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# A Novel WD Repeat Protein Component of the Methylosome Binds Sm Proteins*

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**We have recently described a large (20 S) protein arginine methyltransferase complex, termed the methylosome, that contains the methyltransferase JBP1 (PRMT5) and the pICln protein. The methylosome functions to modify specific arginines to dimethylarginines in the arginine- and glycine-rich domains of several spliceosomal Sm proteins, and this modification targets these proteins to the survival of motor neurons (SMN) complex for assembly into small nuclear ribonucleoprotein (snRNP) core particles. Here, we describe a novel component of the methylosome, a 50-kilodalton WD repeat protein termed methylosome protein 50 (MEP50). We show that MEP50 is important for methylosome activity and binds to JBP1 and to a subset of Sm proteins. Because WD repeat proteins provide a platform for multiple protein interactions, MEP50 may function to mediate the interaction of multiple substrates with the methylosome. Interestingly, all of the known components of the methylosome bind Sm proteins, suggesting that in addition to producing properly methylated substrates for the SMN complex, the methylosome may be involved in Sm protein rearrangements or pre-assembly required for snRNP biogenesis.**

Each spliceosomal snRNP[1] contains a common core comprising an snRNA (U1, U2, U4, or U5) and a seven-member ring of Sm proteins (B, D1, D2, D3, E, F, and G). snRNP cores assemble in the cytoplasm, and the mature snRNPs are subsequently imported into the nucleus where they function in nuclear pre-mRNA splicing (1, 2). The Sm proteins form head-to-tail into a ring structure around the Sm site of the snRNA, which is bound in the center of the ring with at least SmG and SmB directly contacting the RNA (3–6). Although snRNP cores can assemble spontaneously from purified, concentrated components *in vitro* (7, 8), it is becoming increasingly clear that the *in vivo* snRNP assembly pathway is complex and involves two large complexes, the survival of motor neuron (SMN) complex and the methylosome (9–14).

SMN, the spinal muscular atrophy disease gene product, is present in all metazoan cells as a large complex, which, in addition to oligomeric SMN, includes Gemin2 (9), Gemin3 (15), and Gemin4 (16). Reduced levels of or loss-of-function mutations in SMN affect the growth of all cells (17–22) and cause the degeneration of motor neurons, which results in spinal muscular atrophy (23–25). The SMN complex is critical for snRNP core assembly *in vivo* (9–12). The presence of Gemin3, a DEAD box protein putative RNA-dependent ATPase, strongly suggests that the SMN complex plays an active (ATP-dependent) role in snRNP assembly. Direct binding of SMN to the arginine- and glycine-rich (RG) domains of three of the Sm proteins (SmB, SmD1, and SmD3) appears to be required for SMN function in snRNP core assembly (9, 26, 27). Importantly, SMN binds preferentially to these RG domains after they have been post-translationally modified to contain symmetric dimethylarginines (sDMAs) (13).

Protein arginine methyltransferases (PRMTs) produce either asymmetric dimethylarginine (aDMA) (type I enzyme) or sDMA (type II enzyme) and are involved in diverse functions (28–32). The type II PRMT Janus kinase-binding protein 1 or JBP1 (33, 34) (also known as skb1Hs (35), IBP72 (36), and PRMT5 (37)) symmetrically dimethylates Sm protein RG domains (14). We have recently shown that JBP1 is present in and functions in the context of the methylosome, a large 20 S complex that also contains the pICln protein (14). PICln was initially thought to be an ion channel regulator (38) but subsequently has been shown to bind Sm proteins (39) and function in snRNP assembly as a component of the methylosome (14). A fraction of the cytoplasmic RG domain-containing Sm proteins are bound to the methylosome *in vivo*. JBP1 binds Sm protein RG domains, whereas pICln binds the Sm domain (14). PICln is a phosphoprotein (40) and is found also in smaller complexes (4–6 S), at least some of which also contain Sm proteins, suggesting that pICln may regulate Sm protein association with the methylosome (14). After sDMA modification by the methylosome, Sm proteins become high affinity substrates for the SMN complex, which likely completes their assembly into snRNPs cores (13, 14).

Here, we describe a novel 50-kilodalton protein component of the methylosome termed MEP50. We show that MEP50, a novel WD repeat protein, binds to JBP1 and Sm proteins and is

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*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF478464 (MEP50).*

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[1] The abbreviations used are: snRNP, small nuclear ribonucleoprotein; SMN, survival of motor neurons; sDMA, symmetric dimethylarginine; MEP50, methylosome protein 50; GST, glutathione *S*-transferase; nano-ES MS/MS, nanoelectrospray tandem mass spectrometry; NMS, normal mouse serum; flag-JBP1, flag-tagged JBP1.

required for methylosome activity. WD repeat proteins provide a large surface area for multiple protein interactions, suggesting that MEP50 functions to mediate the interaction of protein substrates with the methylosome.

## MATERIALS AND METHODS

*MEP50 Isolation, Plasmid, and Antibody Production*—An expressed sequence tag (GenBank™ accession number AL533305) was obtained from Invitrogen (clone Id: CS0DN003YE12) and found to contain an open reading frame (with multiple in-frame stop codons upstream and downstream), which, upon conceptual translation, contained amino acid sequences that exactly matched all of the peptide sequences obtained by nanoelectrospray tandem mass spectrometry (nano-ES MS/MS) (41). This open reading frame was PCR amplified and cloned into pGV67 (14) (to produce pGV67MEP50) for production of GST-MEP50 fusion protein and into mycpcDNA3 (42) (to produce myc-MEP50pcDNA3) for *in vitro* production of Myc-tagged MEP50 (myc-MEP50). Similarly, constructs for the expression of the GST-MEP50 N terminus (amino acids 1–75) and GST-MEP50 WD domain (amino acids 76–342) were made by inserting the appropriate MEP50 cDNA fragments into pGEX6p2 (Amersham Biosciences, Inc.). All Sm protein constructs used for *in vitro* protein production have been previously described (26). The pICln cDNA was PCR amplified and subcloned into pET28a(+) (Novagen) for *in vitro* protein production, and into pGEX5x (Amersham Biosciences, Inc.) for GST-pICln fusion protein production. JBP1 cDNA (35) was PCR amplified and subcloned into pGV67 for GST-JBP1 fusion protein production. The recombinant proteins GST-D3 and His-SmB were produced as described previously (14, 27). Anti-JBP1 antibody 6G8 was prepared by immunizing Balb/C mice with a C-terminal domain of JBP1 (amino acid 284–637) purified from pGV67 on glutathione-Sepharose and cleaved off the beads with TEV (tobacco etch virus) protease (Invitrogen). Hybridoma production, screening, and ascites fluid production were performed as described previously (43). Polysera against MEP50 were obtained by immunizing Balb/C mice with MEP50 protein purified from pGV67MEP50 on glutathione-Sepharose and cleaved off the beads with TEV protease.

*In Vitro Binding—In vitro* translated [$^{35}$S]methionine-labeled proteins (8 $\mu$l) were incubated with glutathione-Sepharose (Amersham Biosciences, Inc.) immobilized GST fusion proteins (2–3 $\mu$g) in 1 ml of binding buffer (20 mM Tris, pH 7.5, 200 mM NaCl, 0.2 mM EDTA, 0.05% Nonidet P-40, 2 mM dithiothreitol, and 1 tablet/50 ml of Complete EDTA-free protease inhibitor mixture (Roche Molecular Biochemicals)) for 1–2 h at 4 °C. Following five washes with 1 ml of binding buffer, bound proteins were separated by SDS-PAGE and detected by fluorography. GST fusion proteins were purified on glutathione-Sepharose 4B (Amersham Biosciences, Inc.) according to the manufacturer's recommendation.

*Mammalian Cell Culture, Sucrose Gradient Centrifugation, and Immunoprecipitation*—HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cytoplasmic extract was prepared as described previously (44). Sucrose gradient centrifugation, fraction collection, and immunoprecipitation were done as described previously (14). Antibodies used in these experiments were anti-pICln (Transduction Laboratories), anti-Myc (9E10), anti-JBP1 (6G8), and anti-MEP50 polysera.

*Methylosome Activity*—The methylosome was prepared and used to methylate recombinant Sm protein substrates as described previously (14). Approximately 100 ng of purified methylosome (4 $\mu$l) was incubated with 2 $\mu$l of adenosyl-L-[*methyl*-$^3$H]methionine (Amersham Biosciences, Inc.), 3 $\mu$l (600 ng) of recombinant GST-SmD3 or His-tagged SmB (His-SmB), and 21 $\mu$l of phosphate-buffered saline at 30 °C for 45 min. Reactions were supplemented with 5 $\mu$l of anti-MEP50 serum, 5 $\mu$l of normal mouse serum (NMS), or 5 $\mu$l of anti-MEP50 serum that had been pre-incubated with 1.5 $\mu$g of immobilized GST-MEP50 or 5 $\mu$l of anti-MEP50 serum that had been pre-incubated with 1.5 $\mu$g of immobilized GST. After methylation, the mixtures were boiled in SDS sample buffer and resolved by SDS-PAGE. The gels were then treated with Amplify (Amersham Biosciences, Inc.), dried, and exposed to film. The MEP50 GenBank™ accession number is AF478464.

## RESULTS

We have recently shown that the methylosome, which contains the methyltransferase JBP1 and the pICln protein, functions in the cytoplasm to symmetrically dimethylate specific arginines in the RG domains of the Sm proteins. To further study this complex, we produced monoclonal antibodies against JBP1. Mice were immunized with a recombinant protein fragment of JBP1 (amino acid 284–637), and a hybridoma, 6G8, was selected for further analysis (43). To characterize 6G8, *in vitro* produced and radiolabeled flag-tagged JBP1 (flag-JBP1) was incubated with immobilized 6G8 ascites fluid, nonimmune ascites fluid (SP2/0), and NMS. Immunoprecipitated JBP1 was resolved on SDS-PAGE and visualized by fluorography. The 6G8 monoclonal antibody specifically immunoprecipitated JBP1 (Fig. 1*A*). Western blot of HeLa extract revealed that 6G8, but not NMS, detected a protein of about 70 kilodaltons, the expected size of JBP1 (Fig. 1*B*). Thus, 6G8 is a monoclonal antibody that specifically recognizes JBP1.

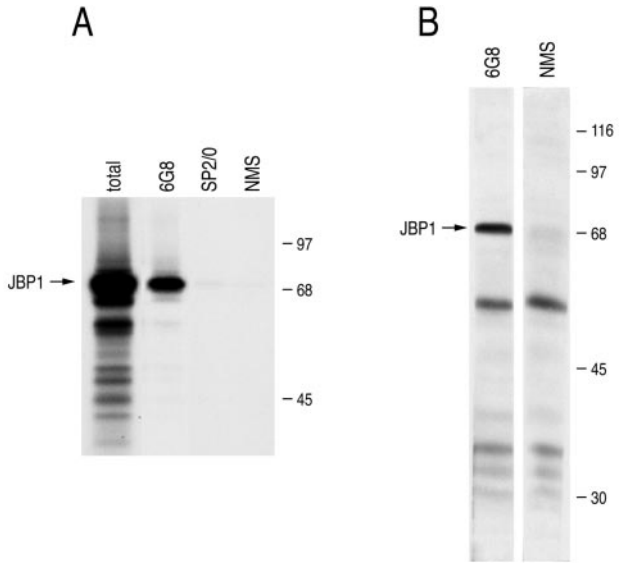
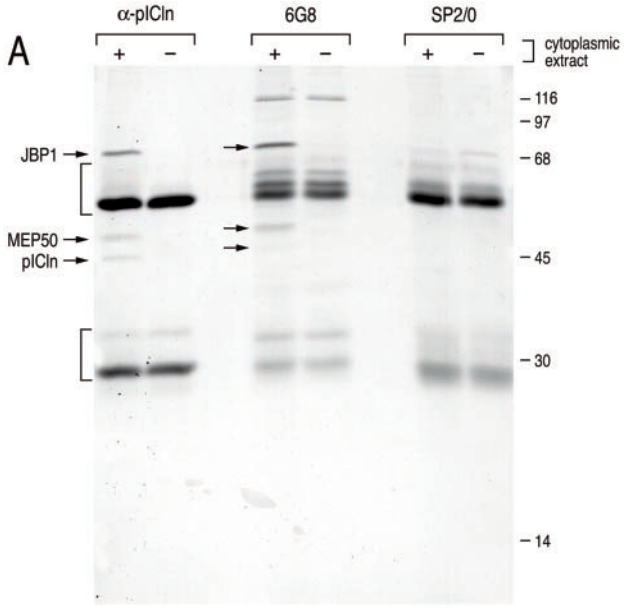



FIG. 1. **6G8 is a specific anti-JBP1 monoclonal antibody.** *A*, *in vitro* produced and radiolabeled JBP1 was incubated with immobilized 6G8 ascites (*6G8*), nonimmune SP2/0 ascites (*SP2/0*), or NMS. Following extensive washing, bound JBP1 was eluted with SDS sample buffer, resolved by SDS-PAGE, and visualized by fluorography. The "*total*" *lane* shows 10% of the protein used in each immunoprecipitation. *B*, HeLa cell lysate was resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with 6G8 ascites or normal mouse serum.

To identify and isolate components of the methylosome, we performed preparative immunoprecipitations from HeLa cytoplasmic extract with anti-JBP1 (6G8) and anti-pICln antibodies ($\alpha$-pICln). After extensive washing, the immunoprecipitates were resolved by SDS-PAGE and stained with Coomassie Blue (Fig. 2*A*). Both anti-JBP1 and anti-pICln antibodies immunoprecipitated JBP1 and pICln as well as a protein of about 50 kilodaltons (MEP50). This 50-kilodalton band was excised from the gel, and five peptide sequences, which did not match any known proteins, were obtained by nano-ES MS/MS (41) (Fig. 2*B*). An expressed sequence tag (GenBank™ accession number AL533305) was found to contain an open reading frame (with several stop codons upstream and downstream) that matched all of the peptide sequences (Fig. 2*B*). Although MEP50 encodes a polypeptide of about 36.7 kilodaltons, it migrated above the 45-kilodalton molecular mass marker by SDS-PAGE (Fig. 2*A* and see below). This is similar to pICln, a protein of about 26.2 kilodaltons that migrates near the 45-kilodalton molecular mass marker. This discrepancy may be due, at least in part, to the fact that pICln and MEP50 are both acidic proteins with a pI of 3.77 and 4.84, respectively. Sequence analysis on the Protein Sequence Analysis server (bmerc-www.bu.edu/psa/) revealed that MEP50 contains six WD repeats (Fig. 2*B*) (45, 46).

To further investigate MEP50 as a potential methylosome component, mice were immunized with the MEP50 protein. Using sera from these mice we detected recombinant MEP50



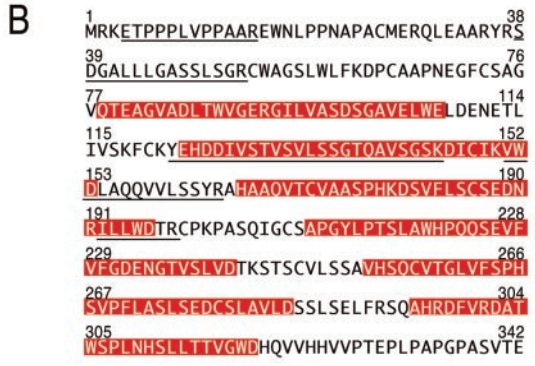




FIG. 2. **Identification of MEP50.** *A*, the indicated immobilized antibodies were used for immunoprecipitation with (+) or without (−) total HeLa extract. Following extensive washing, proteins were resolved by SDS-PAGE and visualized by Coomassie Blue staining. Immunoprecipitated proteins are indicated by *arrows*, and antibody bands are indicated by *brackets*. The positions of the molecular weight markers are indicated on the *right*. Peptide sequences were obtained by nano-ES MS/MS (41). *B*, sequence of MEP50 (GenBank™ accession no. AF478464). *B*, sequence of MEP50. The *underlined* residues correspond to peptide sequences obtained by mass spectrometry analysis, and sequences *outlined* in *orange* are WD repeat domains (see "Results" for detail).

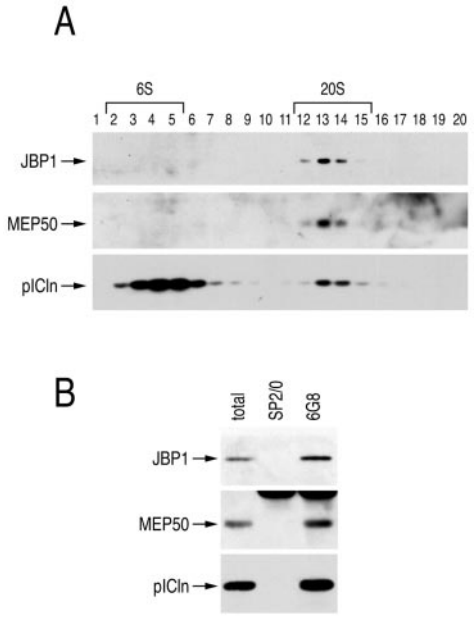



FIG. 3. **MEP50 is a novel component of the methylosome.** *A*, HeLa cytoplasmic extract was prepared and separated on a sucrose gradient. Fractions from the gradient were collected, resolved by SDS-PAGE, and immunoblotted to detect the indicated proteins (*arrows*). *B*, from the gradient fractions immunoblotted in *A*, fractions 12–15 (*20S*) were pooled and immunoprecipitated with anti-JBP1 antibodies (*6G8*) and nonimmune (*SP2/0*) antibodies (as indicated). Proteins were resolved by SDS-PAGE and immunoblotted to detect the proteins indicated by *arrows*.

(data not shown) and MEP50 from HeLa cell extract by Western blotting (Fig. 3*A*). To confirm the association of MEP50 with the methylosome, HeLa cell cytoplasmic extract was prepared and sedimented on a sucrose gradient. Fractions were collected and resolved by SDS-PAGE, and Western blotting was carried out to detect JBP1, pICln, and MEP50. MEP50 sedimented in the same fractions as the methylosome (fractions 12–15) (Fig. 3*A*). Fractions 12–15 containing the methylosome (20 S) were pooled and immunoprecipitated with anti-JBP1 (6G8) antibodies or with nonimmune SP2/0 antibodies. After extensive washing the immunoprecipitates were resolved by SDS-PAGE and immunoblotted to detect JBP1, pICln, and MEP50 (Fig. 3*B*). JBP1, pICln, and MEP50 were detected in anti-JBP1 antibody immunoprecipitate from the 20 S fractions, demonstrating that MEP50 is indeed a novel component of the methylosome. Transiently expressed Myc-tagged MEP50 also associates with the methylosome (data not shown) further confirming that MEP50 is a methylosome component.

We further studied the interactions among the components of the methylosome. *In vitro* produced and radiolabeled JBP1, pICln, and MEP50 were each incubated with immobilized GST fused to JBP1 (GST-JBP1), pICln (GST-pICln), and MEP50 (GST-MEP50) or, as a control, with GST alone. After extensive washing, bound proteins were resolved by SDS-PAGE and visualized by fluorography (Fig. 4). Under our binding conditions, pICln and MEP50 bound JBP1 but did not homo- or hetero-oligomerize. In contrast and as described previously (14, 47), JBP1 did homo-oligomerize. These results further confirm that MEP50 is a *bona fide* component of the methylosome and suggest that oligomerization of JBP1 contributes to the large size of the methylosome.

To test whether MEP50 binds to Sm proteins, the best characterized substrates of the methylosome, *in vitro* produced and radiolabeled SmB, SmD1, SmD2, SmD3, SmE, SmF, and SmG, were each incubated with GST-MEP50, GST-MEP50 WD repeat domain (GST fused to the WD repeat domain of MEP50-(76–342), or GST-MEP50 N terminus (GST fused to MEP50-(1–75)) or, as a control, with GST alone (data not shown). After extensive washing, bound proteins were resolved by SDS-PAGE and visualized by fluorography (Fig. 5). GST-MEP50 and GST-MEP50 WD domain bound most avidly to SmB and SmD2 but also had detectable affinity for SmD3 and SmE. The GST-MEP50 N terminus bound significantly less of these Sm proteins. There was no detectable binding of any of the Sm proteins to GST alone under these binding conditions (data not shown and Refs. 26 and 27). Although it is very unlikely, we cannot rule out the possibility that these interactions may be mediated by components of the reticulocyte lysate. These findings indicate that MEP50 binds to several of the Sm proteins and that these interactions are mediated by the WD repeat domain.

We next wished to determine whether MEP50 plays a role in methylosome activity. To this end, methylosomes were purified from cells transiently expressing flag-JBP1 as described previ-

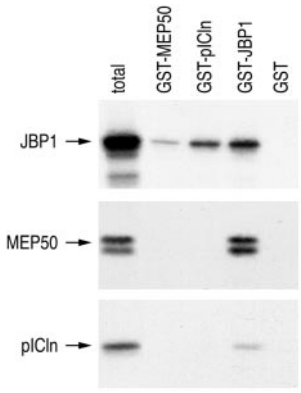



FIG. 4. **All of the methylosome components bind to JBP1.** The proteins indicated by *arrows* were produced and radiolabeled by *in vitro* transcription and translation and incubated with the indicated GST fusion proteins. Following extensive washing, bound proteins were resolved by SDS-PAGE and visualized by fluorography. MEP50 most likely runs as a doublet because translation starts at the Myc tag and the native start codon. The "*total*" *lane* shows 5% of each protein used in each binding.

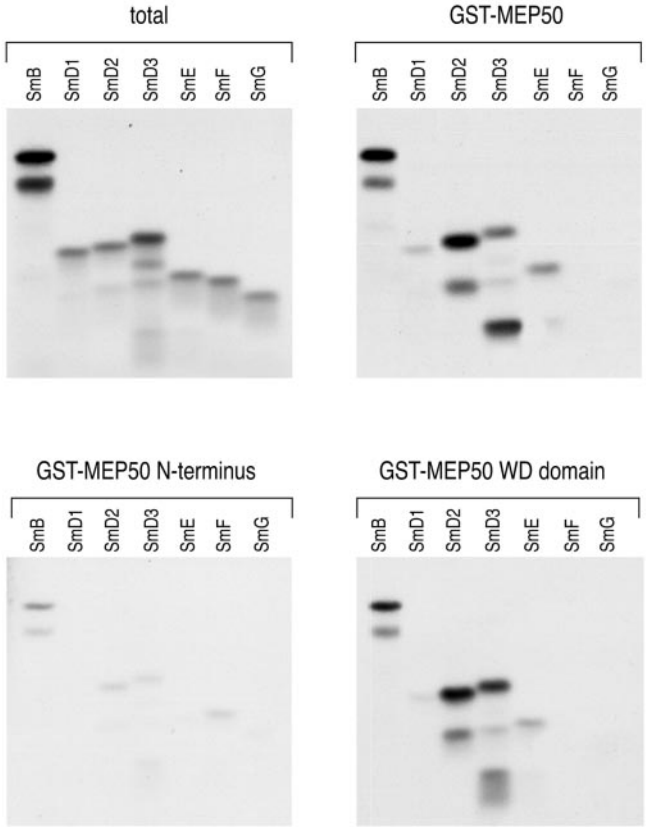



FIG. 5. **MEP50 binds a subset of the Sm proteins.** The indicated proteins were produced and radiolabeled *in vitro* and incubated with the indicated immobilized GST fusion proteins. See "Results" for details. Following extensive washing, bound proteins were resolved by SDS-PAGE and visualized by fluorography. The *total panel* (*top left*) shows 10% of each protein used in each binding.

ously (14) and used to methylate the recombinant Sm protein substrates His-SmB and GST-SmD3 (Fig. 6). Anti-MEP50 antibodies were then used to verify whether they altered the activity of the methylosome, and NMS was used as a negative control. Anti-MEP50, but not NMS, significantly reduced methylosome activity toward the Sm protein substrates. Importantly, the removal of the anti-MEP50 antibodies from the serum by pre-binding it to immobilized GST-MEP50 abolished the inhibitory effect. In contrast, pre-binding of anti-MEP50

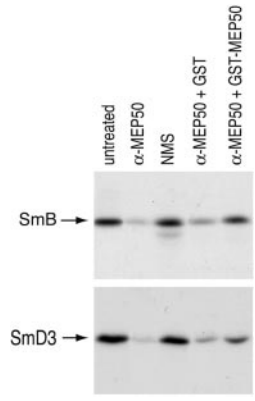



FIG. 6. **MEP50 is important for methylosome activity.** Purified methylosomes were incubated with adenosyl-L-[*methyl*-$^3$H]methionine and the indicated recombinant proteins (*arrows*). Reactions were supplemented with anti-MEP50 serum (*α-MEP50*), NMS, anti-MEP50 serum pre-incubated with immobilized GST-MEP50 (*α-MEP50 + GST-MEP50*), or anti-MEP50 serum pre-incubated with immobilized GST (*α-MEP50 + GST*). The reactions were boiled in SDS sample buffer, resolved by SDS-PAGE, and visualized by fluorography. See "Materials and Methods" for details.

serum with immobilized GST did not significantly reduce the inhibitory effect of the anti-MEP50 antibodies. These results strongly suggest that MEP50 is important for the methyltransferase activity of the methylosome.

## DISCUSSION

The SMN complex mediates the assembly of spliceosomal snRNPs and other RNPs (10–12, 48–50). To carry out these functions, the SMN complex needs to bring together the components of these RNPs and facilitate the proper rearrangements of the individual components. To this end, SMN binds specifically and avidly to RG domains in several of the key protein components of RNPs (26, 48–50). We have recently shown that this interaction depends on a post-translational modification of specific arginines in the RG domains of its cognate proteins (13). For the best characterized protein substrates of the SMN complex, the spliceosomal snRNP Sm proteins, this modification is carried out by a large macromolecular complex termed the methylosome (14). The methylosome contains an arginine protein methyltransferase, JBP1, which converts specific arginines in the RG domains of SmD1 and SmD3 to sDMA, thereby targeting them to the SMN complex for assembly into snRNPs (13, 14). This finding suggested a key role for the methylosome in the process of snRNP assembly, and we therefore wished to characterize the composition and activity of the methylosome in further detail. Here, using co-immunoprecipitation with a monoclonal antibody (6G8) against JBP1, we have identified MEP50, a novel WD repeat protein, as an additional component of the methylosome. We further show that MEP50 binds Sm proteins and is important for methylosome activity. The current inventory of the components of the methylosome thus indicates that it is composed of JBP1, pICln, and MEP50.

Because 6G8, the anti-JBP1 antibody, co-immunoprecipitated only three major proteins (JBP1, pICln, and MEP50) as detected by Coomassie Blue staining, it is likely that all of the major, stably associated methylosome components have thus been identified. It is interesting to note that although this complex is apparently composed of only three proteins it is nevertheless very large. JBP1, but not MEP50 or pICln, readily oligomerizes *in vitro* (Fig. 4), and therefore it is likely that JBP1 contributes significantly to the size of this complex. Indeed, JBP1 forms intermolecular disulfide linkages, suggesting an ordered and regulated oligomerization (47). JBP1 oligomer-

ization may be required for its enzymatic activity. Oligomerization may also allow JBP1 to simultaneously methylate multiple substrates while maintaining them in a specific spatial arrangement and perhaps also to contribute to their arrangement into complexes. Importantly, all of the methylosome components interact with Sm proteins. This is reminiscent of the SMN complex in which SMN, Gemin3, and Gemin4 all bind several of the Sm proteins (9, 15, 16). It is possible that just as the SMN complex functions to facilitate the assembly of the Sm proteins into snRNP cores (10–12), the methylosome as well as producing methylated Sm proteins may also spatially arrange Sm proteins for their efficient assembly into snRNP cores by the SMN complex. In this context, it is particularly interesting that MEP50 interacts with SmD2, a protein that is not a substrate of methylation by the methylosome (data not shown and in Ref. 51); this suggests the possibility of a more general role for the methylosome in snRNP assembly. The interaction of all of the methylosome components with Sm proteins may also be important for the ordered and complete methylation of the many arginines that need to be modified in each RG domain of substrate proteins. In a larger perspective, it is interesting that the critical process of snRNP assembly appears to require the activities of at least two large multiprotein complexes that function sequentially, the methylosome and the SMN complex.

We show that anti-MEP50 antibodies specifically inhibit methylosome activity toward Sm protein substrates. Thus, MEP50 appears to modulate methylosome activity. MEP50 may be important for binding Sm protein substrates and properly positioning them for methylation by JBP1. It is also possible that the presence of MEP50 bound to JBP1 is important for the activity of the enzyme itself. Whichever is the case, it is clear that MEP50 is important for the function of the methylosome to specifically produce Sm proteins with the correct sDMA modification.

WD repeat-containing proteins are unique in that they are functionally diverse while having a common sequence motif, which very likely forms a common structure (45, 52, 53). The structure of a WD repeat protein (the G$\beta$ subunit of heterotrimeric G-proteins) revealed that this class of proteins forms a $\beta$-propeller structure, which apparently creates a stable platform allowing simultaneous interaction with multiple proteins (46, 52, 54, 55). If MEP50 is also able to interact with multiple proteins, it can be expected that it is important for targeting the Sm proteins, as well as other yet unknown proteins, to the methylosome for symmetrical dimethylation.

Only three proteins are known so far to contain sDMA, SmD1, SmD3 (51), and myelin basic protein (56). The identification of proteins that interact with MEP50, JBP1, and pICln may extend the range of substrates of the methylosome as well as of additional sDMA-modified proteins, if such exist. Indeed, JBP1 can symmetrically dimethylate myelin basic protein *in vitro* (34), suggesting that the methylosome may function to symmetrically dimethylate myelin basic protein and raising the interesting possibility that it may play a role in the biogenesis of myelin.

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