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Toward an Assembly Line for U7 snRNPs

*INTERACTIONS OF U7-SPECIFIC Lsm PROTEINS WITH PRMT5 AND SMN COMPLEXES**

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The survival of motor neurons (SMN) complex mediates the assembly of small nuclear ribonucleoproteins (snRNPs) involved in splicing and histone RNA processing. A crucial step in this process is the binding of Sm proteins onto the SMN protein. For Sm B/B′, D1, and D3, efficient binding to SMN depends on symmetrical dimethyl arginine (sDMA) modifications of their RG-rich tails. This methylation is achieved by another entity, the PRMT5 complex. Its pICln subunit binds Sm proteins whereas the PRMT5 subunit catalyzes the methylation reaction. Here, we provide evidence that Lsm10 and Lsm11, which replace the Sm proteins D1 and D2 in the histone RNA processing U7 snRNPs, associate with pICln *in vitro* and *in vivo* without receiving sDMA modifications. This implies that the PRMT5 complex is involved in an early stage of U7 snRNP assembly and hence may have a second snRNP assembly function unrelated to sDMA modification. We also show that the binding of Lsm10 and Lsm11 to SMN is independent of any methylation activity. Furthermore, we present evidence for two separate binding sites in SMN for Sm/Lsm proteins. One recognizes Sm domains and the second one, the sDMA-modified RG-tails, which are present only in a subset of these proteins.

The U7 small nuclear ribonucleoprotein (snRNP)[5] is an essential factor mediating the endonucleolytic 3′-end processing of the replication-dependent, non-polyadenylated animal histone mRNAs (reviewed in Ref. 1). Although this cleavage reaction is biochemically distinct from the transesterifications involved in pre-mRNA splicing (reviewed in Ref. 2), the U7 snRNP particle resembles spliceosomal U snRNPs in various aspects of its structure and biogenesis.

The spliceosomal small nuclear RNAs (snRNAs) contain a conserved single-stranded sequence element, the Sm binding site, which interacts with the seven Sm proteins, B/B′, D1, D2, D3, E, F, and G, to form an ring-shaped heteroheptamer, the so-called Sm core (3–5). In contrast, U7 snRNA has a somewhat degenerate Sm binding site. Earlier studies indicated that, when this U7-specific Sm binding site (AAUUUGUCUAG; U7 Sm WT) was changed to resemble the spliceosomal Sm binding sequence (AAUUUUUGGAG, Sm OPT), the resulting snRNPs were non-functional in histone RNA processing (6, 7). Later, when U7 snRNPs were purified to homogeneity, they were found to contain two U7-specific Sm-like proteins, Lsm10 and Lsm11, which replace Sm D1 and D2 in a U7-specific Sm core (8, 9). The non-functionality of U7 Sm OPT snRNPs could be explained by the findings that U7 Sm OPT RNA forms a standard Sm core containing Sm D1 and D2 and that Lsm11 plays an essential functional role in histone RNA 3′-processing (8–10).

The assembly of Sm core structures occurs in the cytoplasm and is mediated by the multisubunit SMN complex (9, 11, 12). This complex consists of intrinsic components, often referred to as Gemins, and substrate proteins, *i.e.* the Sm/Lsm proteins, which are transferred onto the U snRNA during assembly. We have obtained evidence that the spliceosomal and U7-specific kinds of Sm cores are assembled by separate SMN complexes that contain either Sm D1/D2 or Lsm10/11 along with the five common Sm proteins (9). The presence of these separate and specialized SMN complexes raised the important question of how D1/D2 and Lsm10/11 gain access to the SMN complex and whether they occupy corresponding, mutually exclusive positions within this complex.

Another complex involved in the assembly of at least a subset of Sm proteins into Sm core structures is the PRMT5 complex (also termed methylosome). One of its subunits, the protein methyl transferase PRMT5, catalyzes symmetrical dimethyl arginine (sDMA) modifications within RG repeats found in the Sm proteins B/B′, D1, and D3. It has also been shown that sDMA modifications are essential for binding of these proteins to the SMN complex (13, 14). *In vivo*, the PRMT5 complex may temporarily or permanently associate with the SMN complex to form a larger assembly engine (15).

Here, we have addressed the question of whether the PRMT5 complex is involved in U7 snRNP assembly and how Lsm10/11 are incorporated into the SMN complex prior to U7 snRNP assembly. We find that both Lsm10 and Lsm11 associate with the pICln subunit of the PRMT5 complex *in vitro* and *in vivo*. The basis for this association appears to be a direct binding of both proteins to pICln through their respective Sm domains. However, neither Lsm10 nor Lsm11 appear to be substrates for sDMA modification, and their binding to SMN is independent of any methylation activity. Furthermore, we present evidence that the SMN protein has two binding modes for Sm/Lsm proteins. It can interact with the Sm domains of unmethylated Sm/Lsm proteins such that different members of this protein family can compete with each other for binding. Moreover, the methylated Sm/Lsm proteins exhibit another, strong binding mode, presumably through their sDMA-modified RG repeats, which cannot be competed by Sm domains.

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[5] The abbreviations used are: snRNP, small nuclear ribonucleoprotein; SMN, survival of motoneurons; sDMA, symmetrical dimethyl arginine; PRMT5, protein methyl transferase 5; Lsm, Sm-like protein; snRNA, small nuclear ribonucleic acid; GST, glutathione *S*-transferase; HA, hemagglutinin antigen tag; NP40, Nonidet P40; ECL, enhanced chemiluminescence; SAH, *S*-adenosyl homocysteine; WT, wild type.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmids derived from pcDNA3-HA (8) used for the expression of HA-tagged human Sm B, D1, D2, human Lsm10, or murine Lsm11 and of various truncations of the latter have been described elsewhere (8–10). For the in vitro methylation reactions shown in Fig. 5, a previously described SmB clone (3) and Lsm10 and Lsm11 cDNAs cloned in pET28a were used. For bacterial expression of fusion protein with an N-terminal GST tag, the coding region for the SMN N terminus and tudor domain (amino acids 1–160) was cloned into the pGex4T3 vector (Amersham Biosciences) to yield a plasmid termed GST-SMN-(1–160). The full-length open reading frame of pICln was similarly cloned into pcDNA3-HA and pGEX6P-1 (Amersham Biosciences). All plasmid constructs were verified by DNA sequencing. Details of the constructs are available on request.

GST Pull-down and Competition Assays—To study protein-protein interactions, recombinant proteins were isolated from Escherichia coli BL21 Gold transfected with pGex4T3 (negative control) or with pGex-derived plasmids containing SMN-(1–160) or pICln (see above). Unless indicated otherwise, 1–3 nmol of purified GST or GST fusion proteins were coupled to glutathione-Sepharose 4B beads and incubated with [^{35}S]methionine-labeled proteins obtained by coupled *in vitro* transcription/translation (see below). For competition experiments, 3.7 nmol of bacterially produced heterodimers of SmD1/D2 (16) were used additionally. The beads and proteins were incubated in phosphate-buffered saline supplemented with 0.1% Nonidet P-40 at 4 °C for 2 h while slowly turning on a rotating wheel, washed with phosphate-buffered saline/Nonidet P-40, and the bound and input materials were analyzed by 12% high TEMED SDS-PAGE (17) and detected on a Storm 820 PhosphorImager (Amersham Biosciences).

Coupled in Vitro Transcription/Translation—Protein substrates used for binding were produced by coupled in vitro transcription/translation of pcDNA3-HA-derived plasmids for Lsm10, Lsm11, or Sm B, D1, or D2 in rabbit reticulocyte lysate (TNT kit, Promega) and labeled with [^{35}S]methionine (Hartmann Analytic). To produce unmethylated Sm D1 or Sm B proteins, the reactions contained ~100 nM of the methylation inhibitor S-adenosyl-homocysteine (SAH). Coupled *in vitro* transcription/translation/methylation reactions were performed in rabbit reticulocyte lysate in the presence of 1 μM L-methionine and 2 μCi of *S*-[^{3}H]adenosyl methionine. Reactions were analyzed by 15% high TEMED SDS-PAGE, and dried gels were subjected to fluorography using a Biomax transcreen LE (Kodak).

Interaction Studies in Cell Extracts—Human 293-T cells were cultured as described (8). For transfection, they were grown in 10-cm dishes to 50–60% confluency and transfected with 10 μg of the pcDNA3-HA-derived plasmids complexed with Lipofectamine (Invitrogen, Life Technologies). Cells were harvested 48-h post-transfection. The preparation of small scale whole cell extracts, nuclear or cytoplasmic extracts, and precipitations with biotinylated oligonucleotides complementary to the 5′-ends of U7 or U1 snRNA were performed as described (8, 9). Proteins were analyzed by 12% high-TEMED SDS-PAGE (17), blotted, and probed with appropriate antibodies and developed by the ECL method (Amersham Biosciences).

Antibodies and Immunoprecipitation Experiments—The following antibodies were used: Monoclonal antibodies 7B10 for SMN (18) or Y12 for Sm proteins B/B′, D1, and D3 (19); rabbit antisera or affinity-purified antibodies specific for Lsm11 (9), pICln (14), bacterially produced, non-methylated Sm D1/D2 heterodimer (9), PRMT5 (14), or for WDR77 (alias WD45 or MEP50). For indirect immunodetection in Western blots anti-rabbit or anti-mouse antibodies coupled to horseradish peroxidase (Promega) were used. The HA epitope was detected with anti-HA antibody (Roche Applied Science) directly coupled to horseradish peroxidase.

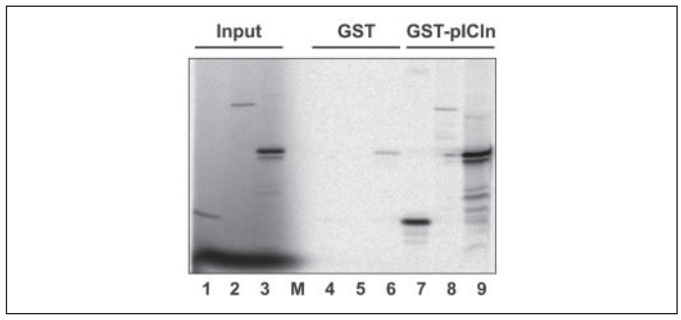

FIGURE 1. **Interactions of Lsm10 and Lsm11 with pICln *in vitro*.** *In vitro* translated [^{35}S]methionine-labeled Lsm10 (*lanes 1*, *4*, *7*), Lsm11 (*lanes 2*, *5*, *8*), and Sm B (*lanes 3*, *6*, *9*) were incubated with GST-pICln (*lanes 7–9*) or GST alone (*lanes 4–6*), immobilized on glutathione-Sepharose beads. *Input* (*lanes 1–3*) show 10% of the amount of *in vitro* translation reactions used in the binding experiments. Proteins binding to the immobilized recombinant proteins were resolved by SDS-PAGE and visualized by a phosphorimaging device. (Molecular Dynamics).

RESULTS

Interaction of Lsm10 and Lsm11 with pICln through Their Respective Sm Domains—In a yeast two-hybrid screen of 174,000 primary transformants using human Lsm10 as the bait, we isolated two full-length cDNA clones encoding the pICln subunit of the PRMT5 complex. These clones produced very strong β-galactosidase signals and did not transactivate the reporter genes in combination with four unrelated proteins used as baits or with the DNA binding domain alone (data not shown). To confirm this interaction and to analyze whether the other U7-specific Lsm protein, Lsm11, might also bind to pICln, we performed in vitro binding experiments. Lsm10, Lsm11, and Sm B were translated in the presence of [^{35}S]methionine in rabbit reticulocyte lysate and then incubated either with GST or with a GST-pICln fusion protein attached to glutathione-Sepharose beads. The beads were precipitated, washed, and their protein content was analyzed by SDS-PAGE and autoradiography. All three proteins clearly bound to GST-pICln (Fig. 1, *lanes 7–9*). For Lsm10 and Lsm11 this binding was caused by the pICln moiety, as no protein bound to GST alone (*lanes 4* and *5*). A trace of Sm B was precipitated by GST (*lane 6*), but the amount precipitated by GST-pICln was much higher, indicating that this previously described interaction (14) also preferentially occurred through the pICln moiety.

To study the relevance of these interactions, we analyzed whether they can also be detected in cell extracts. To this end, extracts from human 293-T cells expressing HA-tagged Lsm10, Lsm11 or various deletion mutants of the latter were subjected to immunoprecipitation by anti-pICln antibodies, and the precipitated material was analyzed by SDS-PAGE and Western blotting with anti-HA antibody. Both Lsm10 (Fig. 2*A*) and full-length Lsm11 (Fig. 2*B*, *top*) were specifically precipitated by the anti-pICln antibody but not by the beads alone. In the large Lsm11 protein, the first Sm motif is preceded by an N-terminal extension of 170 amino acids, and the Sm motifs 1 and 2 that form the Sm domain are separated by 138 amino acids (9). Two different deletions of the N terminus (ΔN104, ΔN140) or a deletion of 77 amino acids from the spacer separating the two Sm motifs (Δsp77) still allowed the interaction with pICln *in vivo* to occur (Fig. 2*B*). Note that all of these deleted proteins are also incorporated into U7 snRNPs in these cells (9). In contrast, an HA-tagged fragment of amino acids 1–136, which does not contain any of the Sm motifs did not associate with pICln *in vivo* (Fig. 2*B*, *N136*). Taken together, these results indicate that Lsm10 and Lsm11

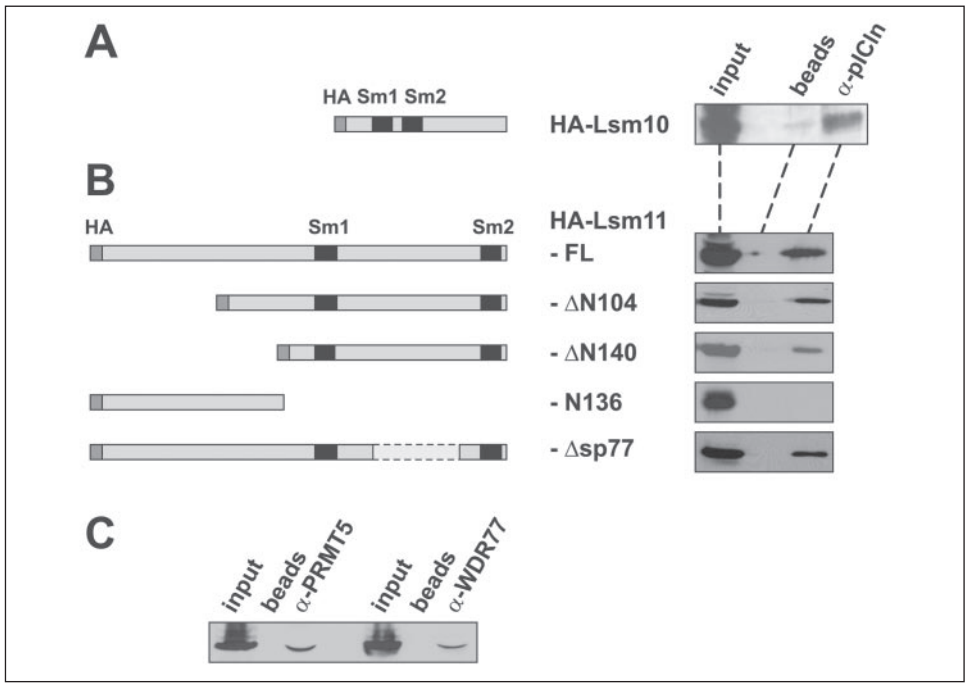

FIGURE 2. **Association of Lsm10 and Lsm11 with pICln, PRMT5, and WDR77 in cell extracts.** Expression plasmids encoding HA-tagged versions of Lsm10 (*A*) or of various parts of Lsm11 (*B*) were transfected into 293-T cells. Whole cell extracts were prepared 48-h post-transfection and subjected to immunoprecipitation with polyclonal anti-pICln antibodies coupled to protein G-Sepharose beads. *C*, similar immunoprecipitation of extract from cells expressing full-length HA-Lsm11 with antibodies against two other components of the PRMT5 complex, PRMT5, and WDR77. The bound proteins were resolved by SDS-PAGE and analyzed by Western blotting with anti-HA antibodies. Each panel shows samples of original extracts from the transfected cells (*input*), control precipitations with protein G-Sepharose beads lacking antibody (*beads*), and precipitations with the specific antibody indicated. An *empty lane* in the Lsm10 panel was loaded with a protein size marker.

interact with pICln both *in vitro* and *in vivo* and that, for Lsm11, this interaction occurs primarily if not exclusively through the Sm domain.

Interaction of Lsm11 with Other Members of the PRMT5 Complex—To test whether the U7-specific proteins interact with the complete PRMT5 complex or only with pICln, we performed a similar immunoprecipitation experiment with extracts from 293-T cells expressing full-length HA-Lsm11 and with antibodies specific for PRMT5 and WDR77. As shown in Fig. 2C, Lsm11 interacts not only with pICln but also with these other subunits of the PRMT5 complex.

Binding of pICln to Free Sm/Lsm Proteins Prior to snRNP Assembly—Because pICln binds Sm and Lsm proteins through their Sm motifs, we wanted to know whether it interacts with these proteins prior to or after their assembly into snRNPs. To address this question, HA-tagged pICln was transiently transfected into human 293-T cells. Nuclear and cytoplasmic extracts from these cells were then precipitated with magnetic streptavidin beads after the addition of biotinylated oligonucleotides complementary to either U7 or U1 snRNAs or without oligonucleotide. The presence or absence of HA-tagged pICln in the precipitates was analyzed by SDS-PAGE and Western blotting with anti-HA antibody. No precipitation of HA-pICln was observed with either the U7- or U1-specific oligonucleotide (Fig. 3A, *top panels*), although the method readily precipitated the Sm B/B' that is associated with both kinds of snRNPs (*lower panels*). Note that the U7 snRNP is less abundant and that, therefore, less B/B' was precipitated.

In control experiments, the expressed HA-pICln was readily precipitated from the cytoplasmic extract with antibodies against Sm proteins (Y12) or against Lsm11 (Fig. 3*B*, *right panel*), indicating that it shows the same interactions as observed for endogenous pICln (see Fig. 2 above). As the Y12 antibody is known to react primarily with the sDMA-modified repeats of Sm B, D1 and D3 (20), we also tested an antibody raised against a bacterially produced, unmethylated heterodimer of Sm D1 and D2. This antibody also precipitated HA-pICln (data not shown).

We note that, although HA-pICln is mostly cytoplasmic, a small fraction of it is also present in the nucleus. However, only traces of the nuclear HA-pICln (which may reflect a low level of cytoplasmic contamination) are associated with Sm proteins or with Lsm11. Taken

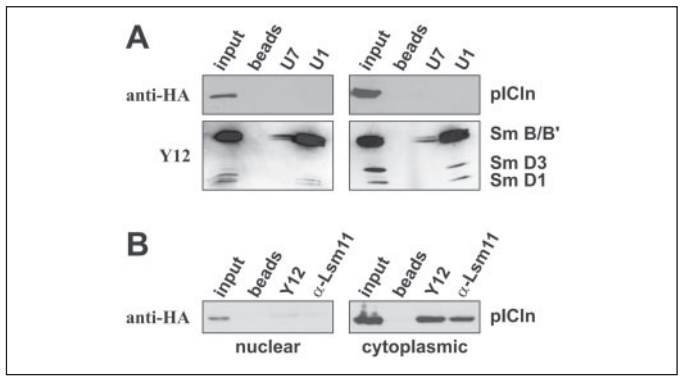

FIGURE 3. **pICln is not associated with mature snRNPs.** HA-tagged pICln was expressed by transient transfection in 293-T cells. Nuclear (*left panels*) and cytoplasmic (*right*) extracts were prepared 48-h post-transfection. *A*, precipitation of the extracts with magnetic streptavidin beads containing biotinylated oligonucleotides complementary to the 5′-ends of either U7 or U1 snRNA. The bound proteins were resolved by SDS-PAGE and analyzed by Western blotting with anti-HA antibodies (*top*) or Y12 anti-Sm antibodies (*bottom*) to reveal the proteins indicated on the *right*. Each panel shows control samples of original extracts from the transfected cells (*input*), as well as control precipitations with streptavidin beads lacking oligonucleotide (*beads*). Note that because of the lower abundance of U7 snRNPs much less Sm B/B′ is precipitated, and Sm D3 is undetectable. *B*, precipitation of the extracts with protein G-Sepharose beads containing monoclonal Y12 anti-Sm antibody or polyclonal anti-Lsm11 serum.

together with the previous results, these findings indicate that pICln (or the PRMT5 complex) does not bind mature assembled snRNPs but only free Sm or Lsm proteins before their assembly into mature snRNPs.

Binding of Lsm10 and Lsm11 to SMN Is Independent of Symmetrical Arginine Dimethylation—Having found that Lsm10 and Lsm11 interact with pICln, which is part of the PRMT5 complex that mediates sDMA modification of RG repeats in certain Sm proteins (13, 14), we asked whether the two U7-specific proteins are substrates for this kind of modification. Human/mouse Lsm11 contain 10/6 RGs and 9/6 GR dipeptides, respectively (9). For human/mouse Lsm10, there are 0/1 RGs and 4/3 GRs (8). However there is only one cluster of RG or GR dipeptides in the N terminus of Lsm11, which is removed by both of the N-terminal deletions described in Fig. 2. It is possible to determine

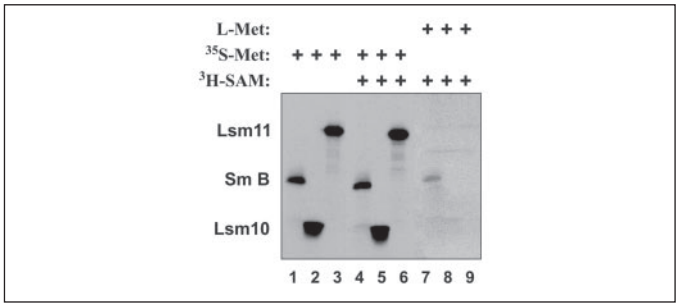

FIGURE 4. **Lack of evidence for symmetrical dimethylarginine modification of Lsm10 and Lsm11.** Sm B (*lanes 1, 4, 7*), Lsm11 (*lanes 2, 5, 8*), and Lsm10 (*lanes 3, 6, 9*) were synthesized by coupled *in vitro* transcription/translation in rabbit reticulocyte lysate. The translation products were either labeled with [^{35}S]methionine (*lanes 1–6*) or synthesized as unlabeled proteins (*lanes 7–9*). Methylation was traced with ^{3}H-labeled *S*-adenosine methionine (*^{3}H-SAM*; *lanes 4–9*) and revealed by fluorography. Note the lack of methylation of the two U7-specific Lsm proteins (*lanes 8–9*), whereas Sm B gets methylated (*lane 7*).

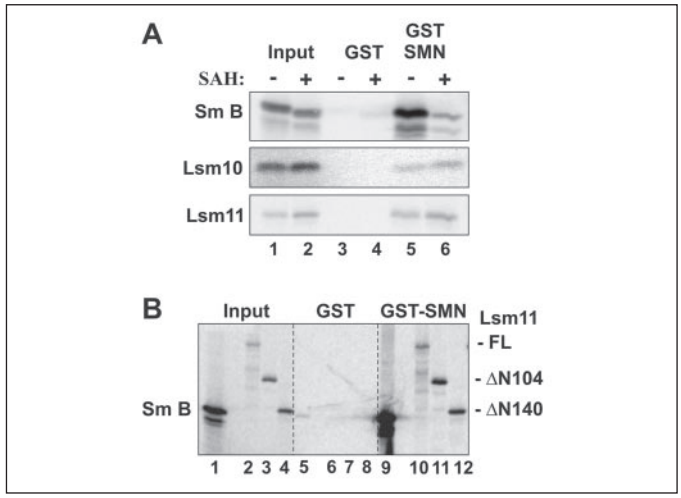

FIGURE 5. **Interaction of Lsm10 and Lsm11 with SMN *in vitro* is independent of sDMA modification.** *A*, interactions of Sm B, Lsm10, and Lsm11 *in vitro* translated in the absence or presence of the methylation inhibitor SAH with GST-SMN-(1–160) (*lanes 5* and *6*) or GST alone (*lanes 3* and *4*). Proteins binding to the immobilized recombinant proteins were analyzed as in Fig. 1. *Input* (*lanes 1* and *2*), show 10% of the amount of *in vitro* translation reactions used in the binding experiments. *B*, comparative binding to GST-SMN-(1–160) of Sm B and various fragments of Lsm11 translated in the absence of SAH.

whether proteins are substrates for methylation by coupled transcription/translation in the presence of tritiated S-adenosyl methionine in rabbit reticulocyte lysate which contains the PRMT5 methylation complex (and possibly PRMT7, which also catalyzes sDMA modifications (21, 22). Such assays were carried out with plasmids encoding Sm B, Lsm10, and Lsm11. Control reactions carried out in the presence of ^{35}S-labeled methionine either without (Fig. 4, *lanes 1–3*) or with *S*-[^{3}H]adenosyl methionine (*lanes 4–6*) proved that all three proteins were efficiently synthesized in the rabbit reticulocyte system. However, only Sm B (*lane 7*), but not Lsm10 (*lane 8*) or Lsm11 (*lane 9*), were detectable by fluorography after translation in the presence of *S*-[^{3}H]adenosyl methionine and unlabeled methionine. These results confirm that Sm B is a methylation substrate. Most importantly, however, they strongly suggest that Lsm10 and Lsm11 are not substrates for sDMA modification, although a weak signal from only one or a few methylated arginines might have escaped detection.

In the case of the Sm/Lsm proteins that are substrates for sDMA modification by the PRMT5 complex (*i.e.* Sm B/B′, D1 and D3, as well as Lsm4), the methylations are important for efficient binding to SMN and the SMN complex (13, 14). This conclusion was reached, among others, by experiments in which the proteins were translated either in the absence or presence of the methylation inhibitor *S*-adenosyl homocysteine (SAH). We therefore analyzed whether translation of Lsm10 and Lsm11 in the presence of SAH had similar effects on the subsequent binding to SMN as was the case for Sm B. As a test for binding, precipitation experiments were performed with recombinant proteins GST-SMN (1–160) or GST (as negative control) that had been immobilized on glutathione beads. Note that the SMN fragment used consists of amino acids 1–160 and contains the binding site for Sm proteins (23).

In such binding assays, all three Sm/Lsm proteins interacted with GST-SMN-(1–160) (Fig. 5*A*, *lane 5*) but not with GST alone (*lane 3*). As expected, the binding of Sm B to SMN was strongly reduced when the *in vitro* translation had been carried out in the presence of SAH (*lane 6*). We also observed a shift in electrophoretic mobility of Sm B that presumably reflected the difference in sDMA modification (*compare lanes 1* and *2 or 5* and *6*). In contrast, the binding of Lsm10 and Lsm11 to GST-SMN-(1–160) was not affected by the inhibition of methylation (*lane 6*), and there also was no detectable mobility shift. Thus methylation of Lsm10 and Lsm11, if it occurs at all, is not important for the interaction of these proteins with SMN.

Next we analyzed whether the two N-terminal truncations described in Fig. 2 could still interact with GST-SMN-(1–160). In these truncation mutants, all RG/GR dipeptide repeats have been deleted. Both Lsm11Δ^{N104} and Lsm11Δ^{N140} specifically bound to GST-SMN-(1–160) (Fig. 5*B*, *lanes 10–12*), but not to the GST control (*lanes 6–8*). Moreover, in immunoprecipitation experiments with extracts from transfected cells similar to those shown in Fig. 2, we found that the same HA-tagged Lsm11 deletions could be precipitated by anti-SMN antibody upon transient expression in 293-T cells (data not shown). Furthermore, we have previously shown that the N-terminally deleted Lsm11 proteins still assemble into U7 snRNPs *in vivo* (9). Therefore these experiments indicate that the N terminus of Lsm11 is not required for interactions with SMN protein or the SMN complex, be it *in vitro* or *in vivo*. Considering all the above results, binding of Lsm10 or Lsm11 to SMN occurs independently of arginine methylation.

Evidence for Separate Binding Sites on SMN for Sm Domains and Methylated RG-rich Tails of Sm/Lsm Proteins—Both the spliceosomal and U7-specific Sm core structures are assembled in the cytoplasm with the help of the SMN complex (9, 11, 12). We have previously obtained evidence for two separate kinds of SMN complexes that contain either Sm D1/D2 or Lsm10/11 and which are devoted to spliceosomal or U7 snRNP assembly, respectively (9). To begin to address the question how these different types of SMN complexes are formed, we analyzed whether the relevant proteins, Sm D1, D2, and Lsm10, Lsm11, bind to SMN in the same way. In particular, we wanted to know whether Sm D1, D2 can compete with Lsm10, Lsm11 for binding to SMN or vice versa.

For this purpose, the binding of these proteins to GST-SMN-(1–160) was analyzed in the absence or presence of an excess of purified recombinant Sm D1/D2 heterodimer used as competitor. As expected, in the absence of competitor all four proteins bound to the immobilized GST-SMN-(1–160) (Fig. 6*A*, *lane 3*) but not to the GST negative control (*lane 2*). Interestingly, however, the recombinant Sm D1/D2 heterodimer competed for the binding to GST-SMN-(1–160) with the *in vitro* translated Lsm11, Lsm10, and Sm D2 proteins, but not with Sm D1 (compare *lanes 4 to 3*).

An important difference between the *in vitro* translated Sm D1 used as binding substrate and the recombinant D1 present in the heterodimer used as competitor resides in the sDMA modification of the C-terminal RG tail. It is possible that SMN may have two binding sites for Sm/Lsm proteins, one interacting with the Sm domain and the other

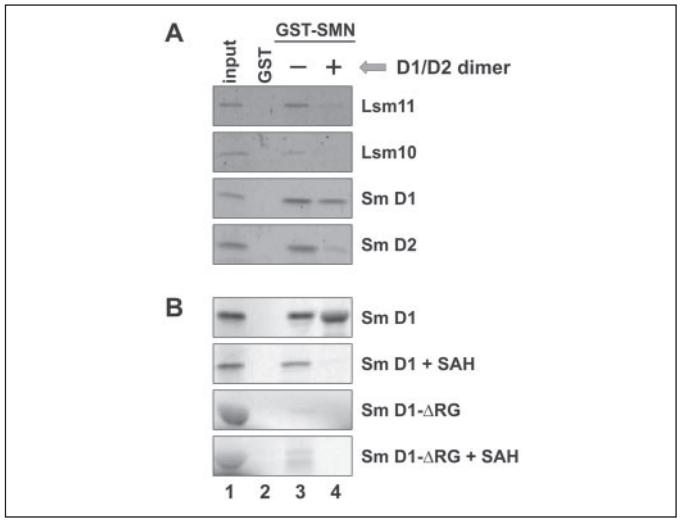

FIGURE 6. **Different binding modes of unmethylated and methylated Sm/Lsm proteins to SMN.** *A*, interactions of *in vitro* translated ^{35}S-labeled Lsm11, Lsm10, SmD1, and Sm D2 with GST (*lane 3;* negative control) or GST-SMN-(1–160) and competition experiments in the absence or the presence of unmethylated Sm D1/D2 heterodimer (*lanes 3* and *4, respectively*). *B*, binding of Sm D1 and Sm D1 deleted of its C-terminal RG-rich tail (*Sm D1-ΔRG*) translated in the absence or presence of the methylation inhibitor SAH to GST or GST-SMN-(1–160) and binding competition using the unmethylated Sm D1/D2 dimer. Proteins binding to the immobilized recombinant proteins were analyzed as in Fig. 1. *Input* (*lane 1*), show 10% of the amount of *in vitro* translation reactions used in the binding experiments.

with the methylated RG-tail (23). Based on this hypothesis, the unmethylated Sm D1/D2 dimer should compete with unmethylated Sm/Lsm proteins, which interact with SMN-(1–160) solely through the Sm domain mode. In contrast, the methylated *in vitro* translated Sm D1 should bind through both the Sm domain and RG-tail modes and therefore be resistant to competition by the D1/D2 dimer.

If this interpretation were correct, the residual binding to SMN-(1–160) of Sm D1 translated in the presence of the methylation inhibitor SAH should be competed by the Sm D1/D2 dimer. The same should be true for the binding to SMN-(1–160) of Sm D1 deleted of its RG-rich C-terminal tail. The experimental verification of these predictions is shown in Fig. 6*B*. The binding of Sm D1 *in vitro* translated in the presence of SAH to GST-SMN-(1–160) is indeed competed by an excess of the unmethylated SmD1/D2 heterodimer (Fig. 6*B*, *second panel*). Moreover, the very weak binding of Sm D1-ΔRG to GST-SMN-(1–160) is efficiently competed by the unmethylated SmD1/D2 heterodimer, irrespective of whether Sm D1-ΔRG has been translated in the presence or absence of SAH (Fig. 6*B*, *third* and *fourth panels*).

Taken together, these data provide strong support for the notion that SMN has two interaction sites for Sm/Lsm proteins. The first one involves the Sm domains and the second involves the methylated RG repeat tails contained in some of these proteins. However, the second interaction appears to be stronger and more important for binding to SMN of those proteins that contain sDMA-modified tails.

DISCUSSION

We have previously shown that the spliceosomal and U7 snRNA-specific Sm core structures are assembled by separate SMN complexes that contain either Sm D1/D2 or Lsm10/11 along with the five common Sm proteins (9). These findings raised the question how D1/D2 and Lsm10/11 gain access to the SMN complex and if their binding to the SMN protein could provide an explanation for these two mutually exclusive SMN complexes. This has been addressed here by analyzing the interactions of Lsm10, Lsm11, and various Sm proteins with pICln and SMN.

Evidence for a Methylation-independent Function of the PRMT5 Complex in snRNP Assembly—Several Sm/Lsm proteins, as well as other substrates acquire sDMA modifications of RG-rich repeats through the action of the PRMT5 complex (13, 14, 20, 24–32). For the Sm proteins B/B′, D1 and D3, these modifications are important for their efficient interaction with SMN, the lead component of the SMN complex involved in snRNP assembly (13, 14). Here we have analyzed, whether Lsm10 and Lsm11, the two proteins that substitute Sm D1 and D2 in the U7-specific core, also interact with the PRMT5 complex and might be substrates for sDMA modification.

Our results clearly demonstrate that both Lsm10 and Lsm11 interact with the pICln subunit of the PRMT5 complex in vitro (Fig. 1) and in cell extracts (Fig. 2). For the large Lsm11 protein, this interaction requires neither the N terminus nor the spacer between the two Sm motifs, but rather seems to depend solely on the Sm domain (Fig. 2*B*). At least Lsm11 also interacts with PRMT5 and WDR77 (Fig. 2*C*) indicating that the interaction is with the entire PRMT5 complex rather than with pICln alone. Moreover, this interaction occurs prior to snRNP assembly (Fig. 3). However, we were unable to detect any sDMA modification of Lsm10 or Lsm11 (Fig. 4), and the binding of both proteins to SMN was not affected by the methylation inhibitor SAH (Fig. 5*A*). Furthermore, the N-terminal part of Lsm11 that contains several RG dipeptides does not contribute significantly to the interaction with SMN *in vitro* (Fig. 5*B*) or in cell extracts (data not shown). We have previously shown that this interaction is not a dead-end product, since these N-terminally truncated proteins get assembled onto U7 snRNA to form snRNPs (9). Taken together, these findings imply that the PRMT5 complex plays a previously unsuspected role in the assembly of Lsm10 and Lsm11 into U7 snRNPs that is independent of its methylation activity.

It is not known how the Sm proteins that are not subject to sDMA modification are recruited to the SMN complex. However our findings may be related to recent evidence demonstrating that the PRMT5 and SMN complexes form a larger structure, which more efficiently assembles U snRNPs than the SMN complex alone (15). Perhaps all Sm/Lsm proteins must bind sequentially to pICln and SMN in this superstructure during the assembly reaction. It is also conceivable that pICln plays an obligatory role in preventing premature assembly of Sm/Lsm oligomers. Recently discovered phosphorylations of pICln and SMN may be involved in this presumptive quality control function (33).

SMN Has Separate Binding Sites for Sm Domains and Methylated RG-rich Repeats—In our studies of the binding of SMN to the Sm D1, D2 and Lsm10, Lsm11 proteins, we have obtained evidence for two separate kinds of interaction. On the one hand, the Sm/Lsm proteins that are not substrates for sDMA modification or whose methylation is prevented by SAH interact with SMN through their Sm domains. This notion was deduced from the binding of the N-terminally truncated Lsm11 proteins to SMN (Fig. 5B). Moreover, the binding of unmethylated Sm D1 was not affected by deletion of the RG-rich C terminus (Fig. 6*B*). In every case analyzed, this Sm-domain-dependent binding could be competed by unmethylated recombinant Sm D1/D2 dimer (Fig. 6, *A* and *B*).

On the other hand, the Sm proteins containing RG-rich tails mainly interact with SMN by virtue of these sDMA-modified structures. This was demonstrated previously by a strongly reduced binding when the tails were deleted or their methylation was prevented (13, 14). Some of our control experiments confirm this for Sm B (Fig. 5*A*) and for Sm D1(Fig. 6*B*). Moreover, methylated RG tail peptides, but not unmethylated ones, have previously been shown to be sufficient for binding to SMN (34, 35).

Relevant for this sDMA-dependent interaction, we have shown that the unmethylated D1/D2 dimer does not compete with methylated Sm D1 for binding to SMN, but competes with unmethylated D1 or D1 deleted of its RG-rich C terminus (Fig. 6*B*). However, because unmethylated D1 and D1-ΔRG still bind to SMN, albeit weakly, this does imply that SMN has two binding sites, one for the Sm domain and the other for methylated RG repeats.

The basis for the interaction of SMN with the methylated RG tails, a negatively charged surface of the protein, has previously been analyzed at the structural level by NMR spectroscopy and x-ray crystallography (23, 36). Interestingly, these studies also revealed a structural similarity between the SMN tudor domain and Sm domains (23). It was therefore speculated that the tudor domain may form a similar intermolecular β4–β5 interface with Sm proteins as is observed between neighboring Sm domains in oligomeric Sm/Lsm structures (5, 16, 37–39). However, this postulated interaction of SMN with Sm domains had not been studied experimentally. Only in one study it was reported that, even though the Sm-like proteins Lsm2, Lsm6, and Lsm7 lack an RG-rich tail, they are still able to bind SMN (34). As discussed above, we have now obtained experimental evidence for this interaction of SMN with Sm domains. Thus, the formation of a high affinity SMN-Sm complex could require multiple and cooperative interactions, involving Sm core and tail binding to the SMN tudor domain, and, possibly, interactions with additional regions of the SMN protein (40, 41) or with other members of the SMN complex.

Although our studies have not yet provided an explanation how the two mutually exclusive SMN complexes dedicated to the assemblies of the U7-specific and spliceosomal Sm core structures are formed, they provide important new insights into the interactions of Sm and Lsm proteins with the PRMT5- and SMN complexes. A full understanding of the specificity of U7 *versus* spliceosomal snRNP assembly will most likely require a deeper insight into the relative arrangements and stoichiometries of the Sm/Lsm proteins with respect to the other members of the SMN complex.

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