SMN, the Product of the Spinal Muscular Atrophy Gene, Binds Preferentially to Dimethylarginine-Containing Protein Targets

Westley J. Friesen, Severine Massenet, Sergey Paushkin, Anastasia Wyce, and Gideon Dreyfuss
Howard Hughes Medical Institute and Department of Biochemistry and Biophysics University of Pennsylvania School of Medicine Philadelphia, Pennsylvania 19104

Summary

The survival of motor neurons protein (SMN), the product of the neurodegenerative disease spinal muscular atrophy (SMA) gene, functions as an assembly factor for snRNPs and likely other RNP complexes. SMN binds to the arginine- and glycine-rich (RG) domains of the snRNP protein SmD1 and SmD3. Specific arginines in these domains are modified to dimethylarginines, a common modification of unknown function. We show that SMN binds preferentially to the dimethylarginine-modified RG domains of SmD1 and SmD3. The binding of other SMN-interacting proteins is also strongly enhanced by methylation. Thus, methylation of arginines is a novel mechanism to promote specific protein-protein interactions and appears to be key to generating high-affinity SMN substrates. It is reasonable to expect that protein hypomethylation may contribute to the severity of SMA.

Introduction

The neuromuscular disease spinal muscular atrophy (SMA) is characterized by degeneration of motor neurons of the spinal cord resulting in muscular weakness and atrophy (Melki, 1997). The survival motor neuron gene (SMN) is present as an inverted repeat on chromosome 5 at 5q13, and over 98% of SMA patients have deletions or mutations of the telomeric copy of the gene (SMN1), resulting in lower levels of SMN protein (LeFebvre et al., 1995) and reviewed in (Burghes, 1997). The SMN protein is found in all metazoan cells in both the nucleus and cytoplasm and is part of a large multiprotein complex. The thus-far-identified SMN complex components include Gemin2 (formerly SIP1) (Liu et al., 1997), the DEAD box RNA helicase Gemin3 (Charroux et al., 1999), and Gemin4 (Charroux et al., 2000). SMN oligomerizes, and its oligomerization greatly enhances its binding to the core snRNP Sm proteins SmB, -D1, and -D3 (Pellizzoni et al., 1999). Several SMN mutants found in SMA patients are defective in oligomerization and in Sm protein binding (Lorson et al., 1998; Buhler et al., 1995; Pellizzoni et al., 1999). All of the seven Sm proteins (B, D1, D2, D3, E, F, and G) that constitute the common core of spliceosomal snRNPs have a conserved Sm domain (Hermann et al., 1995; Seraphin, 1995). SmD1 and SmD3, in addition, have carboxy-terminal arginine- and glycine-rich (RG) domains which are necessary and sufficient for SMN binding (Friesen and Dreyfuss, 2000).

Recently, it was shown that specific arginine residues within these RG domains contain posttranslationally modified symmetrical dimethylarginines (sDMAs), and this modification apparently occurs in the cytoplasm (Brahms et al., 2000). SnRNPs are essential components of the pre-mRNA splicing machinery, and their Sm cores are assembled in the cytoplasm (Luhrmann, 1990). Experiments in Xenopus oocytes have shown that the SMN complex plays a crucial role in snRNP assembly (Fischer et al., 1997; Buhler et al., 1999). In somatic cells, transfection of a dominant-negative SMN mutant deleted of 27 amino terminal amino acids (SMNΔN27) blocks snRNP assembly in the cytoplasm and also inhibits splicing (Pellizzoni et al., 1998) and transcription (Pellizzoni et al., 2001).

Posttranslational methylation of arginines can form monomethylarginine, asymmetrical dimethylarginine (aDMA), or symmetrical dimethylarginine (sDMA) (reviewed by Gary and Clarke, 1998). The aDMA appears to be the more common form (Gary and Clarke, 1998) while sDMA has so far been identified only in a few proteins, including myelin basic protein (MBP) (Baldwin and Carnegie, 1971), SmD1, and SmD3 (Brahms et al., 2000). It is becoming increasingly obvious that many, if not all, RG- and RGG repeat-containing proteins are substrates for protein arginine methyltransferases and receive dimethylation of arginines (Liu and Dreyfuss, 1995; Henry and Silver, 1996; Gary and Clarke, 1998; Brahms et al., 2000). Despite the fact that proteins have been shown to contain dimethylarginines over thirty years ago (Paik and Kim, 1967; Paik and Kim, 1968), little is known about the molecular functions of this post-translational modification. Dimethylation of hnRNP proteins has been suggested to play a role in their nuclear export (Shen et al., 1998), and the methyltransferases CARM1 and PRMT1 are involved in transcriptional activation by a nuclear hormone receptor (Chen et al., 1999). PRMT1 may also have a role in the regulation of interferon signaling pathways (Abramovich et al., 1997). Here, we demonstrate that arginine methylation can enhance and regulate protein-protein interactions. We show that SMN, a protein that plays a critical role in snRNP core particle assembly in vivo, binds preferentially to the sDMA-modified forms of SmD1 and SmD3 as well as to other methylated protein substrates.

Results

We have previously shown that the carboxy-terminal RG domains of SmD1 and SmD3 are necessary and sufficient for in vitro binding of these proteins to SMN (Friesen and Dreyfuss, 2000). To further investigate the interaction of these regions, HeLa cell extract was subjected to affinity chromatography with immobilized glutathione-S-transferase (GST) fused to full-length SmD1 (GST-D1), the carboxy-terminal 29 amino acids of SmD1 (GST-D1c29), full-length D3 (GST-D3), and the carboxy-terminal 32 amino acids of SmD3 (GST-D3c32). GST
fused to the SMN binding protein Gemin2 (GST-Gemin2) was used as a positive control, and GST alone was used as a negative control. Retained proteins were resolved by SDS-PAGE, Western blotted onto a membrane, and probed with the SMN-specific antibody 2B1 (Figure 1A). Despite the fact that recombinant SMN binds directly to these recombinant Sm proteins and carboxy-terminal fragments, none of them bound SMN in HeLa cell extract. Bacterially produced SmD1 and SmD3 do not contain the sDMAs found in the RG domains of mammalian SmD1 and SmD3 (Brahms et al., 2000), suggesting that in the context of native cell extract sDMA posttranslational modification may be important for Sm protein association with the SMN complex. To directly test this, peptides corresponding to the carboxy-terminal 29 and 32 amino acids of SmD1 and SmD3, respectively, without (Biotin-D1c29 and Biotin-D3c32) or with (Biotin-D1c29-sDMA and Biotin-D3c32-sDMA) the specific sDMA modifications formed in vivo, were synthesized (Figure 1B). As an additional control a D3c32 peptide (Biotin-D3c32-aDMA) with aDMA in place of sDMA was also synthesized. Each peptide or biotin alone was immobilized on streptavidin sepharose and used for binding of HeLa cell extract. After extensive washing of the beads and SDS-PAGE, the amount of SMN bound was determined by Western blotting (Figure 1C). Strikingly, only the symmetrically methylated RG peptides bound SMN, indicating that native SMN binds preferentially to symmetrically dimethylated SmD1 and SmD3 in cell extracts.

To further investigate SMN binding to sDMA-modified targets, His-tagged SMN bound to Gemin2 (Gemin2/His-SMN) was tested for direct interaction with immobilized SmD3 peptides (Figure 2A). Gemin2/His-SMN was used for these experiments rather than SMN alone because Gemin2 stabilizes and solubilizes recombinant SMN in its oligomeric form, and Gemin2 does not bind to Sm proteins (Charroux et al., 1999, 2000; Pellizzoni et al., 1999). As measured by Western blotting to detect protein association with the SMN complex, significantly more SMN bound to the sDMA-modified RG peptide than to the unmethylated RG peptide, demonstrating that SMN has higher affinity for the sDMA-containing form of SmD3. To obtain a more quantitative estimate of the difference in SMN’s affinity for sDMA-modified versus unmodified RG peptide, increasing amounts of immobilized GST-Gemin2/His-SMN were incubated with a constant amount of each peptide which had been radiolabeled by preincubation with [35S]streptavidin (Figure 2B). All of the SMN amounts tested bound significantly more of the sDMA-modified RG peptide than the unmodified RG peptide. Immobilized SMN (8.1 nanomoles) bound 16-fold more
SMN Binds Preferentially to Methylated Proteins

Figure 2. SMN Binds Methylated SmD3 with Higher Affinity Than Unmethylated SmD3

(A) 0.75 nanomole of the indicated streptavidin sepharose-immobilized peptides or biotin was incubated with Gemin2/His-SMN. After washing, retained His-SMN (arrow) was detected with anti-T7 tag antibody. The total lane shows 50% of the recombinant protein used in each binding.

(B) The indicated amounts of GST-Gemin2-immobilized His-SMN (nanomoles) were incubated with 2.6 nanomoles of each peptide, which had been radiolabeled with [35S]streptavidin. Following washing, the amount of retained [35S]-labeled peptide (nanomole) was measured by scintillation counting. Black bars are for SMN binding to D3c32-sDMA, and white bars are for SMN binding to D3c32. Error bars represent standard error of the mean for at least three measurements.

(C) 293 cells transiently expressing myc-D3 were either treated (+) or not treated (-) with the protein methyltransferase inhibitor Adox. Cytoplasmic extracts were immunoprecipitated with the anti-SMN monoclonal antibody 2B1 (α-SMN) and nonimmune antibody (SP2/0) as indicated, and retained proteins were Western blotted to detect the indicated proteins (arrows). The total lanes show 10% of the extract used in each immunoprecipitation.

Radiolabeled sDMA-modified RG peptide than radiolabeled unmodified RG peptide, demonstrating that under these conditions SMN has at least a 16-fold higher affinity for the modified RG peptide over the unmodified RG peptide. It is likely that the difference in binding affinity is actually higher, but the conditions for this assay preclude using higher volumes of beads. Taken together, the results presented in Figures 1 and 2A and 2B demonstrate that the methylation state of Sm proteins affects their ability to interact with the SMN complex. These data demonstrate that protein-protein interactions can be regulated by arginine methylation.

To examine the role of methylation on SmD3 interaction with the SMN complex in vivo, myc-tagged SmD3 (myc-D3) was expressed by transient transfection in 293 cells. After transfection, cells were either treated with the protein methyltransferase inhibitor, periodate oxidized adenosine (Adox), or left untreated. Treatment of cells with Adox produces lysates which contain undermethylated proteins (Najbauer and Aswad, 1990; Li et al., 1998). Cytoplasmic cell extracts were produced from each cell population, and anti-SMN-specific antibody or nonimmune antibody (SP2/0) immunoprecipitates were prepared and detected by Western blotting with SMN, Gemin2, and myc-D3-specific antibodies (Figure 2C). We did not use anti-Sm monoclonal antibodies to detect native Sm proteins because the methylation state of SmD1 and SmD3 (and therefore possibly SmB) has been shown to affect their reactivity with these antibodies (Brahms et al., 2000). Anti-SMN antibody (2B1) immunoprecipitated similar amounts of SMN and Gemin2 from untreated or Adox-treated cells. In contrast, 2B1 coimmunoprecipitated significantly less myc-D3 from Adox-treated cells than from untreated cells, supporting the conclusion that arginine methylation of SmD3 is required for (or at least strongly enhances) its interaction with SMN.

In addition to Sm proteins, SMN interacts with many other RGG domain-containing proteins, including fibrillarin, hRNP U (Liu and Dreyfuss, 1996), and RNA helicase A (Pellizzoni et al., 2001), two of which have been shown to receive dimethylation of arginines (Christensen and Fuxa, 1988; Liu and Dreyfuss, 1995). Thus, we considered it likely that preference for dimethylated arginine-modified targets (asymmetrical as well as symmetrical DMA) could extend to other SMN binding proteins. To test this, 293 cells were treated with the methyltransferase inhibitor Adox or left untreated. Both treated and untreated cells were metabolically labeled with [35S]methionine. Total extracts prepared from these cells were then incubated with GST alone or His-SMN bound to GST-Gemin2. Following extensive washing, SMN-bound proteins were resolved by SDS-PAGE and visualized by fluorography (Figure 3A). GST-Gemin2 shows no binding and serves to better present SMN (data not shown). Four proteins, p120, p100, p72 and p22, which bound SMN when in their normal methylation state (no Adox treatment), had dramatically reduced SMN binding when in a hypomethylated state (Adox treatment). To assure that proteins with reduced SMN binding after Adox treatment (hypomethylated state) are indeed capable of receiving methylation, total cell extract prepared from Adox-treated 293 cells was labeled with the methyl donor S-adenosyl-L-[methyl-3H]methionine (3H-SAM) and bound to GST alone or to His-SMN immobilized on GST-Gemin2. The SMN bound, 3H-labeled proteins were resolved by SDS-PAGE and visualized by fluorography (Figure 3B). Four bands of the same molecular mass as those whose SMN binding is reduced by hypomethylation (p120, p100, p72, and p22) were observed, demonstrating that these four proteins do indeed receive methylation. These results demonstrate that, in addition to SmD1 and SmD3, SMN binds preferentially to other proteins after they are posttranslationally methylated.

Discussion

The results presented here establish the mechanism by which SMN, the product of the SMA disease gene, recognizes its targets, and demonstrate a novel molecular function for dimethylation of arginines. SMA is one
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can serve as an entirely novel mechanism to promote specific protein-protein interactions. The dimethylated arginines must form a unique surface to mediate the interaction of RG domains with SMN, constituting a novel mode of interaction between the surfaces of two proteins. Many of the other substrates of SMN have RG domains similar to the two substrates we analyze here in detail, SmD1 and SmD3 (Liu and Dreyfuss, 1996; Pellizzoni et al., 2001). We suggest that arginine methylation is, therefore, a key to substrate recognition by the SMN complex.

SMN can discriminate between the RG domains from different proteins (Friesen and Dreyfuss, 2000), and arginine methylation now emerges as a new mechanism for enhancing SMN substrate selection. Our view that protein arginine methylation is a general mechanism by which high-affinity Sm protein substrates are generated and targeted to the SMN complex is presented in Figure 4. It illustrates our understanding of the role of methylation of Sm proteins in promoting their interaction with SMN. This is presently the best-characterized interaction of SMN with protein substrates, but we suggest that the same general considerations apply to SMN’s interactions with its many other RGG-containing targets (Liu and Dreyfuss, 1996; Pellizzoni et al., 2001). Except for a few proteins, in which the methylation results in sDMAs, the majority of RGG-containing proteins receive the more common aDMAs (Gary and Clarke, 1998). Given the results shown in Figure 3, we consider it likely that SMN also interacts preferentially with many proteins that receive the asymmetric modification. The DMA modification of SmD3 and SmD1 (and possibly SmB) targets these proteins to the SMN complex, which then assembles them, along with the other Sm proteins (which also bind to the SMN complex), on snRNA to form an snRNP core particle (Figure 4). By regulating the binding of an Sm protein to the SMN complex, it appears likely that Sm protein methylation serves to regulate snRNP core particle assembly. According to this view, protein arginine methyltransferases serve as key regulators of RNP assembly processes, providing an entry point for regulatory signals, akin to the role of protein kinases.

Interestingly, SMN in cell extracts discriminates more effectively, and apparently strictly, between the sDMA-modified and unmodified SmD3 RG peptides than recombinant SMN, such that it does not display any measurable binding to the unmodified RG peptide (compare Figure 1C and Figure 2A). It is possible that SMN from mammalian cells may be more correctly folded or receive a posttranslational modification that could account for the observed difference in binding. Alternatively, other components of the SMN complex (e.g., Gemin3, Gemin4, etc.) may increase SMN’s ability to distinguish between sDMA-modified and unmodified Sm proteins. It is also conceivable that there is another protein (or proteins) which preferentially binds to the unmodified form of the RG peptide, effectively competing with SMN. Although it is of lower affinity, the binding of recombinant SMN to unmethylated recombinant RG peptide or in vitro translated SmD3 permitted the identification of the RG domain as the binding region for SMN (Friesen and Dreyfuss, 2000). The key role of methylation in the interaction of Sm
Figure 4. Arginine Methylation of Sm Proteins Is Important for Their Binding to the SMN Complex and Subsequent Inclusion into snRNP Core Particles

Schematic depicting the posttranslational sDMA modification of the SmD1 and SmD3 RG-rich domains by a yet-to-be-identified protein arginine methyltransferase. After sDMA modification, SmD3, SmD1, and possibly SmB associate with the SMN complex and along with the other Sm proteins are assembled on snRNA to form an snRNP core particle.
strate recognition by the SMN complex, and that the processes of RNP assembly may be regulated by protein arginine methyltransferases.

Experimental Procedures

Affinity Chromatography and Recombinant Protein Purification and Binding

Affinity chromatography and binding were done with Glutathione-S-transferase (GST) fusion proteins (3 μg for Sm proteins and fragments or 5 μg of His-SMN bound on GST-Gemin2) immobilized on Glutathione Sepharose 4B (Amer sham) with 100–150 μl (3–4 mg protein) of cell extract or 200–300 ng of His-SMN/Gemin2 in binding buffer (50 mM Tris [pH 7.5], 200 mM NaCl, 0.2 mM EDTA, 0.05% NP-40, 2 mM DTT, and protease inhibitor cocktail) at 4°C. After incubation for 1–3 hr, binding reactions were washed five times with 1.25 ml binding buffer. For radiolabeled peptide binding to immobilized SMN, peptides were radiolabeled by preincubation for 15 min with [35S]Streptavidin (Amersham) (2.6 nanomoles of peptide per 26 nanomoles [35S]streptavidin).

Increasing amounts of His-SMN immobilized on GST-Gemin2 were incubated with 2.6 nanomoles of peptide in 500 μl of binding buffer for 30 min at 4°C. After washing, reactions were extracted in 5 ml of EcoLette scintillation fluid (ICN) and counted in a Packard Tri-Carb 2100TR Scintillation counter. Gemin2 alone retained less than 0.025 nanomole of either peptide, and the highest amount of SMN used (8.1 nanomoles) retained less than 0.025 nanomole [35S]streptavidin-labeled biotin (data not shown). Sigma Genosys synthesized the SmD3 peptides, and the SmD1 peptides were synthesized by the Protein Chemistry Laboratory of the Medical School of the University of Pennsylvania supported by core grants of the Diabetes and Cancer Centers (DK-19525 and CA-16520). BL21 cells expressing GST-Gemin2 and His-SMN were resuspended in 20 ml of purification buffer (20 mM Tris [pH 7.5], 300 mM NaCl, 0.2 mM EDTA, and protease inhibitor cocktail) lysed by sonication, and purified on 750 μl Glutathione Sepharose 4B (Amer sham) according to the manufacturer’s recommendation. His-SMN/Gemin2 was cleaved off the beads by incubation overnight at 4°C with 10 U TEV protease (GIBCO-BRL) per 3 μg of GST-Gemin2.

Cell Culture and Extract Preparation

For transient expression of myc-tagged SmD3 (myc-D3), 293 cells were transfected with 5 μg mycD3pcDNA3 using the CalPhos Mammalian Transfection Kit (Clontech Laboratories) according to the manufacturer’s recommendation. Cells were treated with 100 μM periodate-oxidized adenosine (Adox) (Sigma) for 20 hr, 32 hr after transfection. Cells were labeled with 20 μCi per ml of [35S]methionine (Amersham) during Adox treatment. Cell fractionation was done as described (Sioni et al., 1997).

Immunoprecipitation and Western Blotting

Immunoprecipitation, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting were done as previously described (Liu et al., 1997). Antibodies used were monoclonal anti-SMN (2B1) (Liu and Dreyfuss, 1996); monoclonal anti-Gemin2 (2E17) (Liu et al., 1997); monoclonal anti-myc (9E10); and monoclonal anti-T7 tag (Invitrogen).

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