

Spliceosome Sm proteins D1, D3, and B/B' are asymmetrically dimethylated at arginine residues in the nucleus[☆]

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Abstract

We report a novel modification of spliceosome proteins Sm D1, Sm D3, and Sm B/B'. L292 mouse fibroblasts were labeled in vivo with [³H]methionine. Sm D1, Sm D3, and Sm B/B' were purified from either nuclear extracts, cytosolic extracts or a cytosolic 6S complex by immunoprecipitation of the Sm protein-containing complexes and then separation by electrophoresis on a polyacrylamide gel containing urea. The isolated Sm D1, Sm D3 or Sm B/B' proteins were hydrolyzed to amino acids and the products were analyzed by high-resolution cation exchange chromatography. Sm D1, Sm D3, and Sm B/B' isolated from nuclear fractions were all found to contain ω -N^G-monomethylarginine and symmetric ω -N^G,N^{G'}-dimethylarginine, modifications that have been previously described. In addition, Sm D1, Sm D3, and Sm B/B' were also found to contain asymmetric ω -N^G,N^{G'}-dimethylarginine in these nuclear fractions. Analysis of Sm B/B' from cytosolic fractions and Sm B/B' and Sm D1 from cytosolic 6S complexes showed only the presence of ω -N^G-monomethylarginine and symmetric ω -N^G,N^{G'}-dimethylarginine. These results indicate that Sm D1, Sm D3, and Sm B/B' are asymmetrically dimethylated and that these modified proteins are located in the nucleus. In reactions in which Sm D1 or Sm D3 was methylated in vitro with a hemagglutinin-tagged PRMT5 purified from HeLa cells, we detected both symmetric ω -N^G,N^{G'}-dimethylarginine and asymmetric ω -N^G,N^{G'}-dimethylarginine when reactions were done in a Tris/HCl buffer, but only detected symmetric ω -N^G,N^{G'}-dimethylarginine when a sodium phosphate buffer was used. These results suggest that the activity responsible for the formation of asymmetric dimethylated arginine residues in Sm proteins is either PRMT5 or a protein associated with it in the immunoprecipitated complex.

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The spliceosome is a nuclear complex that catalyzes the splicing of pre-mRNA in eukaryotes. Each spliceosome snRNP is made up of snRNAs (U1, U2, U4/U6,

and U5) bound to a unique set of proteins as well as a shared set of seven Sm proteins (B/B', D1, D2, D3, E, F, and G) [1–4]. Structural data show that the seven Sm proteins form a ring/doughnut structure with a positively charged interior that binds directly to the highly conserved Sm site on the snRNA [5].

The seven Sm core proteins have a key role in snRNP biogenesis. In mammalian cells, the Sm proteins assemble with snRNAs containing an Sm sequence motif in the cytoplasm [6]. Newly synthesized snRNP proteins

[☆] Abbreviations: snRNP, small nuclear ribonucleoprotein; AdoMet, S-adenosyl-L-methionine; [³H]AdoMet, S-adenosyl-[methyl-³H]-L-methionine; HA, hemagglutinin; PRMT5, protein arginine methyltransferase 5.

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are stored in pools of partially assembled RNA-free complexes [7–10]. Hypermethylation of the m⁷G cap of the snRNAs requires a proper assembly of the Sm proteins on the U1, U2, U4, and U5 snRNAs [11,12]. The snRNP is then imported into the nucleus.

The Sm proteins D1, D3, and B/B' contain a C-terminal rich in arginine and glycine residues that is conserved in most eukaryotic organisms except yeast. Both Sm D1 and Sm D3 contain a RG dipeptide repeat at their C-termini with clusters of nine (for D1) or four (for D3) alternating arginine and glycine residues [13]. Sm B/B' contains several GRG triplets flanked by proline residues [13]. It has been shown by mass spectrometry and sequencing of the C-terminus of these Sm proteins that these RG dipeptide regions in Sm D1 and Sm D3 and the GRG triplets in Sm B/B' contain symmetric dimethylated arginine residues [13,14]. Other known RNA binding proteins (hnRNP1, fibrillarin, and nucleolin) have also been shown to contain methylated arginine residues [15,16]. However, these proteins all contain asymmetrically dimethylated arginine residues.

It has been shown that the protein arginine methyltransferase 5 (PRMT5/JBP1), a type II methyltransferase, in complex with pICln and two novel factors, can catalyze the methylation of Sm proteins [17,18]. There is also evidence that this symmetrical dimethylation of arginine residues by PRMT5 in these Sm proteins is important for regulating the snRNP assembly [19]. SMN, a protein involved in spinal muscular atrophy, makes up part of a complex containing the Sm proteins and is a critical factor in the assembly of Sm proteins onto snRNA. The binding of Sm proteins to SMN is enhanced upon symmetrical dimethylation of arginine residues of Sm D1, Sm D3, and Sm B/B' [19].

Here we show biochemically that Sm D1, D3, and B/B' purified from nuclear fractions contain asymmetric ω -N^G,N^G-dimethylarginine residues in addition to the already reported symmetric ω -N^G,N^{G'}-dimethylarginine residues. However, cytoplasmic SmB/B' and Sm D1 purified from a cytoplasmic 6S complex were found to contain only symmetric ω -N^G,N^{G'}-dimethylarginine residues. *In vitro* assays using a hemagglutinin-PRMT5 immunoprecipitated from HeLa cells detected the presence of a second activity. Asymmetric dimethylarginine residues in addition to the suspected symmetric dimethylarginine residues were detected in Sm D1 and D3 when the *in vitro* reactions were done in a Tris/HCl buffer. However, when these same reactions were repeated using a sodium phosphate buffer, this second activity was no longer present.

Experimental procedures

In vivo labeling of Sm D1, Sm D3, and Sm B. L929 mouse fibroblasts were grown in suspension culture as described previously [10].

Two hundred fifty milliliters of cells at 50×10^4 cells/ml was concentrated into 25 ml of 100% methionine-free medium and labeled for 3 h with 100 μ Ci/ml [³H]methionine (75 Ci/mmol). Labeled nuclear and cytoplasmic Sm proteins were isolated as described previously [10]. Soluble fractions were made by rinsing cells in phosphate buffered saline and then resuspending them in CSK buffer (100 mM NaCl, 3 mM MgCl₂, and 100 mM Pipes, pH 6.8) which contained 0.5% Triton X-100. Cells were vigorously vortexed at 4 °C. To separate the nuclei and attached remnants from the cytoplasmic supernatant the lysed cells were centrifuged at 2000g for 3 min. The supernatant was collected and then the nuclear pellet was extracted in 0.3 M NaCl, 2 mM MgCl₂, 20 mM Tris/HCl, pH 8.0. The suspension was rocked for 10 min at 25 °C. The suspension was then centrifuged at 10,000g for 5 min and the supernatant was collected. The cytoplasm or nuclear soluble fractions were immunoprecipitated with Y12 anti-Sm mAb crosslinked to protein A-Sepharose. Immunoprecipitation was repeated a second time. To isolate the 6S complex 1 ml of the cytoplasmic supernatant was layered on a 7.5–25% linear sucrose gradient prepared in CSK buffer and centrifuged for 20 h at 40 K at 4 °C in a Beckman SW 41 rotor as previously described [20]. Gradients were collected in 12 equal fractions. Sedimentation markers of bovine serum albumin (4S), catalase (11S), and β -galactosidase (19.5S) were run in a parallel gradient and detected in a continuously recording UV spectrophotometer. Fractions containing the 6S complex were immunoprecipitated with Y12 anti-Sm mAb crosslinked to protein A-Sepharose. All immunoprecipitants were then run on a 13% polyacrylamide gel containing 3 M urea. Bands containing the individual Sm proteins were excised from gel for amino acid analysis.

In vitro labeling of Sm D1, Sm D3, and Sm B. C-terminal His-tagged human Sm proteins were expressed in and purified from BL21 *Escherichia coli* as previously described [22]. Briefly, cells were pelleted and resuspended in 0.1 M Tris, pH 8.0, 0.3 M KCl containing 10 μ g/ml lysozyme, 20 μ g/ml benzamidine, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A, 10 μ g/ml DNase 1, and 0.5 mM PMSF. The mixture was then brought to 20% glycerol, sonicated for 30 s, and centrifuged at 15,000 rpm for 15 min at 4 °C. To every 50 ml of supernatant was added 1 ml of packed nickel-NTA agarose beads (Qiagen) and the mixture was rotated for 2 h. After several washes in GTK buffer (10% glycerol, 20 mM Tris, pH 8.0, 100 mM KCl, and 0.1 mM EDTA) containing 17 mM imidazole, the His-tagged protein was eluted with GTK buffer containing 0.4 M imidazole. Fractions were analyzed on Coomassie stained SDS-PAGE gels and peak fractions were pooled, dialyzed and further purified on an AKTA (Pharmacia) MonoS column and stored in GTK buffer. Proteins were judged to be at least 50% pure by Coomassie staining. HA-PRMT5 was cloned and purified as described before [23]. HA-JBP1 (PRMT5) was immunoprecipitated with anti-HA antibody from the HeLa-HA-SPMT cell line as described previously [23]. Sm D1, Sm D3, or Sm B was added to the HA-PRMT5 (immunoprecipitated from 1×10^7 HeLa cells/reaction) bound to protein A/G beads in 10 mM Tris, pH 7.5, or 50 mM sodium phosphate, pH 7.5, followed by incubation at 37 °C for 2 h with 5 μ l *S*-adenosyl-L-[methyl-³H]methionine ([³H]AdoMet; Amersham-Pharmacia Biotech; 72.0–79.0 Ci/mmol, 1 mCi/ml in dilute HCl:ethanol (9:1), pH 2–2.5) in a final volume of 35 μ l.

Chemical analysis of methylated Sm D1, Sm D3, and Sm B. The *in vitro* reactions were mixed with 20 μ g bovine serum albumin as a carrier protein and an equal volume of 25% (w/v) trichloroacetic acid in a 6 \times 50-mm glass vial and incubated at room temperature for 30 min. The precipitated protein was then centrifuged at 4000g for 30 min at 25 °C, and the supernatant was drawn off and discarded. After air-drying the pellets were subjected to acid hydrolysis in a Waters Pico-Tag vapor-phase apparatus in vacuo for 20 h at 110 °C using 200 μ l of 6 N HCl. For *in vivo* labeled Sm D1, Sm D3, and Sm B, excised gel slices were placed in a 6 \times 50-mm glass vial. One hundred microliters of 6 N HCl was added to each 6 \times 50-mm glass vial and the samples were hydrolyzed in vacuo for 20 h at 110 °C. The hydrolyzed samples were resuspended in 50 μ l water and mixed with 1.0 μ mol of

each of the standards ω - N^G -monomethylarginine (ω -MMA; Sigma product M7033; acetate salt) and asymmetric ω - N^G, N^G -dimethylarginine (ADMA; Sigma product D4268; hydrochloride) for amino acid analysis by column chromatography. 500 μ l of citrate dilution buffer (0.2 M Na^+ , pH 2.2) was added to the hydrolyzed samples before loading onto a cation exchange column (Beckman AA-15 sulfonated polystyrene beads; 0.9-cm inner diameter \times 10-cm column height) equilibrated and eluted with sodium citrate buffer (0.35 M Na^+ , pH 5.27) at 1 ml/min at 55 $^{\circ}\text{C}$.

Results and discussion

Evidence for a novel type of methylation of Sm proteins was previously reported [13]. Experiments in which recombinant D1 and a synthetic peptide was methylated in vitro by either HeLa cytosolic S100 extract or nuclear extract showed that only the cytosolic extract produced the symmetric ω - N^G, N^G -dimethylarginine residues. The nuclear extract catalyzed a novel methylation event that was not recognized by an antibody for symmetric ω - N^G, N^G -dimethylarginine residues [13]. In light of their results we decided to see if another type of methylation could be detected in Sm proteins in vivo. L929 mouse fibroblasts were labeled as described in the “Experimen-

tal procedures” section and the nuclei were isolated. The soluble nuclear fraction was then immunoprecipitated with an anti-Sm antibody and the immunoprecipitant was electrophoresed on a gel. Stained bands containing the B, D3, and D1/D2 Sm proteins were excised, acid-hydrolyzed, and fractionated on a cation-exchange column. Amino acid analysis of the B/B', D3, and D1/D2 Sm proteins detected radioactivity that eluted just ahead of the symmetric ω - N^G, N^G -dimethylarginine standard and the ω - N^G -monomethylarginine standards, which are the positions expected for the [^3H]methyl derivatives of these two modified arginine residues. This result is in agreement with previous studies [13,14]. However, we were also able to detect asymmetric ω - N^G, N^G -dimethylarginine residues in all three nuclear proteins (Fig. 1). These modifications have not been described previously.

We next wanted to ask if the asymmetrically dimethylated arginine residues were also found in Sm proteins in the cytoplasm or if this methylation event was specific to nuclear Sm proteins. L929 mouse fibroblasts were labeled with [^3H]methionine. Sm proteins were immunoprecipitated from the cytoplasm of lysed cells. The immunoprecipitant was run on a polyacrylamide gel with urea and the stained band containing the Sm B pro-

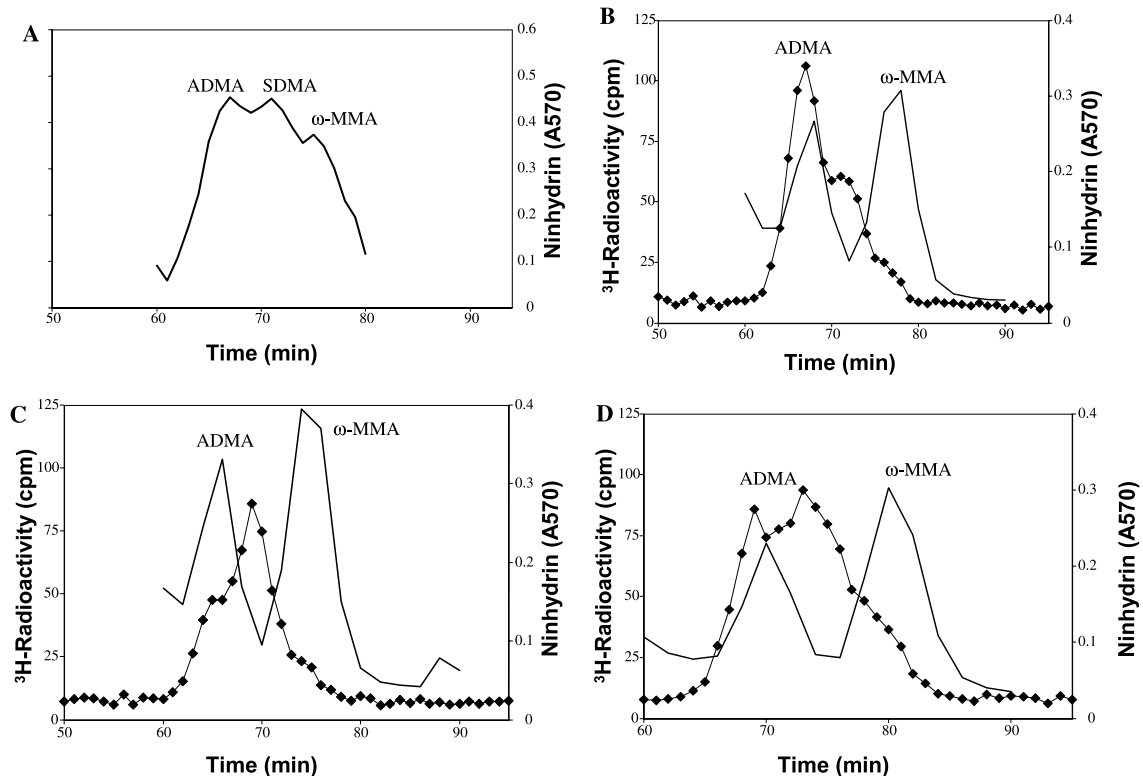


Fig. 1. Sm D1, Sm D3, and Sm B from mouse fibroblasts are asymmetrically dimethylated at arginine residues in the nucleus. (A) Chromatograph of ω - N^G -monomethylarginine (ω -MMA), ω - N^G, N^G -dimethylarginine (ADMA), and ω - N^G, N^G -dimethylarginine (SDMA, Sigma D0390). (B–D) L929 cells were labeled in vivo with [^3H]methionine and nuclear Sm D1/D2 (B), Sm D3 (C), and Sm B (D) proteins were isolated, acid-hydrolyzed, and analyzed by high resolution cation exchange chromatography after mixing with the standards ω -MMA and ADMA as described in “Experimental procedures.” ^3H -radioactivity (\blacklozenge) was determined by counting a 200- μ l aliquot of every fraction diluted with 400 μ l water in 5 ml fluor (Safety Solve; Research Products International) three times for 3 min. The unlabeled amino acid standards were analyzed using a ninhydrin assay (—) with 100- μ l aliquots of every other fraction [23].

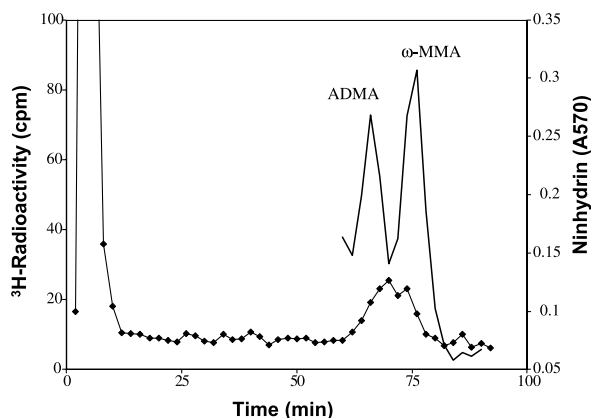


Fig. 2. Cytosolic Sm B/B' contains symmetric ω - $N^G, N^{G'}$ -dimethylarginine and ω - N^G -monomethylarginine in vivo. L292 cells were labeled in vivo and cytoplasmic Sm B was isolated and acid-hydrolyzed as described in "Experimental procedures." Samples were then run on the cation exchange column as described in Fig. 1 legend.

tein was excised, acid-hydrolyzed, and analyzed as previously described. As shown in Fig. 2, cytosolic Sm B contained only symmetric ω - $N^G, N^{G'}$ -dimethylarginine and ω -monomethylarginine.

The assembly of the SnRNP core particle occurs in the cytoplasm. As Sm proteins become newly synthesized they are stored in pools of partially assembled RNA-free complexes. These partially assembled complexes include a 6S particle of [D1, D2, (E, F, G)₂], a 20S particle of B, D3, and a 70 kDa protein, and 2S–6S particles made up of only the Sm B protein [11,22]. In looking at the methylation states of Sm B and Sm D1 from 6S particles (see "Experimental procedures"), Sm D1 and Sm B/B' isolated from 6S complexes were found to contain symmetric ω - $N^G, N^{G'}$ -dimethylarginine residues (Fig. 3). These results, along with the observations described above, suggest that there is a novel methylation of Sm proteins that occurs in the nucleus and not the cytoplasm.

It has been previously reported that PRMT5 can form symmetrically dimethylated arginine residues in vitro using MBP, GST-GAR [23], and the Sm proteins D1, D3, and B/B' as a substrate [17,18]. Further analysis of PRMT5's activity suggested a new activity present in immunoprecipitants of HA-tagged PRMT5 purified from HeLa extracts. The Sm proteins D1, D3, and B were incubated with HA-PRMT5 in the presence of [³H]AdoMet and 10 mM Tris/HCl, pH 7.5. Proteins were acid hydrolyzed and analyzed for the presence of methylarginine derivatives by cation exchange chromatography (Fig. 4). Control reactions were also analyzed with Sm protein and [³H]AdoMet or HA-PRMT5 and [³H]AdoMet. In all control reactions we did not detect any of the methylarginine derivatives. However, in the reactions containing either Sm D1 or Sm D3 and HA-PRMT5 we detected asymmetric ω - $N^G, N^{G'}$ -dimethylarg-

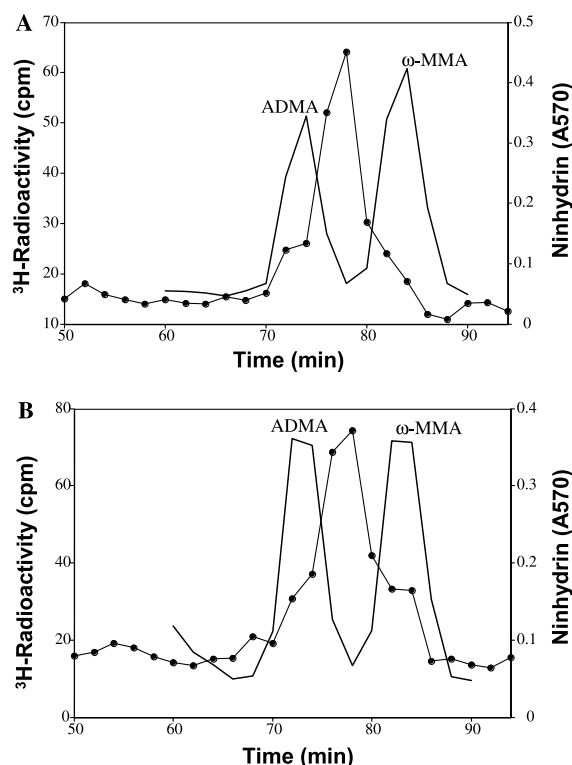


Fig. 3. Sm B/B' and Sm D1 from cytosolic 6S complexes contain symmetric ω - $N^G, N^{G'}$ -dimethylarginine and ω - N^G -monomethylarginine in vivo. L292 cells were labeled in vivo and 6S complexes were isolated as described in "Experimental procedures." Purified Sm B/B' and Sm D1 were then acid-hydrolyzed and analyzed as described in "Experimental procedures" and Fig. 1 legend. ³H-radioactivity is indicated by (●) and the ninhydrin assay is indicated by (—).

inine, symmetric ω - $N^G, N^{G'}$ -dimethylarginine, and ω - N^G -monomethylarginine. With Sm B only symmetric ω - $N^G, N^{G'}$ -dimethylarginine, and ω - N^G -monomethylarginine were formed. Interestingly, when these same reactions are repeated but in 50 mM sodium phosphate (pH 7.5) instead of 10 mM Tris/HCl, we only see the formation of monomethylarginine and symmetric dimethylarginine when Sm D3 was used as substrates (Fig. 5). In previous studies, Flag-PRMT5 purified from 293 cells was shown to symmetrically dimethylate residues in a GST fusion of the last 32 amino acids in the C-terminus of Sm D3 [17]. These results suggest that either a contaminating arginine methyltransferase is immunoprecipitated with PRMT5, which is responsible for the asymmetric dimethylation of arginine residues in Sm D1 and Sm D3, or PRMT5 is capable of catalyzing both reactions.

Here we have presented evidence that Sm D1/D2, Sm D3, and Sm B/B' are asymmetrically dimethylated in the nucleus but not in the cytoplasm. The function of this methylation event has yet to be determined. Other RNA binding proteins, such as hnRNP1, fibrillarin, nucleolin, and Sam68 have also been shown to be asymmetrically dimethylated at arginine residues [15,16].

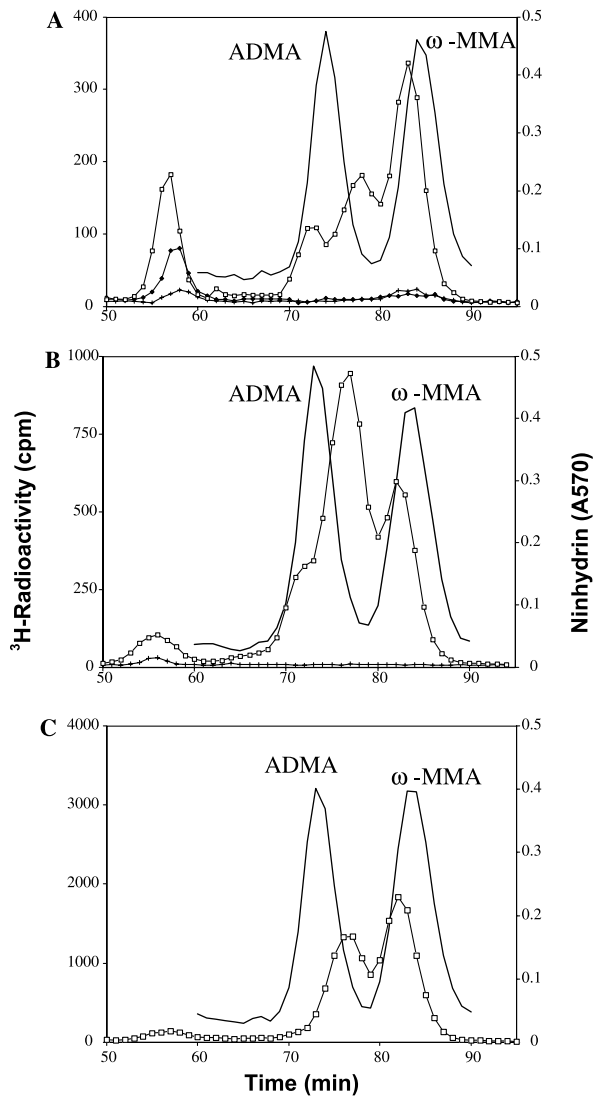


Fig. 4. Amino acid analysis of Sm D1, Sm D3, and Sm B modified by the PRMT5 methyltransferase. Sm D1 (A), Sm D3 (B), or Sm B (C) was methylated by HA-PRMT5 in vitro with [^3H]AdoMet in the presence of 10 mM Tris/HCl, pH 7.5 (□) (see “Experimental procedures”). Controls were performed in the absence of HA-PRMT5 (A,B, +), and the absence of the Sm protein (A, ◆). ^3H -radioactivity was determined by counting a 200 μl aliquot of every other fraction diluted with 400 μl water in 5 ml fluor (Safety Solve; Research Products International) three times for 3 min. Unlabeled amino acid standards (ADMA, $\omega\text{-N}^{\text{G}},\text{N}^{\text{G}}$ -dimethylarginine and $\omega\text{-MMA}$, $\omega\text{-N}^{\text{G}}$ -monomethylarginine) were analyzed using a ninhydrin assay with 100 μl aliquots of every other fraction (solid lines).

The hnRNPs Npl3p, Hrp13p, and Nab2p are all involved in the shuttling of mRNAs across the nuclear membrane [23,24]. Arginine methylation of these proteins has been shown to be necessary for the export of these proteins out of the nucleus [15,25,26]. The methylation of Sam68 localizes this RNA-binding protein to the nucleus [16]. Deletion of the methylation sites or the use of methylase inhibitors causes Sam68 to localize to the cytoplasm [16].

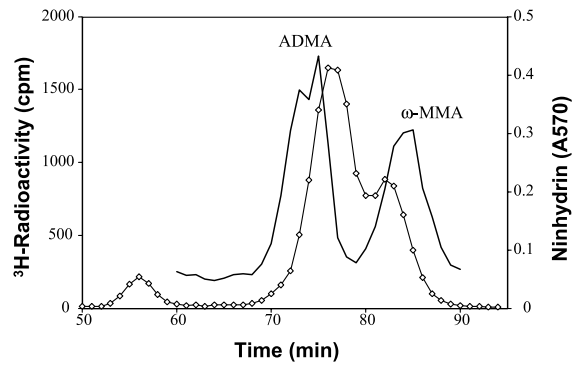


Fig. 5. Sodium phosphate affects the methylation of Sm D3 by the HA-PRMT5 immunoprecipitant. Sm was methylated by HA-PRMT5 in vitro with [^3H]AdoMet in the presence of 50 mM sodium phosphate, pH 7.5 (see “Experimental procedures”). Labeled proteins were precipitated with trichloroacetic acid and acid-hydrolyzed as described under “Experimental procedures,” and analyzed by cation exchange chromatography as described in Fig. 1 legend.

The C-termini of the yeast *Saccharomyces cerevisiae* Sm proteins are different than their mammalian homologs. *S. cerevisiae* Sm D1, Sm D3, and Sm B proteins do not contain the multiple (G)RG repeats at the C-terminus. It has been previously suggested that the C-terminal tails of Sm D1, Sm D3, and Sm B in mammalian cells have additional functions, contributing to a biological process that does not occur in yeast [27]. There are no data in yeast that support a cytoplasmic phase for yeast snRNPs [27]. Therefore, it has been proposed that the (G)RG repeats in mammalian Sm D1, Sm D3, and Sm B play a role in the nuclear imports of snRNPs [27]. It is possible that methylation of these (G)RG repeats acts as a signal to import these proteins into the nucleus. Further studies will need to be done to determine if the asymmetric dimethylation of arginine residues in the Sm proteins is a signal to keep the spliceosome in the nucleus, a signal for the spliceosome to be transported into the nucleus or has some other function.

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