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Symmetrical dimethylation of arginine residues in spliceosomal Sm protein B/B′ and the Sm-like protein LSm4, and their interaction with the SMN protein

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ABSTRACT

Arginine residues in RG-rich proteins are frequently dimethylated posttranslationally by protein arginine methyltransferases (PRMTs). The most common methylation pattern is asymmetrical dimethylation, a modification important for protein shuttling and signal transduction. Symmetrically dimethylated arginines (sDMA) have until now been confined to the myelin basic protein MBP and the Sm proteins D1 and D3. We show here by mass spectrometry and protein sequencing that also the human Sm protein B/B′ and, for the first time, one of the Sm-like proteins, LSm4, contain sDMA in vivo. The symmetrical dimethylation of B/B′, LSm4, D1, and D3 decisively influences their binding to the Tudor domain of the "survival of motor neurons" protein (SMN): inhibition of dimethylation by S-adenosylhomocysteine (SAH) abolished the binding of D1, D3, B/B′, and LSm4 to this domain. A synthetic peptide containing nine sDMA-glycine dipeptides, but not asymmetrically modified or nonmodified peptides, specifically inhibited the interaction of D1, D3, B/B′, LSm4, and UsnRNPs with SMN-Tudor. Recombinant D1 and a synthetic peptide could be methylated in vitro by both HeLa cytosolic S100 extract and nuclear extract; however, only the cytosolic extract produced symmetrical dimethylarginines. Thus, the Sm-modifying PRMT is cytoplasmic, and symmetrical dimethylation of B/B′, D1, and D3 is a prerequisite for the SMN-dependent cytoplasmic core-UsnRNP assembly. Our demonstration of sDMAs in LSm4 suggests additional functions of sDMAs in tri-UsnRNP biogenesis and mRNA decay. Our findings also have interesting implications for the understanding of the aetiology of spinal muscular atrophy (SMA).

Keywords: mRNA decay; posttranslational modification; protein arginine methyltransferase (PRMT); protein sequencing; sDMA; spinal muscular atrophy (SMA); spliceosome; UsnRNP

INTRODUCTION

The Sm and the Sm-like (LSm) proteins make up an evolutionarily conserved family of RNA-associated proteins that occur in eukaryotes. The seven Sm proteins B/B′, D1, D2, D3, E, F, and G are found in association with various spliceosomal m_3G-capped UsnRNAs. They are involved in several steps of the biogenesis of the UsnRNPs (reviewed by Will & Lührmann, 2001, and see below) and may also be involved in the splicing process itself (Padgett et al., 1983; Segault et al., 1995; Zhang & Rosbash, 1999; Zhang et al., 2001). Of the nine known LSm proteins (LSm1 to LSm9), two different RNA complexes have been described: LSm2–8 form a complex with U6 snRNA and mediate the integration of U6 snRNA into the U4/U6.U5 tri-snRNP (Achsel et al., 1999; Mayes et al., 1999; Salgado-Garrido et al., 1999; Vidal et al., 1999), whereas LSm1–7 are involved in the decay of cytoplasmic mRNA (Bouveret et al., 2000; Tharun et al., 2000; for a review, see He & Parker, 2000). The Sm and LSm proteins display strong similarities in sequence, especially in respect of two

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Abbreviations: JBP1: Janus kinase-binding protein 1; MBP: myelin basic protein; PRMT: protein arginine methyltransferase; (s/a)DMA: (symmetrical/asymmetrical) dimethylarginine; SAH: S-adenosylhomocysteine; SMA: spinal muscular atrophy; SMN: survival of motor neurons.

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stretches, one 32 and the other 14 amino-acid residues in length, which are termed Sm motifs 1 and 2 (Cooper et al., 1995; Hermann et al., 1995; Séraphin, 1995). Furthermore, the Sm motifs and their flanking sequences appear to fold into similar three-dimensional structures termed Sm domain, as revealed by X-ray crystallography of several eucaryotic and archaebacterial proteins (Kambach et al., 1999; Achsel et al., 2001; Mura et al., 2001).

In the biogenesis of UsnRNPs, the Sm proteins bind to the conserved Sm sites of the m^7G-capped snRNAs U1, U2, U4, and U5 after these have been exported to the cytoplasm. The result is the formation of the so-called UsnRNP core. This core has at least two functions: (1) it is the binding site for an as yet unidentified methyltransferase that hypermethylates the m^7G cap of the UsnRNA to give the m_3G cap (Mattaj, 1986; Plessel et al., 1994; Seipelt et al., 1999), and (2) together with the m_3G cap, it forms a bipartite nuclear localization signal for the reimport of the UsnRNAs into the nucleus (Fischer & Lührmann, 1990; Hamm et al., 1990; Fischer et al., 1993). The UsnRNP core also helps to recruit the snRNP-specific proteins (Nelissen et al., 1994).

The assembly of the UsnRNP core has been investigated in detail in vitro. Thus, each Sm protein interacts in vitro with one or more of the other Sm proteins, giving rise to three stable, RNA-free heteromeric complexes of E.F.G, D1.D2, and B/B′.D3. All of these interactions are mediated by the Sm domains (Hermann et al., 1995; Lehmeier et al., 1994; Raker et al., 1996). The complexes D1.D2 and E.F.G bind cooperatively to the Sm site of the UsnRNAs, resulting in the so-called subcore (Raker et al., 1996). The addition of the B/B′.D3 complex to this subcore leads to the stable core UsnRNP. Whereas the in vitro assembly of core UsnRNPs using purified Sm proteins and UsnRNAs occurs spontaneously, the assembly of the core in vivo is facilitated by a protein complex that contains, among others, several Sm proteins and the survival of motor neurons (SMN) protein (Fischer et al., 1997; Liu et al., 1997; Pellizzoni et al., 1998; Bühler et al., 1999; Charroux et al., 2000; Meister et al., 2000). The SMN protein has been shown to bind directly to Sm proteins (Bühler et al., 1999; Pellizzoni et al., 1999). Interestingly, mutations in the SMN protein have been shown to cause the motoneuron disease spinal muscular atrophy (SMA; Lefebvre et al., 1995). It is thus conceivable, though as yet unproven, that the aetiology of SMA involves a malfunction in the biogenesis of UsnRNPs. Consistent with this, a number of pathological mutations in protein SMN have been shown to disrupt its interaction with the Sm proteins (Bühler et al., 1999; Pellizzoni et al., 1999).

The interaction between the Sm proteins and SMN involves its so-called Tudor domain, comprising amino-acid residues 91–145 (Bühler et al., 1999; Selenko et al., 2001). This domain suffices to bring about the interaction with the Sm proteins in vitro. Additionally, the C-terminal YG box domain that mediates homooligomerization of SMN appears to enhance its interaction with several Sm proteins (Pellizzoni et al., 1999). Sm proteins prepared by translation in vitro showed various degrees of affinity with SMN: The strongest association was with the Sm proteins D1, D3, and B/B′, whereas D2 and E reacted only weakly, and F and G not at all (Liu et al., 1997; Pellizzoni et al., 1999; Friesen & Dreyfuss, 2000). A common feature of Sm proteins D1, D3, and B/B′ is the presence of a C-terminal region containing several RG dipeptides; these are most conspicuous in D1 and D3, with clusters of, respectively, nine and four alternating arginines and glycines, whereas B/B′ contains several GRG triplets flanked by proline residues, raising the possibility that SMN binds primarily to these regions. Consistent with this, Friesen and Dreyfuss (2000) showed that the C-terminal extensions of B/B′, D1, and D3 were necessary for the interaction with SMN. These authors also found that LSm4, the LSm protein most strongly interacting with SMN in vitro, is bound by its RG-rich C-terminus. We have recently shown that the alternating RG dipeptides of D1 and D3 contain symmetrically dimethylated arginines (sDMA; Brahms et al., 2000), which raises the interesting possibility that symmetrical dimethylation of the Sm proteins modulates the association with SMN and thus the biogenesis of the UsnRNPs.

The posttranslational symmetrical dimethylation of arginine is very rare in humans, and had previously only been described for the myelin basic protein (Baldwin & Carnegie, 1971) and D1 and D3 (Brahms et al., 2000); dimethylated arginines in proteins with RG-rich regions are usually dimethylated asymmetrically (Najbauer & Aswad, 1990; for review, see Gary & Clarke, 1998). We were therefore interested in learning more about the function of this unusual posttranslational modification. By mass spectrometry and protein sequencing, we could show that the human proteins B/B′ and LSm4, like D1 and D3, are dimethylated symmetrically in vivo. The symmetrical dimethylation of B/B′, D1, D3, and LSm4 is crucial for their binding to the SMN protein. This is indicated by our findings that either inhibition of dimethylation by S-adenosylhomocysteine (SAH) of in vitro-translated Sm proteins B/B′, D1, D3, and LSm4 or addition of a synthetic peptide containing nine sDMA-glycine dipeptides specifically abolished binding of the Sm proteins to SMN. While this work was being prepared for publication, Dreyfuss and coworkers (Friesen et al., 2001) observed that sDMAs in D1 and D3 enhance the affinity to SMN. We further established an in vitro methylation assay, thus providing evidence that the symmetrical dimethylation of the Sm proteins takes place in the cytoplasm of HeLa cells. These findings have interesting implications for the understanding of UsnRNP biogenesis, mRNA decay, and the aetiology of SMA.

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RESULTS

The Sm protein B/B′ and the Sm-like protein LSm4 are symmetrically dimethylated in vivo

The proteins B/B′ and LSm4 both contain several RG dipeptides in their C-terminal regions, although these are less extensive than in D1 (with nine consecutive RG dipeptides) or D3 (with four). Protein B/B′ contains no repeating RG pairs, but it has five GRG triplets (arginines 112, 147, 172, 181, and 209), along with two GR pairs (arginines 25 and 108) and three RG pairs (arginines 73, 132, and 228; Fig. 1A). Protein LSm4 has three GRG triplets involving arginines 102, 109, and 125, and two GRGRG pentapeptides involving arginines 88, 90, 115, and 117 (Fig. 1B). In addition, it contains a single RG pair (Arg62) and a single GR pair (Arg134). We investigated the possibility that these proteins might be dimethylated.

To obtain human B/B′ protein for Edman sequencing, the proteins of affinity-purified HeLa UsnRNPs were separated by high-TEMED SDS-PAGE and the B/B′ bands were excised from the gel and electroeluted. Peptide fragments were prepared by digestion with trypsin and isolated by HPLC. One peptide obtained (denoted $B/B'_{95–132}$) contained amino acid residues 95–132, and another ($B/B'_{133–228}$) contained residues 133–228. Both peptides contained internal arginines, suggesting that these might be methylated (at least asymmetrical DMA is not recognized by trypsin; Lischwe et al., 1985). By microsequencing the peptide $B/B'_{95–132}$, we showed that the internal arginines 108 and 112 were indeed symmetrically dimethylated (Fig. 2). Peptide $B/B'_{133–228}$ was sequenced from its N-terminal Gly133 to Met195; arginines 147, 172, and 181 were all found to be symmetrically dimethylated. Finally, fragment $B/B'_{133–228}$ was cleaved with cyanogen bromide, yielding the dodecapeptide Gly204–Met215, which was sequenced; its internal Arg209 was also found to be symmetrically dimethylated. Of the other arginine residues with a neighboring glycine, Arg132 and Arg25 are presumably not dimethylated, as these were recognized by trypsin. Results are summarized in Figure 1C.

Human LSm4 protein was prepared by purification of the human U6-associated LSm complex from nuclear tri-snRNPs (Achsel et al., 1999) and subsequent fractionation of the LSm proteins using high-TEMED SDS-PAGE. The bands containing LSm4 were excised and digested with protease EndoLysC. Although protein LSm3 usually comigrates with LSm4, the peptides of LSm4 could be separated by HPLC and identified by mass spectrometry.

Results are summarized in Table 1. Methylation was found in fragment LSm4–7 (dimethylation of both arginines in the fragment, at positions 88 and 90) and in fragment LSm4–9 (dimethylation of all five arginines, at positions 102, 109, 115, 117, and 125). In contrast, arginines at positions 55, 62, 70, and 134 were found not to be methylated. No result was obtained for Arg41 and Arg48, as fragment LSm-3 did not yield a measurable peak in the mass spectrometer. Thus, all the GRG and GRGRG tracts in LSm4 are fully dimethylated, whereas arginines with one glycine neighbor only and those with none are unmethylated.

Edman sequencing of fragments LSm4–7 and LSm4–9 was performed, to determine whether the methylation of the arginines was symmetrical or asymmetrical (data not shown). In fragment LSm4–7, both arginines (residues 88 and 90) were found to be symmetrically methylated. In fragment LSm4–9 arginines 102 and 109 were also found to be symmetrically dimethylated. The methylation patterns of arginines 115, 117, and 125 could not be determined, as the small quantities of fragment LSm4–9 available did not allow sequencing of all its 30 amino-acid residues. The results are summarized in Figure 1C.

Symmetrical dimethylation of the Sm proteins B/B′, D1, D3, and the Sm-like protein LSm4 is a prerequisite for efficient interaction with SMN

It has already been shown that recombinant SMN binds to certain Sm and LSm proteins prepared by translation in vitro, and that the binding of SMN was strongest to proteins B/B′, D1, D3, and LSm4 (see Introduction; Liu et al., 1997; Pellizzoni et al., 1999; Friesen & Dreyfuss, 2000). Because all of these proteins are symmetrically dimethylated in vivo, it seemed possible that the symmetrical methylation influences the interaction with SMN. Binding experiments were performed using the following potential binding partners:

1. The SMN deletion mutant $SMN_{1–160}$, which has a functional Tudor domain (Bühler et al., 1999). As a negative control for $SMN_{1–160}$, the Tudor-domain point mutant $SMN_{1–160}$-E134K was used; this well-characterized mutant is incapable of binding to Sm proteins (Bühler et al., 1999; Selenko et al., 2001). The deletion mutants were chosen, because these, in contrast to the full-length SMN, were soluble when expressed as fusion proteins.
2. Proteins B/B′, D1, D3, or LSm4 prepared by translation in rabbit reticulocyte lysate. As the methylation status of the proteins prepared in vitro in this way was unknown, the translation was performed both in the presence and in the absence of the methyltransferase inhibitor SAH.

Figure 3A shows the result of pull-down experiments in which the respective Sm proteins, prepared by translation in rabbit reticulocyte lysate, were exposed to immobilized $SMN_{1–160}$. In each of the first four boxes, the

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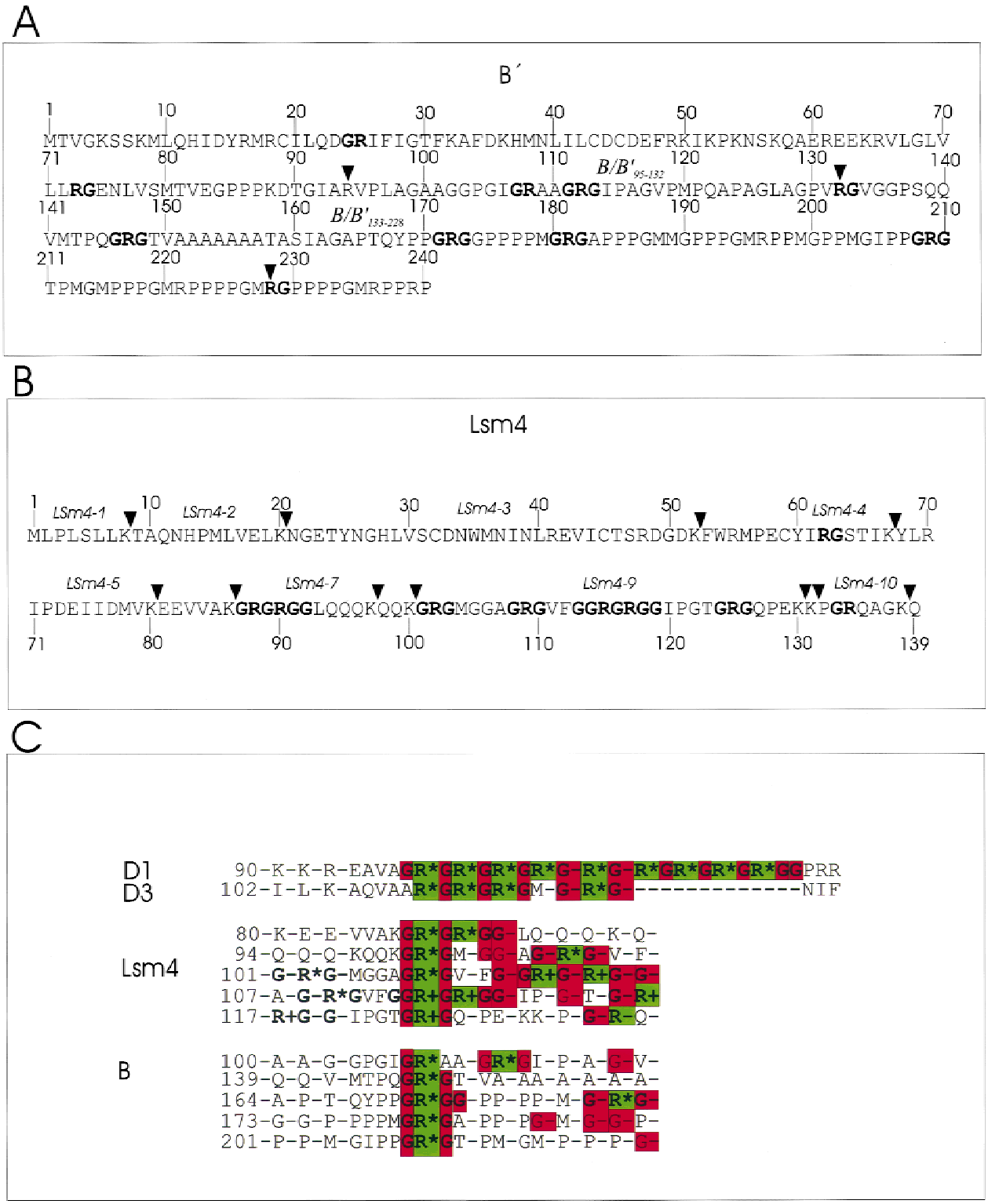

FIGURE 1. **A:** Sequence of the B′ protein. The tryptic peptides that were sequenced are indicated by arrowheads. Glycine/arginine-rich sequence elements (at least one GR or RG) are shown in bold type. **B:** Sequence of LSm4. The positions at which LSm4 was cleaved by EndoLysC are indicated by arrowheads. Glycine/arginine-rich sequence elements (at least one GR or RG) are shown in bold type. The proteolytic peptides that were obtained (e.g., LSm4–4, LSm4–7, LSm4–9, and LSm4–10) are indicated. **C:** Alignment of the symmetrically dimethylated arginines in the Sm proteins D1, D3, B/B′, and the Sm-like protein Lsm4. Symmetrically dimethylated arginins are indicated by *, dimethylated arginines are indicated by +. Arginines are shown in green; glycines are shown in red.

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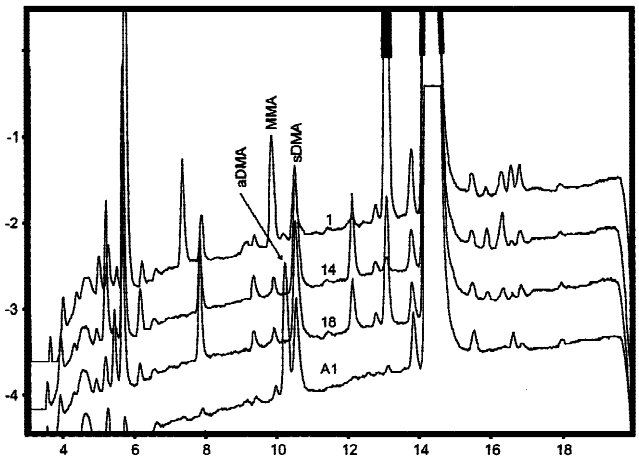

FIGURE 2. Sequencing of the tryptic B/B′$_{95–132}$ fragment of B/B′. The elution profiles from sequencing steps 14 and 18 (corresponding to arginines 108 and 112 in the wild-type B/B′ sequences) are shown aligned with control runs that used a mixture of monomethylarginine (MMA) and symmetrical dimethylarginine (sDMA, trace 1), or a mixture of asymmetrical dimethylarginine (aDMA) and sDMA (trace A1).

left-hand lane indicates the amount of the respective Sm protein bound to the immobilized SMN$_{1–160}$: protein B (lane 1), D3 (lane 4), D1 (lane 7), and LSm4 (lane 10). However, when the translation in vitro was conducted in the presence of SAH, the binding was dramatically reduced (lanes 2, 5, 8, and 11). This provides prima facie evidence that efficient binding had a requirement for methylation of the arginines.

The following control experiments were performed to rule out alternative explanations for the dramatic decrease in yield of the pull-down experiments described above.

1. The SAH was added after the translation reaction instead of during it (Fig. 3A, lanes 3, 6, 9, and 12). For proteins B, D3, and LSm4, the posttranslational addition of SAH had no effect; for protein D1, the yield was reduced (lane 9, contrast lane 7), but not as strongly as when the SAH was added cotranslationally (lane 8). We infer that the reduced binding is indeed due to lack of methylation of the B, D1, D3, and LSm4 translates and not due to blocking of the binding by SAH.
2. The yields of the translation reactions in reticulocyte lysate were compared in the absence and presence of SAH (Fig. 3A, lanes 13–20). In each case, SAH failed to influence the yield of the translation in vitro.
3. Instead of SMN$_{1–160}$, the Tudor point mutant SMN$_{1–160}$-E134K was immobilized for pull-down experiments. This mutant bound none of the four proteins, either with or without SAH present (Fig. 3A, lanes 21 and 22). The inhibition experiments with SAH thus indicate that methylation of arginines is necessary for the binding to SMN-Tudor.

The specificity of the methylation requirement was examined by competition studies. The binding of proteins D1, D3, LSm4, and B/B′ to immobilized SMN$_{1–160}$ was conducted in the presence of increasing quantities of an icosamer CG-(RG)$_9$ in which the arginines were uniformly nonmethylated (in the following designated as (RG)$_9$), symmetrically dimethylated ((sDMA-G)$_9$) or asymmetrically dimethylated ((aDMA-G)$_9$). Only (sDMA-G)$_9$ interfered with the binding (Fig. 3B); (aDMA-G)$_9$ and nonmethylated (RG)$_9$ had no effect on it. Thus, the binding to SMN$_{1–160}$ (and by implication to SMN) requires not only methylation of arginines, but, specifically, their symmetrical dimethylation. Moreover, because the peptide consisted exclusively of sDMA-G dipeptide repeats, such a motif appears to be sufficient for binding.

The methylation of the Sm proteins takes place in the cytoplasm and is independent of the assembly status of the D1 protein

The recognition of SMN by Sm proteins only when they are symmetrically dimethylated suggests a functional relationship between the dimethylation and the Sm–SMN interaction; indeed the involvement of SMN in spliceosome assembly and the splicing reaction has already been proposed (see Introduction). It is of interest to know at what assembly stage and at what cellular compartment the Sm proteins are dimethylated,

TABLE 1. Calculated and observed molecular masses (in daltons) of protonated fragments of LSm4.

Protein fragment	Calculated molecular mass, unmodified	Calculated molecular mass with *n* DMAs	Molecular mass found
LSm4–2 (9–20)	1,380.73	—	1,380.80
LSm4–4 (53–67) + acrylamide	1,957.94	—	1,958.05
LSm4–5 (68–80)	1,604.87	—	1,605.04
LSm4–7 (87–97)	1,184.66	1,240.72 ($n = 2$)	1,240.77
LSm4–9 (101–130)	2,840.48	2,980.64 ($n = 5$)	2,980.80
LSm4–10 (131–138)	841.50	—	841.52

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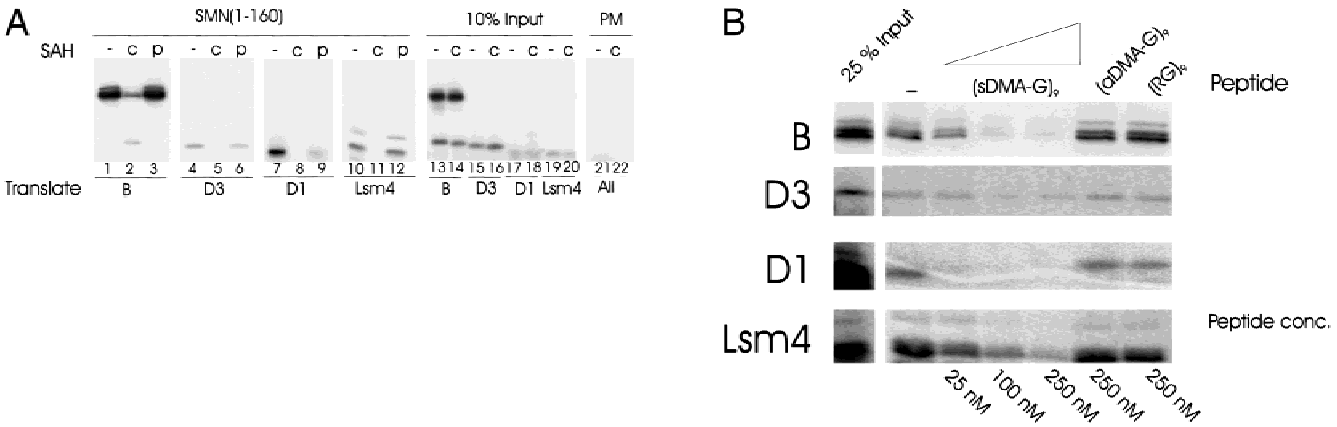

FIGURE 3. Pull-down experiments with the immobilized SMN deletion mutant $SMN_{1–160}$. **A:** Lane 1 shows the binding of protein B to $SMN_{1–160}$; the binding was greatly reduced when the methyltransferase inhibitor SAH had been added cotranslationally in the synthesis of protein B (c, lane 2) but not when it had been added posttranslationally (p, lane 3). Lanes 4–12 show corresponding comparisons for proteins D3, D1, and LSm4. Lanes 13–20 show 10% of the yields of the in vitro syntheses of the four proteins; the cotranslational addition of 0.4 mM SAH does not affect these yields. Lanes 21 and 22 are analogous to lanes 1 and 2, but the immobilized protein was $SMN_{1–160}$-E134K (termed PM). None of the four proteins was bound, with (lane 21) or without (lane 22) cotranslationally added SAH. **B:** Excerpts from gels in binding experiments analogous to those in **A**, lanes 1, 4, 7, and 10 are shown: left: without addition; center: binding in the presence of increasing concentrations (0–250 nM) of a symmetrically dimethylated D1-C-terminal peptide $(sDMA\text{-}G)_9$; second from right: in the presence of 250 nM of the analogous asymmetrically dimethylated peptide $(aDMA\text{-}G)_9$; far right: in the presence of 250 nM of the analogous nonmethylated peptide $(RG)_9$.

and this might also indicate at which point in the reaction cycle of the Sm proteins the interaction with SMN commences.

For this purpose, we employed a recombinant D1 fusion protein expressed in *Escherichia coli* and known to be nonmethylated (Brahms et al., 2000). This protein was incubated with (1) HeLa cytosolic extract S100 or (2) HeLa nuclear extract in the presence of [^{14}C]-methyl-S-adenosylmethionine. The methylated product was detected by fluorography after SDS-PAGE (Fig. 4A). When the entire product was analyzed (Fig. 4A, lanes 1 and 2), both incubations were seen to have led to methyl-

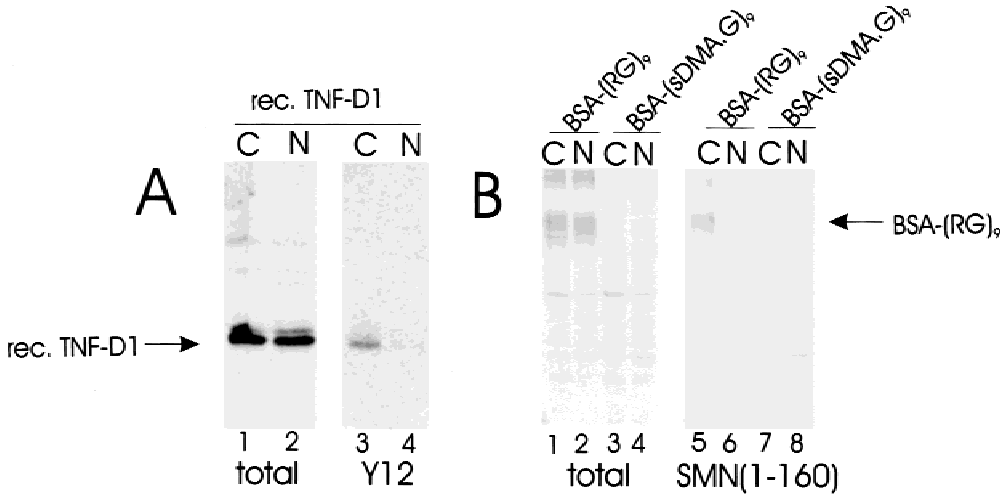

FIGURE 4. **A:** 500 ng TNF-D1 fusion protein were incubated with 0.125 μCi [^{14C}]-SAM and HeLa S100 cytosolic extract (C) or HeLa nuclear extract (N). In lanes 1 and 2 (total), 10% of the reaction mixture was applied to the gel; the rest was immunoprecipitated with immobilized antibody Y12 and the material bound was analyzed (lanes 3 and 4). **B:** 2 μg BSA-conjugated $(RG)_9$ peptide or $(sDMA\text{-}G)_9$ peptide were incubated with 0.125 μCi [^{14C}]-SAM and HeLa S100 cytosolic extract (C) or HeLa nuclear extract (N). In lanes 1–4 (total), 10% of the reaction mixture was applied to the gel; the rest was precipitated with immobilized $SMN_{1–160}$ and the material bound was analyzed (lanes 5–8).

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ation. However, when the radioactively methylated D1 fusion protein was exposed to immobilized antibody Y12, which is known to bind only to symmetrically dimethylated protein D1 (Brahms et al., 2000), then only the product of incubation with the cytosolic extract was precipitated (Fig. 4A, lane 3), whereas a similar quantity of the product of incubation with the nuclear extract was not (Fig. 4A, lane 4). We infer that the nuclear extract produced a different methylation pattern, and that the methyltransferase that recognizes protein D1 is located in the cytoplasm.

The substrate for methylation (the D1 fusion protein) was present in great excess over the endogenous Sm proteins in the extract (this was confirmed by immunoblotting; data not shown). Therefore, the methylation can take place at the stage of single, free Sm proteins. To substantiate this, we used an $(RG)_9$-BSA conjugate as methylation substrate. This conjugate does not possess an Sm domain and can therefore not form complexes with other Sm proteins (see Introduction). However, it was methylated by both cytosolic and nuclear extracts (Fig. 4B, lanes 1 and 2). The analogous $(sDMA\text{-}G)_9$-BSA was not methylated (Fig. 4B, lanes 3 and 4), showing that the methylation took place on the $(RG)_9$ unit and not on the BSA moiety. The respective products of methylation of $(RG)_9$-BSA by cytosolic and nuclear extracts were subjected to pull-down experiments with immobilized $SMN_{1–160}$. Only the product of incubation of $(RG)_9$-BSA with cytosolic extract yielded a radioactive methyl derivative capable of binding to $SMN_{1–160}$ (Fig. 4B, lanes 5–8); this confirms the result obtained with the D1 fusion protein, namely, that the correct dimethylation takes place in the cytosolic extract.

We infer that the methylation of D1, and by implication of other Sm proteins, takes place in the cytoplasm. Moreover, the specific recognition of the conjugate, in vitro methylated in cytosolic extracts, substantiates our finding that SMN exclusively binds to symmetrically dimethylated arginines.

SMN interacts with fully assembled UsnRNPs via symmetrical dimethylarginines

To gain an understanding of the function of protein SMN, it will be necessary to know in what assembly status the Sm proteins can bind to it: (1) as monomers, (2) in RNA-free protein complexes, or (3) as constituents of fully assembled snRNPs. The first has here been shown to be the case by the precipitation by SMN of proteins B, D1, D3, and LSm4 prepared separately in vitro. The second has been demonstrated by the interaction of SMN with the RNA-free Sm-protein complexes D1.D2 and B.D3 (Bühler et al., 1999). It remained to be seen whether the interaction with SMN is also possible in fully assembled snRNPs.

By pull-down binding experiments (Fig. 5), we were able to show that at least one of the symmetrically dimethylated C-termini is accessible in assembled UsnRNP particles. Whereas $SMN_{1–160}$ efficiently precipitated the U1 and U2 snRNPs and the U4/U6.U5 tri-snRNP (Fig. 5, lane 1), the same snRNPs were not recognized by the point mutant $SMN_{1–160}$-E134K (Fig. 5, lane 8). This interaction of SMN with complete snRNPs is thus also mediated by the Tudor domain. The interaction, like that of the monomers, takes place through the symmetrically dimethylated arginines, as we showed by specifically inhibiting the precipitation with the $(sDMA\text{-}G)_9$ oligopeptide (Fig. 5, lanes 4 and 5); the analogous nonmethylated and asymmetrically dimethylated oligopeptides did not inhibit the binding (Fig. 5, lanes 2, 3, 6, and 7).

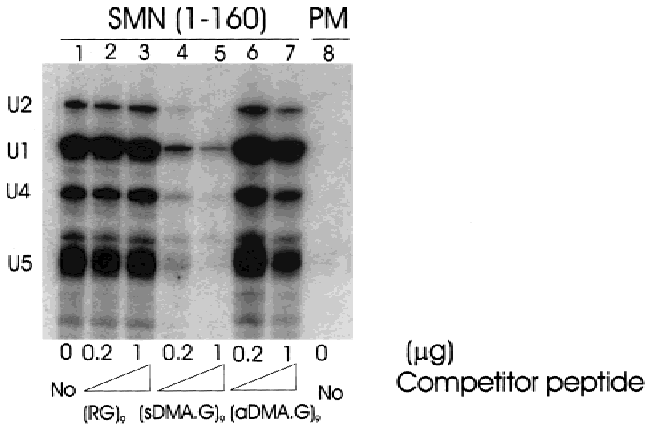

FIGURE 5. Binding of UsnRNPs to immobilized $SMN_{1–160}$. The bound material was detected by pCp-labeling of coprecipitated UsnRNAs (Brahms et al., 1997). Lane 1: no addition; the positions of the bound U1, U2, U4, and U5 snRNAs are shown on the left. Lanes 2 and 3: addition of the oligopeptide $(RG)_9$ as a competitor; lanes 4 and 5: addition of $(sDMA\text{-}G)_9$; lanes 6 and 7: addition of $(aDMA\text{-}G)_9$; lane 8: as lane 1 but with immobilized $SMN_{1–160}$-E134K.

DISCUSSION

Arginine residues in glycine- and arginine-rich protein sequences are frequently methylated posttranslationally by protein arginine methyltransferases (PRMTs). The methylation pattern most frequently seen is asymmetrical dimethylation (Najbauer & Aswad, 1990); this modification appears to be important for protein shuttling and signal transduction (for review, see McBride & Silver, 2001). The rarer symmetrical dimethylation is less well understood, and known cases were confined until recently to the myelin basic protein (MBP) and the Sm proteins D1 and D3. We show here that this list also includes the Sm protein B/B′ and, for the first time, one of the Sm-like proteins, LSm4. Thus, the symmetrical dimethylation of at least four proteins may play a major part in the biogenesis and/or function of the spliceosome. Protein LSm4 is of particular interest as it is also a constituent of the cytoplasmic complex responsible for the decapping of mRNA in the process of mRNA

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decay. Consequently, the symmetrical dimethylation of arginine could play a part in mRNA turnover, and possibly be implicated in the pathogenesis of SMA, a common hereditary autosomal disease (see below).

The Tudor domain of SMN binds to the symmetrically dimethylated arginine residues of D1, D3, B/B′, and LSm4

We have shown previously that a symmetrically dimethylated C-terminal peptide of D1 is recognized by the monoclonal antibody Y12, whereas the corresponding nonmethylated and asymmetrically dimethylated peptides are not recognized (Brahms et al., 2000). Here we show that the binding of D1, D3, B/B′, and LSm4 to SMN is likewise dependent upon symmetrical dimethylation of the arginines (Fig. 3). An oligopeptide consisting exclusively of alternating glycine and symmetrically dimethylated arginine residues was able to inhibit the binding (Fig. 3B); asymmetrical and nonmethylated arginine containing peptides did not interfere. Moreover, when D1, D3, B/B′, and LSm4 are synthesized by translation in rabbit reticulocyte lysate, the translation products can be prevented from interacting with SMN by cotranslational inhibition of methylation; this not only demonstrates that methylation of Sm proteins is necessary for SMN-binding but furthermore indicates the presence of a type II (i.e., sDMA generating) methyltransferase in the lysate.

Our data show that the sDMA binding domain is located on the Tudor domain of SMN. The point mutant $SMN_{1–160}$-E134K was unable to bind any of the symmetrically dimethylated proteins or intact UsnRNPs. This mutation lies at the surface of the Tudor domain (Selenko et al., 2001) and is known to disrupt SMN-Sm interactions (Bühler et al., 1999). A recent NMR study (Selenko et al., 2001) showed that residue E134 of SMN interacts directly with a model oligopeptide that contained the RG dipeptide cluster of protein D1.

In the NMR study, and also in earlier pull-down experiments employing recombinant D1, D3, and B proteins (Pellizzoni et al., 1999; Friesen & Dreyfuss, 2000) the SMN ligands were not methylated. However, in all of these experiments, the concentrations used (e.g., 0.1 to 0.3 mM) were orders of magnitudes higher than in the experiments with in vitro translated proteins; under the conditions described above (e.g., ~100 pM in Fig. 3A), symmetrical dimethylation was a necessary condition for observing binding at all. It is thus evident that symmetrical dimethylation strongly increases the binding constant to SMN. While this work was being prepared for publication, Dreyfuss and coworkers (Friesen et al., 2001) observed that sDMAs in D1 and D3 enhance the affinity to SMN. Our data show that this holds true for all four (L)Sm proteins symmetrically dimethylated in vivo.

Thus, symmetrical dimethylation of arginine residues can modulate protein–protein interactions in a strong and specific manner.

Symmetrically dimethylated sites show a common sequence element

By definition, PRMTs of type I catalyze the asymmetrical dimethylation of arginine residues in proteins, whereas PRMTs of type II catalyze the corresponding symmetrical dimethylation. Type I PRMTs act preferentially at RGG triplets with the consensus sequence FGGRGGF (for review, see Gary & Clarke, 1998). For type II PRMTs, no consensus sequence has yet been established. In proteins D1 and D3, all the symmetrically dimethylated arginines were found in alternating $(RG)_n$ tracts. Although such tracts are also found in protein LSm4 (Fig. 1), this protein is also symmetrically dimethylated at two positions for which the only common sequence element is the GRG triplet (Fig. 1C). B/B′ contains no alternating $(RG)_n$ tracts and is methylated at five GRG triplets and at one GRA triplet.

To generalize from these observations, the shortest sequence element at which symmetrical dimethylation takes place is the triplet GRG, either within $(RG)_n$ repeats or on its own. This generalization includes proteins D1, D3, LSm4, and B/B′. Interestingly, D3 contains a GRG tripeptide in position 97 that had not been investigated for possible methylation before (Brahms et al., 2000). If GRG was the minimal consensus, this tripeptide should also be dimethylated. Indeed, in mass spectrometry we could detect a mass of 14,100, consistent with N-terminally acetylated D3 with five dimethylarginines (data not shown). It is therefore likely that an additional dimethylarginine is located in position 97 of D3. All 25 occurrences of the triplet GRG in these four proteins are dimethylated, and, with the exception of three arginines in LSm4 and one in D3 for which no data are available, all are symmetrically dimethylated. The symmetrically dimethylated arginine of MBP is also flanked by glycines, in the sequence $_{104}$GKGRG$_{108}$ (Baldwin & Carnegie, 1971); moreover, replacement of G106 or G108 by bulky residues inhibited the methylation in vitro (Rawal et al., 1995). Our in vitro methylation studies provide evidence that GRG tripeptides are the optimal substrates (Fig. 4B). However, there is a single instance each of symmetrical dimethylation of the arginine in the triplet GRA (protein B/B′, at Arg108) and in the triplet ARG (protein D3, at Arg110). This could mean either that the consensus sequence is not absolute, or else that the real consensus sequence is (G/A)R(G/A). As there are no other GRA or ARG triplet sequences in any of these four proteins, or indeed in any other Sm or LSm protein, no decision can be made between these alternatives at present.

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Our methylation experiments with cytosolic and nuclear extract show that the cytoplasm of HeLa cells contains a type II methyltransferase that dimethylates symmetrically the arginines in $(RG)_n$ tracts, both in protein D1 and in synthetic oligopeptides (Fig. 4). The nuclear extract also contained a methyltransferase active towards these substrates, but this did not give a symmetrically dimethylated product. This points to the existence of further factors that determine the specificity and kind of methylation that takes place in the cell. Consistent with this, Rawal et al. (1995) showed that two partially purified Type I methyltransferases (from calf and rat) dimethylated a peptide with alternating (RG) units asymmetrically, with high efficiency. Conversely, Branscombe et al. (2001) showed that a partially purified Type II methyltransferase, PRMT5 (also termed JBP1), was able to dimethylate symmetrically peptides with the RGG box—the "classical" signal for asymmetrical dimethylation. There are therefore clearly further determinants of the methylation pattern in vivo, not least because there exist asymmetrically dimethylated RGG boxes that contain internally alternating RG dipeptides (e.g., guinea-pig bFGF; Gary & Clarke, 1998).

One of these determinants is clearly the compartmentalization of the cell. The only known type II methyltransferase, PRMT5 (JBP1; Pollack et al., 1999; Branscombe et al., 2001; Rho et al., 2001), has been shown to reside exclusively in the cytoplasm (Rho et al., 2001), whereas Type I methyltransferases are present in the cytoplasm and the nucleus (Mowen et al., 2001, and references therein). It is thus possible that PRMT5 is the methylase responsible for the symmetrical dimethylation of the (L)Sm proteins. In addition to cellular compartmentalization, it is probable that other protein factors also provide specificity. This is supported by the fact that no methyltransferase has yet been isolated biochemically. For example, the Type II methyltransferase that methylates MBP can be separated into two electrophoretically distinct bands of 72 kDa and 100 kDa (Ghosh et al., 1988). The 72-kDa band may be PRMT5. The 100-kDa band, as yet unidentified, could be just such a "specificity factor." PRMT5 has also been shown to interact with the pICln protein (Krapivinsky et al., 1998). As pICln interacts not only with PRMT5, but also with the Sm proteins (Pu et al., 1999), it is easily conceivable that pICln is a specificity factor that provides for the symmetrical dimethylation of Sm proteins by PRMT5.

The symmetrical dimethylation occurs at an early point in the biogenesis of UsnRNP

The Sm proteins are involved in all stages of the biogenesis of the snRNPs, starting from their own association into hetero-oligomers (see Introduction). The interaction between them takes place by way of the Sm domains, as has been shown by X-ray crystallography (Kambach et al., 1999) and by mutagenesis (Hermann et al., 1995; Camasses et al., 1998). Each of the four proteins D1, D3, LSm4, and B/B′, can be methylated by Type II methyltransferase alone (Fig. 3), and in the case of D1, the C-terminal region suffices for correct methylation (Fig. 4). It is thus probable that methylation in vivo occurs soon after—or even during—the biosynthesis of these proteins on the ribosome. Thus, a necessary condition for the interaction of these proteins with protein SMN is fulfilled, and it is therefore possible that the binding to SMN occurs at this early stage, supporting a role for SMN in the assembly of the Sm core complex (see Introduction).

This in turn raises the question of when the SMN dissociates from the snRNP. The specific interaction of SMN with the completely assembled UsnRNPs (Fig. 5) shows that at least one of the symmetrically dimethylated C-termini is accessible in completely assembled UsnRNPs, and suggests that the SMN–Sm interaction can take place at any stage of, and perhaps throughout, the cytoplasmic assembly process. In agreement with previous injection studies in oocytes (Fischer et al., 1997), the dissociation is thus likely to occur directly before or after the snRNP enters the nucleus.

In vitro, Sm cores can be assembled spontaneously, even in the absence of detectable quantities of SMN (Sumpter et al., 1992; Raker et al., 1996). The function of SMN in the assembly in vivo is thus unclear; it is possible that the assembly proceeds more rapidly or accurately in the presence of SMN. Additional functions of sDMAs like promoting the import of core UsnRNPs into the nucleus (similar to the asymmetrically dimethylated STAT1 protein; Mowen et al., 2001) or in splicing cannot be excluded.

The symmetrical dimethylation may play a part in mRNA turnover

LSm4 is the first of the Sm-like proteins to show symmetrical dimethylation of arginines in vivo. This is of particular interest, as the LSm proteins take part not only in the assembly of the tri-snRNP in the nucleus, but also in the cytoplasmic decapping of mRNA. Cytoplasmic and nuclear LSm complexes differ in their composition: whereas the U6-associated LSm-complex in the nucleus contains proteins LSm2–8, the cytoplasmic LSm complex contains the proteins LSm1–7 and, at least in yeast, the decapping factors Dcp1 and Pat1/Mrt1 (Bouveret et al., 2000; Tharun et al., 2000). Two-hybrid assays evidence an interaction between LSm4 and LSm1, and also between LSm4 and LSm8 (Uetz et al., 2000). Our finding that SMN interacts with LSm4 in vitro, raises the interesting possibility that it may also regulate the association of LSm4 to LSm1 or LSm8 by way of its binding to the sDMA residues in vivo. sDMA and SMN could thus be important in mRNA turnover as well as in the maturation of UsnRNPs.

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Possible role of symmetrical dimethylarginines as determinants in the pathogenesis of SMA

In this work, we provide evidence that sDMAs may be important for the pathogenesis of SMA, a neurodegenerative disorder caused by mutations in SMN. Because of the participation of SMN in the biogenesis of UsnRNPs it is generally assumed that the pre-mRNA-splicing apparatus of SMA patients is dysfunctional (for review, see Jablonka et al., 2000). Our demonstration of an in vitro interaction between SMN and LSm4, mediated by sDMA residues, introduces additional possible explanations of the pathogenesis of SMA. Symmetrical dimethylarginines and SMN can be involved both in the biogenesis of UsnRNPs and in the decay of mRNA (see above). Nerve cells could be more severely affected by such defects in the RNA-processing machinery than other cell types. The assumption of such a dosage effect is supported by the observation that the severity of the disease is correlated with the quantity of SMN expressed in SMA patients (Lefebvre et al., 1997). Therefore, to understand this pathogenesis more fully, it will be necessary to clarify whether SMN interacts with LSm4 also in vivo and to investigate the effect of SMN mutations on the biogenesis of UsnRNPs and mRNA decay in different cell types.

MATERIALS AND METHODS

Purification, mass spectrometry, and protein sequencing of human Sm B/B′ and LSm4

B/B′ proteins

HeLa UsnRNPs were purified by anti m_3G-cap immunoaffinity chromatography from nuclear extracts and subsequently fractionated by high-TEMED SDS-PAGE (Lehmeier et al., 1990). Coomassie blue-stained bands of B/B′ were electroeluted and digested with trypsin: 140 μg B/B′ were dissolved in 50 μL of a 100 mM Tris-HCl, pH 8.0/10% acetonitrile solution and incubated with 3.6 μg of trypsin (Promega) at 37 °C overnight. The resulting peptide mixture was fractionated by HPLC on a Vydac C4 (1.00 × 250 mm) column as described previously (Brahms et al., 2000). Manually recovered peptide fractions were then sequenced by Edman degradation using a Procise 492 sequencer equipped with an on-line 140C phenylthiohydantoin analyzer (Perkin Elmer). To distinguish between sDMA, aDMA, and MMA, combinations of these were applied as standards in a first sequencing cycle (Brahms et al., 2000).

LSm4

A human tri-UsnRNP-associated LSm-complex was purified (Achsel et al., 1999). Briefly, tri-UsnRNPs were purified by anti m_3G-cap immunoaffinity chromatography of HeLa nuclear extracts and subsequent glycerol-gradient centrifugation. An RNA-free complex of the proteins LSm2 to LSm8 was obtained from tri-UsnRNPs by glycerol-gradient centrifugation under high-salt conditions (700 mM NaCl) and subsequent MonoS chromatography. The LSm proteins were separated by SDS-PAGE and a Coomassie blue-stained band containing LSm3 and LSm4 was excised. After in-gel digestion with endoLysC, 10% of the digest was used directly for MALDI-MS (Voyager DE STR, Applied Biosystems), using α-cyano-hydroxycinnamic acid as matrix. The rest of the digest was separated on a C4 Vydac column (see above) and the resulting peptide peaks were sequenced by Edman degradation.

Peptide synthesis and conjugation to BSA

Peptides with the sequence CGRGRGRGRGRGRGRGRG containing symmetrically [designated as $(sDMA\text{-}G)_9$], asymmetrically [$(aDMA\text{-}G)_9$], or nonmodified [$(RG)_9$] arginines were synthesized by Michael Krause at the Institut für Molekularbiologie und Tumorforschung in Marburg, Germany. Fmoc-protected sDMA and aDMA derivatives for peptide synthesis were purchased from Bachem, Heidelberg, Germany. BSA–peptide conjugates were produced by crosslinking the $(RG)_9$, $(aDMA\text{-}G)_9$, and $(sDMA\text{-}G)_9$ peptides to BSA (Sigma) via their N-terminal cysteine. The crosslinking reaction with SulfoSMCC (Pierce) was performed according to the manufacturer's instructions.

Pull-down assays and competition studies

$SMN_{1–160}$ and $SMN_{1–160}$-E134K GST-fusion proteins were prepared as described previously (Bühler et al., 1999). In vitro translation reactions were carried out in rabbit reticulocyte lysate (Promega) using as templates D1, D3, B′, and LSm4 mRNAs prepared by transcription in vitro (Raker et al., 1996; Achsel et al., 1999). To inhibit cotranslationally arginine methylation, 0.4 mM S-adenosylhomocysteine (Sigma) were included in the in vitro translation reaction.

GST-SMN constructs (250 ng per assay with in vitro-translated proteins, 1 μg for pull-downs with UsnRNPs) were coupled to glutathione Sepharose (20 μL per assay) for 2–4 h at 4 °C in a head-over-tail rotor. After three washes with SMN binding buffer (20 mM HEPES-KOH, pH 7.9, 200 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% IGEPAL CA-630 (Sigma), 0.5 mM DTE, 0.25 mM PMSF) the beads were incubated with the indicated amounts of peptides for 30 min in 250 μL SMN binding buffer at 4 °C on a head-over-tail rotor (for the competition experiments). Subsequently, 3–10 μL of Sm proteins (approximately 10–20 fmol) prepared by translation in vitro or 10 μg of affinity-purified HeLa UsnRNPs were added, and incubation was continued for 1 h. After five washes with SMN binding buffer, the beads were dried, resuspended in protein loading buffer (50 mM Tris-HCl, pH 6.8, 50 mM DTT, 10% glycerol, 2% SDS, 0.025% bromphenol blue), fractionated by SDS-PAGE, and analyzed by fluorography. Precipitated snRNPs were analyzed by phenol/chloroform extraction of the beads and subsequent 3′-end labeling of the coprecipitated UsnRNAs with pCp (as described in Brahms et al., 1997).

Methylation in vitro

A recombinant His_6-TNF-D1 fusion protein was obtained, as described previously (Brahms et al., 2000). Preparation of BSA–peptide conjugates is described above.

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Routinely, a total volume of 25 μL was used in each methylation assay. A typical reaction mixture consisted of 40% (v/v) HeLa nuclear extract (Dignam et al., 1983) or 40% (v/v) HeLa S100 extract (HeLa cytosolic extract in 20 mM HEPES–KOH, pH 7.9, 3 mM $MgCl_2$, 150 mM KCl, 1 mM DTT, 0.25 mM PMSF, centrifuged at 100,000 $\times$ *g* for 2 h), 125 nCi of [^{14}C]-S-adenosylmethionine (Amersham/Pharmacia), 50 mM HEPES–KOH, pH 7.9, and 500 ng His_6-TNF-D1 or BSA–peptide conjugate. The reaction mixtures were incubated at 30 °C for 2 h and either directly subjected to SDS-PAGE or further analyzed in pull-down assays using glutathione–Sepharose-immobilized $SMN_{1–160}$ (1 μg/assay) or protein G–Sepharose immobilized mAb Y12 (procedure for pull-down: see above). Incorporation of [^{14}C]-methyl groups was detected by SDS-PAGE and subsequent fluorography using a Molecular Dynamics Phosphorimager.

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