.

Schmitt AO, Specht T, Beckmann G, Dahl E, Pilarsky CP, Hinzmann B, Rosenthal A. 1999. Exhaustive mining of EST libraries for genes differentially expressed in normal and tumour tissues. Nucleic Acids Res 27:4251–4260.

van Dijk TB, Gillemans N, Stein C, Fanis P, Demmers J, van de Corput M, Essers J, Grosveld F, Bauer UM, Philipsen S. 2010. Friend of Prmt1, a novel chromatin target of protein arginine methyltransferases. Mol Cell Biol 30:260–272.

Wolf SS. 2009. The protein arginine methyltransferase family: An update about function, new perspectives and the physiological role in humans. Cell Mol Life Sci 66:2109–2121.