Histone Methyltransferases in *Aspergillus nidulans:* Evidence for a Novel Enzyme with a Unique Substrate Specificity†

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ABSTRACT: We have studied enzymes involved in histone arginine methylation in the filamentous fungus *Aspergillus nidulans*. Three distinct protein arginine methyltransferases (PRMTs) could be identified, which all exhibit intrinsic histone methyltransferase activity when expressed as glutathione *S*-transferase (GST) fusion proteins. Two of these proteins, termed RmtA (*a*rginine *m*ethyl*t*ransferase *A*) and RmtC, reveal significant sequence homology to the well-characterized human proteins PRMT1 and PRMT5, respectively. Native as well as recombinant RmtA is specific for histone H4 with arginine 3 as the methylation site. Furthermore, methylation of histone H4 by recombinant RmtA affects the acetylation by p300/CBP, supporting an interrelation of histone methylation and acetylation in transcriptional regulation. The second methyltransferase, named RmtB, is only distantly related to human/rat PRMT3 and must be considered as a member of a separate group within the PRMT family. The 61 kDa protein, expressed as a GST fusion protein, exhibits a unique substrate specificity in catalyzing the methylation of histones H4, H3, and H2A. Unlike human PRMT3, the *Aspergillus* enzyme lacks a Zn-finger domain in the amino-terminal part indicating functional differences of RmtB. Furthermore, phylogenetic analysis indicated that RmtB together with other fungal homologues is a member of a separate group within the PRMT proteins. The existence of in vivo arginine methylation on histones as demonstrated by site-specific antibodies and the high level and specificity of PRMTs for individual core histones in *A. nidulans* suggests an important role of these enzymes for chromatin modulating activities.

Posttranslational covalent modifications of core histones, such as acetylation, methylation, ubiquitination, and phosphorylation, represent key elements for the modulation of chromatin structure and function (*1*−*5*). Histones can be methylated on lysine as well as arginine residues, preferentially on the amino-terminal tails of histones H3 and H4. Protein arginine methylation was previously linked to signal transduction and RNA metabolism (*6*, *7*). Many methylated proteins such as nucleolin (*8*), fibrillarin (*9*), hnRNP A1 (*10*), and the poly(A)-binding protein II (*11*) were found to be associated with RNA. However, histones were also identified as physiological targets for arginine methylation. PRMT1 (*12*, *13*) and the coactivator-associated arginine methyltransferase 1 [CARM1 (*14*)][1] were shown to specifically methylate histones H4 and H3, respectively. Furthermore, direct evidence linking arginine methylation with transcription was found, since CARM1 interacts and cooperates with the steroid hormone receptor coactivator GRIP-1, a member of the p160 family of coactivators (*14*). PRMT1 also interacts with p160 coactivators, indicating a cooperative mode of action to enhance nuclear receptor functions (*15*). Together, these results indicate that arginine methylation of histones has an essential role in transcriptional regulation.

Protein arginine *N*-methyltransferases (PRMTs) catalyze the transfer of methyl groups from *S*-adenosylmethionine to the guanidino nitrogen atoms of arginine residues (*6*). As a result, mono- or dimethylated arginine is formed, where dimethylation can exist in a symmetric or asymmetric configuration. Depending on the methylation pattern, PRMTs are classified into type I enzymes that catalyze asymmetric dimethylation and type II enzymes that cause symmetric dimethylation.

The PRMT family includes seven known members which vary in size, subcellular location, and substrate specificity (*12*, *14*, *16*−*19*). However, only for PRMT1 (*13*) [RMT1/HMT1 from yeast (*20*, *21*)], PRMT4/CARM1 (*22*), and PRMT5/JBP1 (*19*, *23*) has histone methyltransferase activity been reported. For PRMT1 a variety of different substrates were described. Besides its ability to methylate RNA-binding proteins, PRMT1 can also methylate Arg 3 on histone H4 in vitro and in vivo. Regulation of gene activity is thought to be mediated by specific methylation of H4 (*13*). CARM1 preferentially uses histone H3 as substrate with methylation sites Arg 2, Arg 17, and Arg 26 (*22*). PRMT5/JBP1, the only known member of class II enzymes, is able to methylate histones H4 and H2A besides the non-histone proteins fibrillarin and myelin basic protein (*19*, *23*). In contrast to the gene-activating properties of PRMT1 and CARM1/

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[1] Abbreviations: CARM1, coactivator-associated arginine methyltransferase 1; PRMT, protein arginine *N*-methyltransferase; RmtA, arginine methyltransferase A; [^{3}H]SAM, *S*-adenosyl-L-[*methyl*-^{3}H]-methionine; HMT, histone methyltransferase; HAT, histone acetyltransferase; JBP1, Janus kinase-binding protein; GST, glutathione *S*-transferase.

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PRMT4, PRMT5/JBP1 was shown to negatively control cyclin E1 transcription and cellular proliferation (*24*). However, not all PRMTs display histone methylating activity. The presence of a Zn finger in PRMT3 was reported to be responsible for the specific recognition of RNA-associated methyl-accepting proteins in RAT1 fibroblasts (*25*).

Although the number of enzymes involved in protein methylation is increasing, the function of this modification is not cleared up. Within the family of PRMTs a broad spectrum of different substrates has been reported, indicating a high level of complexity. Therefore, the study of the responsible enzymes and the analysis of substrate specificities represent important steps for understanding the functions of these proteins in cellular regulation. Furthermore, recent observations have shown that different histone modifications at distinct sites can positively or negatively affect other modifications and thus affect the state of transcription. For example, methylation at Lys 9 in the histone H3 tail by SUV39H1 inhibits phosphorylation of Ser 10 by Ipl1/aurora, a modification that is important during mitosis. Additionally, phosphorylation of Ser 10 positively affects the histone acetyltransferase GCN5 to acetylate Lys 14, events that are correlated with the activation of transcription (*26*). An interplay between Arg 3 methylation and lysine acetylation of histones was also shown for PRMT1, emphasizing its important role in transcriptional activation (*15*). Therefore, these findings suggest that also other HMTs exhibiting alternative substrate specificities may play important roles for gene regulatory processes although not yet determined.

We have analyzed the *Aspergillus nidulans* genome for putative PRMTs and fungal protein extracts for enzymes that are involved in histone methylation. Three distinct PRMTs were identified which all exhibit in vitro histone methyltransferase (HMT) activity as recombinant proteins. Two of these proteins, termed RmtA and RmtC, reveal significant sequence homology to the well-characterized proteins PRMT1 and PRMT5, respectively. However, another less conserved methyltransferase named RmtB is present in *Aspergillus* which shows slight structural similarities with human/rat PRMT3 but exhibits a unique substrate specificity and may represent a novel type of PRMT. RmtB expressed as a GST fusion protein leads to pronounced methylation of core histones preferentially of H4 and to a lesser extent of H3 and H2A. Furthermore, site-specific antibodies confirmed Arg 3 on H4 and Arg 26 on H3 as methylation sites resulting in a methylation pattern similar to that observed for *Aspergillus* core histones in vivo, indicating a physiological role of RmtB in this fungus. In *Aspergillus* protein extracts, endogenous RmtA was identified as the predominant in vitro activity using histones as substrates. Both purified native and recombinant RmtA proteins possess histone H4 Arg 3 specificity. Furthermore, methylation of H4 by GST−RmtA influences subsequent acetylation, supporting the role of this modification in context with histone acetylation for transcriptional regulation.

EXPERIMENTAL PROCEDURES

Cultivation of Organism. A. nidulans strain A4 (Glasgow wild type) provided from the Fungal Genetic Stock Center (Kansas City, KS) was grown in minimal medium according to ref *27* for 21 h at 37 °C.

Cloning of rmtA, rmtB, and rmtC. Total *Aspergillus* RNA was used to prepare cDNA using Superscript reverse transcriptase (Life Technologies, Inc.) according to the manufacturer's instructions employing an oligo-dT_{17} standard primer. For amplification of fragments of *rmtA* from the cDNA of *A. nidulans* degenerate oligodeoxynucleotide primers were used. The forward primer was 5′-GGNATH-CAYGARGARATG-3′, based on the amino acid sequence GIHEEM, and the reverse primer was 5′-ACNCAYTG-GAARCARAC-3′, based on THWKQT. A product of 750 bp was isolated and cloned into the pGEM-T vector (Promega). A full-length genomic copy of *rmtA* was obtained using cDNA PCR products to screen a genomic library of *A. nidulans* DNA. For amplification of the 3′ end of *rmtA*, the 3′ rapid amplification of cDNA ends (RACE) protocol of ref *28* was performed. The first PCR was performed with the forward primer 5′-ATACCGTCGAGCTCAAG-3′ and an adapter primer of sequence 5′-GACTCGAGTCGACATCGA-3′. For "nested PCR", the primer 5′-GACTAGAGTACAC-GATGGA-3′ (forward) and the dT4 adapter primer 5′-GACTCGAGTCGACATCGATTTT-3′ (reverse) were used. Amplified products were cloned into the pGEM-T vector (Promega).

To obtain *rmtB* and *rmtC* sequences, the Whitehead genome database (Whitehead Institute, http://www-genome.wi.mit.edu/) was searched for homologous genes in *A. nidulans*. Matches were analyzed for putative start and stop codons to engineer specific primers for the amplification of the coding sequences. *rmtB* primer sequences were 5′-CGCGGATCCATGTCCGCTCCCTCTGCT-3′ (forward) and 5′-CCGCTCGAGTTACTGTAACGCCCAGTTCT-3′ (reverse), respectively, and primers used for the amplification of *rmtC* were 5′-CGCGGATCCATGCTCACTACTCCAATT-3′ (forward) and 5′-CCGGAATTCCTACATAAGACAC-CCATC-3′ (reverse), respectively. Amplified products were cloned into the pGEM-T vector (Promega), and DNA sequencing of recombinant plasmid clones was accomplished by the dideoxynucleotide chain termination method (*29*). The nucleotide sequences of *rmtA*, *rmtB*, and *rmtC* have been submitted to GenBank (accession numbers AY272042, AY375304, and AY375305, respectively).

Computational Methods. WUBLASTP and BLASTX were used for the identification of protein coding regions by database similarity search. Multiple alignments were produced using CLUSTAL W 1.8 (*30*), and the graphic representation of the alignment was done with SeqVu 1.1 (Garven Institute of Medical Research, Sydney, Australia) for the Macintosh Computer. We used the SMART simple modular architecture research tool (http://smart.embl-heidelberg.de/) for the analysis of the sequences for characteristic domain structure motifs. The phylogenetic tree was generated on the basis of the alignment in CLUSTAL W.

Preparation of GST Fusion Proteins and Production of Polyclonal Antibodies. For the expression of GST fusion proteins in *Escherichia coli*, the coding sequences were cloned into pGEX-5X-1 and pGEX.6P.1 expression vectors for GST−RmtA and GST−RmtB/C, respectively (Amersham Pharmacia Biotech). Proteins were expressed in BL21 cells in LB medium. Cultures of 250 mL with an A_{600} of 0.4 were induced with a final concentration of 1 mM isopropyl β-D-thiogalactopyranoside and grown for 4 h at 37 °C. After centrifugation of cells at 4000*g*, the pellet was resuspended

in 6 mL of GST binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) containing one protease inhibitor tablet (Complete; Roche, Mannheim, Germany) per 50 mL of buffer. For cell lysis, lysozyme was added at a final concentration of 5 mg/mL binding buffer, and cells were passed through a French press with a pressure setting of 1000 psi. The resulting lysate was centrifuged at 20000*g* for 10 min at 4 °C. GST fusion proteins were purified from soluble extracts by binding to a GST−HiTrap column (Amersham Pharmacia Biotech). Proteins were eluted with 50 mM Tris-HCl and 10 mM reduced glutathione, pH 8.0, and assayed for histone methyltransferase activity. For production of polyclonal antibodies against recombinant RmtA in rabbits cleavage of the GST tag was performed using factor Xa (Amersham Pharmacia Biotech) according to the manufacturer's instructions. IgG antibodies were purified by protein G immunoaffinity chromatography (Amersham Biosciences, Uppsala, Sweden). Recombinant p300-GST was purchased from Upstate Biotech.

Purification of RmtA. Protein Extraction. The fungal mycelia were collected and washed in a sintered glass filter, thoroughly dried with filter paper, and immediately frozen in liquid nitrogen for subsequent lyophilization. Five grams of lyophilized mycelia was ground to powder in an IKA grinding machine, and the powder was suspended in 40 mL of buffer A which contained 15 mM Tris-HCl, pH 8.0, 10 mM NaCl, 0.25 mM EDTA, 1 mM β-mercaptoethanol, 10% (v/v) glycerol, and one protease inhibitor tablet (Complete; Roche, Mannheim, Germany) per 50 mL of buffer. The mixture was stirred for 15 min on ice and then centrifuged for 30 min at 35000*g* at 4 °C.

SourceQ Chromatography (SQ). The supernatant was applied onto a 10 mL Source 15Q anion-exchange FPLC column (1.6 × 15 cm; Amersham Biosciences, Uppsala, Sweden), equilibrated with buffer A. Elution of proteins was performed with 50 mL of a linear gradient from 10 to 500 mM NaCl in buffer A at a flow rate of 1 mL/min. Fractions of 1.5 mL were collected and assayed for HMT activity. Peak fractions were pooled and concentrated to a final volume of 1 mL by centrifugation (2500*g*, 4 °C) using an Amicon Centriprep-10.

Size Exclusion Chromatography (Superdex 200). Concentrated fractions of the Source 15Q column were applied onto a Superdex 200 FPLC column (2.5 × 100 cm, 120 mL; Amersham Biosciences, Uppsala, Sweden), equilibrated with 100 mM NaCl in buffer A. The flow rate was maintained at 1 mL/min, and fractions of 1.5 mL were collected and assayed for HMT activity. For estimation of the molecular weight of the native enzymes, the Superdex 200 column was calibrated with proteins of known molecular weight.

Polylysine−Agarose Chromatography. Fractions with HMT activity of the S200 column were pooled and directly loaded onto a polylysine−agarose (Sigma Chemical Co., St. Louis, MO) column (1.5 × 20 cm, 5 mL) equilibrated with buffer A at a flow rate of 0.8 mL/min. Elution was performed with 50 mL of a linear gradient from 1 mM to 1 M NaCl in buffer A; fractions of 1.5 mL were collected and assayed for HMT activity. Fractions with high HMT activity were pooled and desalted by passage through a Sephadex G-25 gel filtration column (2.5 × 15 cm; Amersham Biosciences, Uppsala, Sweden).

Histone−Agarose Chromatography. Desalted protein fractions were applied onto a histone−agarose (Sigma Chemical Co., St. Louis, MO) column (1.5 × 20 cm, 5 mL) equilibrated with buffer A at a flow rate of 0.8 mL/min. Proteins were eluted with 50 mL of a linear gradient from 10 to 500 mM NaCl in buffer A, and fractions of 1 mL were collected. Fractions with maximum HMT activity were pooled and desalted with buffer B [10 mM potassium phosphate, pH 8.0, 1 mM β-mercaptoethanol, 10% (v/v) glycerol] by passage through a Sephadex G-25 gel filtration column.

Hydroxyapatite Chromatography. Desalted protein fractions were applied onto a hydroxyapatite Bio-Scale CHT-I (Bio-Rad, Richmond, CA; 2 mL) column, equilibrated with buffer B. Elution of proteins was performed with 40 mL of a linear gradient from 10 to 500 mM potassium phosphate. The flow rate was 1 mL/min. Fractions containing HMT activity were used for immunoblotting experiments.

Histone Acetyltransferase (HAT) and Histone Methyltransferase Assay (HMT). HAT and HMT activities were assayed with chicken erythrocyte core histones as substrate. Additionally, HMT activity was also tested with recombinant histone H3 and H4 purchased from Sigma Chemical Co. (St. Louis, MO). For determination of HAT activity, 50 μL of samples was incubated with 2.5 μL of histone substrate (20 μg) and 5 μL of [^{14}C]acetyl-CoA (20 μCi/mL) in a final volume of 57.5 μL for 1 h at 30 °C. To avoid chemical acetylation, NaCl was added to a final concentration of 300 mM. For determination of HMT activity, 50 μL of samples was incubated with 2.5 μL (20 μg) of chicken core histones and 1 μg of recombinant H3 and H4, respectively, and 1 μL of [^{3}H]-*S*-adenosyl-L-methionine (SAM) (0.55 μCi) for 45 min at 30 °C. HAT and HMT reactions were stopped by TCA precipitation (25% final concentration), and samples were kept on ice for 20 min. Whole sample volumes were collected onto glass fiber filters (Whatman GF/F) preincubated with 25% TCA. Filters were washed three times with 3 mL of 25% TCA and then three times with 1 mL of ethanol. After the filters were dried for 10 min at 70 °C, radioactivity was measured by liquid scintillation spectrophotometry (3 mL of scintillation cocktail).

Specificity of Histone Acetyltransferases and Histone Methyltransferases. For determination of substrate specificity, HAT and HMT assays were performed as described above, but TCA precipitates were centrifuged and pellets containing labeled histones were dissolved directly in Laemmli sample buffer. Histones were analyzed by SDS−polyacrylamide gel electrophoresis as previously described (*31*), and the incorporation of radiolabel into histones was detected by fluorography performed as described (*32*).

Immunoblotting. Sample aliquots were electrophoresed in precast 14% polyacrylamide gels (Novex) at 35 mA for 1.5 h at room temperature. Gels were blotted onto nitrocellulose membrane (Schleicher and Schuell) at 350 mA for 2 h, and membranes were blocked with 5% (w/v) skim milk in TBS/Tween (20 mM Tris-HCl, pH 7.6, 140 mM NaCl, 0.1% Tween) for 2 h. Membrane strips were incubated with antibodies in 5% TBS/Tween at 4 °C overnight. After washing, strips were incubated for 2 h with alkaline phosphatase-conjugated secondary anti-rabbit Ig (Amersham Pharmacia Biotech), and immunodetection was performed using the ready to use BCIP/NBT color development

Table 1: *A. nidulans* PRMT Family[a]

enzyme	coding sequence	number/length of introns	amino acids	MW	contig in database	human orthologues
RmtA	1038	3 (52, 63, 61 bp)	345	39365.83	1.68	PRMT1
RmtB	1629	3 (125, 54, 58 bp)	542	61041.79	1.51	
RmtC	2445	2 (61, 53 bp)	814	89922.05	1.4	PRMT5

[a] Coding and genomic sequences and intron information of the corresponding genes. The molecular weight (MW) was calculated by the Expasy ProtParam tool (http://us.expasy.org/tools/protparam.html). Contig in the database was retrieved from the Whitehead Institute assembly of the *Aspergillus nidulans* genome in June 2003 (http://www-genome.wi.mit.edu/annotation/fungi/aspergillus/index.html). Amino acid sequence identities of the *Aspergillus* sequences with their human homologues were calculated with the MultiAlign program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page= /NPSA/npsa_clustalw.html).

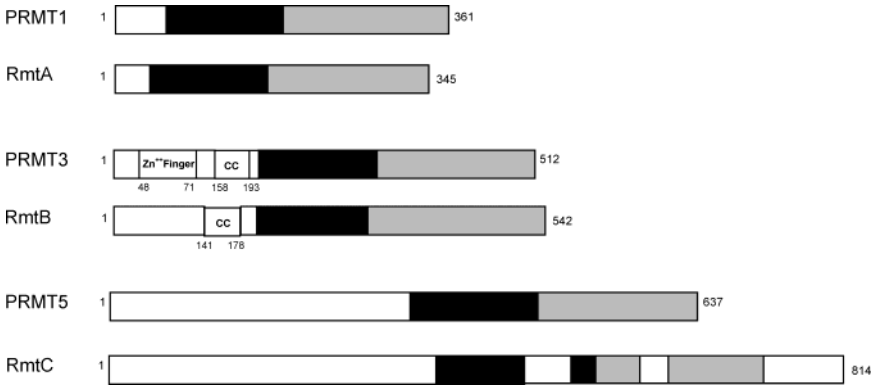

FIGURE 1: Schematic representation of *A. nidulans* and mammalian PRMT proteins. Highly and less conserved regions are highlighted in black and gray, respectively. The sequence alignment was performed using the NPS@: Network Protein Sequence Analysis program (*47*; http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html). Amino acid numbers of each protein are indicated. The zinc-finger and the coiled-coil (CC) domains were determined using SMART (http://smart.embl-heidelberg.de/) and are indicated by amino acid numbers. The schematic representation is not drawn to scale.

substrate (Promega).

Specific antibodies used were anti-RmtA (see Preparation of GST Fusion Proteins) for the identification and analysis of RmtA in *A. nidulans* and anti-dimethyl histone H4 (R3), anti-mono/di/trimethyl histone H4 (K20), anti-di/trimethyl histone H3 (K4), anti-mono/di/trimethyl histone H3 (K9) (Abcam), and anti-dimethyl histone H3 (R17 and R26) antibodies (Upstate) for determination of site specificity of recombinant and native PRMTs.

Extraction of Aspergillus Histones and Analysis of H3 and H4 Methylation. Frozen lyophilized mycelium (1 g) was ground to powder in an IKA grinding machine, and the powder was suspended in 50 mL of homogenization buffer (10 mM PIPES, pH 6.9, 5 mM $CaCl_2$, 5 mM $MgSO_4$, 0.5 M sucrose, 1 mM PMSF, 10 mM β-mercaptoethanol). The suspension was homogenized by five strokes at 1000 rpm in a Potter−Elvehjem homogenizer and centrifuged for 5 min at 400*g*. The supernatant was filtered and centrifuged for 20 min at 6000*g*. The supernatant was discarded, and the pellet was resuspended in 1 mL of water. Histones were extracted by the addition of 5 mL of 0.5 M hydrochloric acid and gentle stirring overnight. The extract was cleared by centrifugation at 14000*g* for 20 min. For precipitation of histones, 48 mL of acetone was added to the supernatant and was kept at −20 °C overnight. Precipitated proteins were centrifuged at 2000*g* for 10 min. The pellet was washed with acetone, vacuum-dried, and dissolved in water. Aliquots of nuclear extracts were electrophoresed in precast 14% polyacrylamide gels and blotted onto nitrocellulose membrane. Membrane strips were incubated with anti-dimethyl histone H4 (R3) and anti-dimethyl histone H3 (R17 and R26) antibodies (Upstate), and immunodetection was performed as described above.

RESULTS

Identification of PRMT Genes. The PRMT family includes six members which share a conserved catalytic core but only minor amino acid conservation outside of the core domain. We have searched the Whitehead *A. nidulans* genome database for sequences that match PRMT sequences. Three loci could be identified, and the coding sequences were analyzed and putative open reading frames refer to *rmtA* for PRMT1 and *rmtC* for PRMT5 homologous genes in *A. nidulans*. For the third sequence, *rmtB*, no unequivocal match with any known PRMT was obtained. However, a BLAST search of protein sequence databases revealed the closest relationship of RmtB to a hypothetical protein related to protein arginine *N*-methyltransferase 3 of *Neurospora crassa* and rat PRMT3 with only 35% identity for the latter enzyme. Performing the BLAST search with RmtA indicated its closest relationship to a putative PRMT of *Schizosaccharomyces pombe* followed by Hmt1p of *Saccharomyces cerevisiae*. Finally, searching protein sequence databases with RmtC, the highest degree of conservation was obtained for a protein related to the SHK1 kinase-binding protein in *N. crassa*, a homologue of human PRMT5. The characteristics of the *A. nidulans* PRMT family members are shown in Table 1.

Using the "SMART simple modular architecture research tool", we furthermore analyzed the sequences for characteristic domain structure motifs and compared them with those of the human homologues (Figure 1). The most remarkable difference could be observed when we compared the structures of RmtB and PRMT3, which exhibited the

highest identity to RmtB after the BLAST search. A C_2H_2 zinc-finger motif was described in the amino-terminal tail of PRMT3. The zinc finger was reported to be responsible for the specific recognition of RNA-associated methyl-accepting proteins in RAT1 fibroblast cells (*25*). Its position locates between Cys48 and His69. However, no such zinc-finger motif is present in the amino-terminal tail of RmtB nor in the putative RmtB homologues of *N. crassa* or *Aspergillus fumigatus* (data not shown). Analyzing the sequence of RmtB, a "coiled-coil" (CC) sequence motif was the only apparent structural motif identified within the N-terminal region, located at amino acids 148−173. Interestingly, further structural analysis revealed this CC domain also in human and rat PRMT3 genes (Figure 1). No remarkable structural differences were observed for RmtA and RmtC.

RmtA, RmtB, and RmtC Are Histone-Specific Arginine Methyltransferases. Core histones are substrates for PRMT1, CARM1/PRMT4, and PRMT5 in vitro and in vivo. To analyze the substrate specificities of PRMT family members in *Aspergillus*, we generated GST fusion proteins and analyzed recombinant proteins for their ability to methylate core histones. Remarkably, all purified recombinant proteins revealed significant HMT activity with chicken erythrocyte core histones as substrate (Figure 2A).

To further substantiate this finding and to identify the core histone species accepted by these enzymes, assay products were analyzed by fluorography. In the case of RmtA, intense methylation of H4 was observed but no other histone species was labeled (Figure 2B, left column). When we analyzed the substrate specificity of GST−RmtB, radiolabel was incorporated predominantly into H4 but also into histones H3 and H2A (Figure 2B, middle column). Although only a minor portion of RmtC protein was expressed into the soluble fraction of cell lysates, the purified protein exhibited significant HMT activity (Figure 2A), and label was incorporated into H4 and to a less extent into H2A (Figure 2B, right column). Results of Figure 2B could be confirmed when recombinant H4 and H3 were used as substrates (data not shown). None of the three GST fusion proteins yielded detectable methylation of H2B (Figure 2B). Note that the HMT activity of GST−RmtB is levels above that of all other known recombinant protein arginine methyltransferases except PRMT1 and its homologues (like GST−RmtA).

To determine the site specificities of GST−RmtA, GST−RmtB, and GST−RmtC, we used site-specific antibodies against dimethylated H4 Arg 3, H3 Arg 17, and H3 Arg 26. The immunoblot of Figure 2C demonstrates that all three recombinant proteins methylate H4 at Arg 3. Additionally, GST−RmtB also methylates H3 at Arg 26. No immunosignals were obtained for GST−RmtB with antibodies specific for H3 Arg 17 (data not shown) and for GST−RmtA or GST−RmtC with antibodies specific for H3 Arg 26 (Figure 2C). As negative control, immunoreactions were also performed with anti-H4 Lys 20, anti-H3 Lys 4, and anti-H3 Lys 9 antibodies, but no immunosignals were detectable (data not shown).

Purification of RmtA. The identification of the three putative PRMT genes prompted us to analyze protein extracts of *Aspergillus* for endogenous histone methyltransferase activities. Total protein extracts from mycelia were prepared and fractionated by SourceQ chromatography. Chromatographic fractions were tested for HMT activity. A single enzyme activity peak eluted at a salt concentration of 200 mM NaCl (Figure 3A).

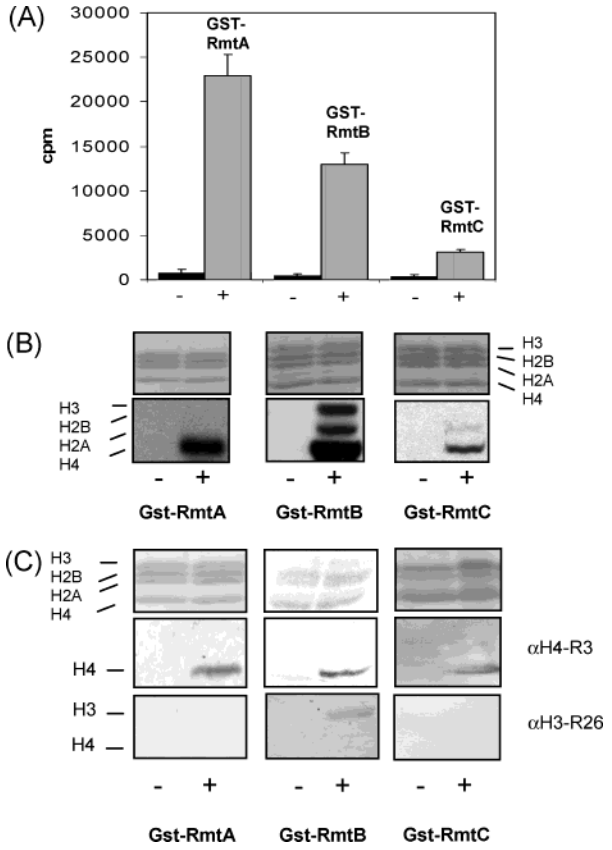

FIGURE 2: In vitro activity and substrate specificity of GST−PRMT fusion proteins. (A) For determination of HMT activity, aliquots of GST−RmtA, GST−RmtB, and GST−RmtC proteins were incubated with chicken erythrocyte core histones and [^{3}H]-*S*-adenosyl-L-methionine as described. As a control (−), the HMT assay was performed with extracts of noninduced cells containing the expression plasmid. Standard deviation values are indicated for five independent determinations. (B) Fluorography. Aliquots of enzyme activity assays in (A) were electrophoresed and analyzed by fluorography. The position of histone species is indicated, and Coomassie blue stained gels are shown. (C) Immunoblot. Aliquots of enzyme activity assays in (A) were electrophoresed and analyzed by immunoblotting with antibodies against H4 Arg 3 and H3 Arg 26, respectively. The position of histone species and the Ponceau red stained blots are shown. Key: (−) noninduced; (+) induced. Immunoblots represent typical results obtained in three independent experiments.

To test for the substrate specificity, aliquots of the enzyme activity assay were analyzed by fluorography. Figure 3A shows that within the enzyme peak two unresolved activities with different specificities seem to be present: in the first part of the peak, corresponding to fractions 20−24, with respect to histones only H4 was methylated. Thereby, radiolabel incorporation into H4 matches with the peak of enzyme activity. No histone H3-specific HMT activity could be detected. To rule out the possibility that preexisting modifications on the chicken erythrocyte histones interfere with an H3-specific enzyme, we also performed HMT assays with recombinant H3 and H4 as substrates. Again, incorporation could only be detected into H4 but not into H3 (data not shown). Immunoblots of the chromatographic fractions with antibodies against recombinant RmtA revealed that the enzymatic activity observed in fractions 20−28 is obviously

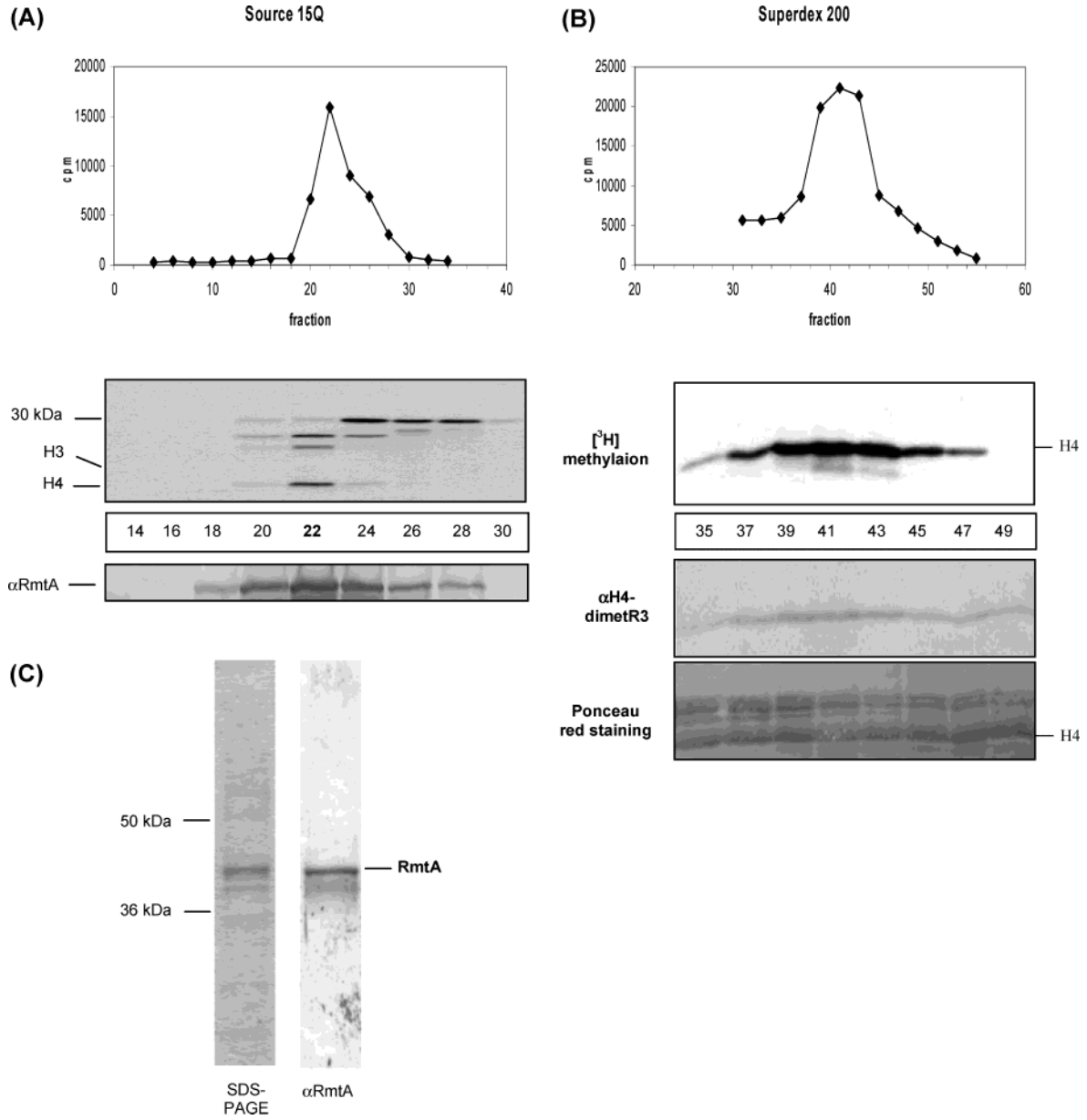

FIGURE 3: Purification of RmtA. (A) Histone methyltransferase activity was determined in fractions after S15Q chromatography (upper part). An aliquot of the activity assay was subjected to SDS−PAGE with subsequent fluorography (middle part) and immunoblotting (lower part), respectively. The position of H4 and H3 and a molecular mass marker protein are shown on the fluorogram, and the position of RmtA on the immunoblot is indicated. (B) Histone methyltransferase activity of fractions derived from the S200 gel filtration chromatography was determined. Lower panels show a fluorogram of HMT activity assays using chromatographic fractions and an immunoblot with anti-dimethyl H4 (Arg 3) antibodies. As a loading control, the Ponceau red stained blot is shown. The position of histone H4 is indicated. (C) SDS−PAGE and immunoblotting of proteins after hydroxyapatite chromatography. Aliquots of the peak fraction of the hydroxyapatite column (data not shown) were electrophoresed, and the gel was stained with Coomassie blue (SDS−PAGE) and analyzed by immunoblotting with antibodies against recombinant RmtA (αRmtA). The position of the RmtA protein on the immunoblot and two molecular mass marker proteins are shown.

due to RmtA since it can be detected in all fractions exhibiting HMT activity (Figure 3A). The right part of the peak (shoulder from fractions 24−28) methylated an additional protein of 30 kDa. However, the protein remains to be identified. Known non-histone substrates for PRMT1 include nucleolin (*8*), fibrillarin (*9*), several hnRNPs (*6*), poly(A)-binding protein II (*11*), interleukin enhancer binding factor 3 (ILF3) (*33*), and STAT1 (*34*).

For determination of the molecular size of native HMT and for further characterization, the enzyme activity peak (fractions 20−28) was subjected to Superdex 200 size exclusion chromatography (Figure 3B). The H4-specific enzymatic activity corresponds to a molecular mass of 250000 Da, indicating that the isolated enzyme is either present as a component of a multiprotein complex or, alternatively, is built up as a homohexamer.

To determine the site specificity of the purified enzyme, we used chromatographic fractions of the Superdex 200 containing HMT activity (Figure 3B) for immunoblotting experiments with antibodies against methylated H4 Arg 3 and H4 Lys 20, respectively. Only the H4 Arg 3 antibodies detected specific dimethylation on H4 in fractions within the HMT activity peak (fractions 39−43 in Figure 3B; data not shown), ruling out the possibility that we had purified a member of the SET-domain family of methyltransferases which methylates H4 at Lys 20 (*35*, *36*). Weak staining of

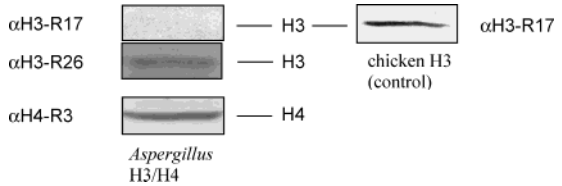

FIGURE 4: Arginine methylation on H3 and H4 occurs in vivo. For the analysis of the in vivo methylation pattern of *Aspergillus* histones, equal amounts of nuclear extracts were subjected to SDS−14% PAGE and blotted onto nitrocellulose membranes. Membrane strips were incubated with antibodies directed against H4 Arg 3, H3 Arg 17, and Arg 26, respectively. As a positive control for the H3 Arg 17 antibody, chicken erythrocyte core histones were used for immunoblotting (right blot). Antibodies used and positions of H3 and H4 are indicated. Immunodetection was performed with alkaline phosphatase conjugated secondary antibody.

H4 in fractions lacking methyltransferase activity is due to premethylation of chicken erythrocyte core histones.

After extensive purification of the enzyme activity by sequential chromatography with polylysine−agarose, histone−agarose, and hydroxyapatite (data not shown) we finally analyzed the peak fraction of the hydroxyapatite by SDS−PAGE and immunoblotting (Figure 3C). The purified sample contained a major protein band migrating at an apparent molecular mass of 43 kDa in Coomassie blue stained gels, somewhat higher than the predicted molecular mass of 39 kDa of isolated *rmtA* (Table 1). The immunoblot of the same figure demonstrates that the RmtA antibody (see Experimental Procedures) specifically immunoreacted with the purified protein (Figure 3C).

Arginine Methylation on H3 and H4 Occurs in Vivo. To test whether physiologically meaningful arginine methylation on histones H3 and H4 is carried out by PRMTs in *Aspergillus*, nuclear extracts were subjected to SDS−PAGE and immunoblotting using site-specific antibodies. As shown in Figure 4, histone H4 is methylated at Arg 3, and antibodies directed against H3 Arg 26 detected methylation of H3 at this site. However, Arg 17 on H3 is no methylation site for endogenous PRMTs since no immunoreaction could be observed with the corresponding antibody (Figure 4). These data provide evidence for arginine methylation of *Aspergillus* histones that occurs in vivo.

Methylation of H4 and H3 by RmtA and RmtB Interferes with Acetylation by p300. Recently, an interplay between H4 Arg 3 methylation and H4 Lys acetylation was reported (*15*). These findings prompted us to analyze whether methylation of H4 by recombinant RmtA has an effect on subsequent acetylation in our experimental system. When recombinant H4 was methylated by GST−RmtA in vitro and subsequently incubated with the recombinant HAT p300, a significantly higher incorporation of radiolabel into H4 could be observed than in the control reaction (Figure 5A). However, when the experiment was performed with recombinant H4 that had been acetylated by p300 in vitro (Figure 5B), subsequent methylation by RmtA was strongly reduced. To rule out the possibility that RmtA and p300 positively or negatively affect enzymatic activities in these experiments, both enzymes were incubated with histones before addition of either SAM or acetyl-CoA. However, no interference could be observed (data not shown).

We also analyzed the effect of GST−RmtB methylation of recombinant H3 and H4 on subsequent acetylation and vice versa. Interestingly, methylation of H3 by GST−RmtB had a negative effect on subsequent acetylation by p300 (Figure 5C, left panel), and a slightly enhanced acetylation pattern could be observed for premethylated H4 (Figure 5C, right panel). No significant effects on methylation could be observed when H3 and H4 were first acetylated by p300 and were then methylated by GST−RmtB (Figure 5D). Whether the lack of an acetylation effect on methylation might be simply due to the proposed unique structural and enzymatical properties of RmtB has to be determined.

DISCUSSION

A common feature of all identified PRMTs is the presence of a highly conserved core region containing an *S*-adenosylmethionine binding motif. Outside this domain, sequence conservation is weak. Sequence analysis of *Aspergillus* RmtB has revealed the existence of the conserved core domain, in line with protein methyltransferase activity. This was confirmed by the substrate specificity of the recombinant RmtB, which catalyzes the methylation of histones H4, H3, and H2A, a property not reported for histone methyltransferases so far. Although a BLAST search has revealed weak sequence homology to human/rat PRMT3, several aspects argue for the classification of RmtB as a distinct member of the protein arginine methyltransferase family. *Aspergillus* RmtB exhibits low sequence conservation, and most of these similarities are located in the conserved methyltransferase motif. The unique position of RmtB is also suggested by the phylogenetic tree based on the alignment of a set of available PRMT sequences of different organisms (Figure 6). Sequence analysis suggests that within the PRMT family rather diverse (sub)classes exist; whereas PRMT5 homologous proteins and mammalian PRMT1 homologues have diverged into two distinct classes, fungal RmtB homologues are more distantly related to other human and rat PRMTs, respectively. Furthermore, human and rat PRMT3 proteins seem to be more closely related to the PRMT1 class rather than to RmtB enzymes of fungi, which are clearly separated in this phylogenetic tree. Altogether, this suggests that RmtB of *A. nidulans* rather represents a member of a separate class than a subclass within the PRMT3 family of enzymes.

The most intriguing feature of this enzyme is its unique substrate specificity. RmtB accepts three different core histone molecules as substrates. Besides histone H4, histones H3 and H2A are also substrates in vitro. This is the first example of a protein methyltransferase exhibiting such a diverse histone specificity. CARM1 was found to methylate H3, H2A, and H2B in vitro when recombinant histones were used as substrates; however, this enzyme was only capable of H3 methylation in the presence of core histones (*14*). The observation that non-histone proteins seem to be a poor substrate for RmtB further supports our view that histones are the main target for RmtB. When we used an *Aspergillus* protein extract as a substrate source for recombinant RmtB, only one single 32 kDa protein was labeled (data not shown). In this context it is important to note that for PRMT3 no histone specificity was reported so far but, instead, a set of RNA-associated substrates in RAT1 cell extracts was identified (*25*). Furthermore, as a special feature, a zinc-finger domain in the amino-terminal region of PRMT3 was demonstrated to be required for substrate recognition. A zinc finger is also present in human and *S. pombe* PRMT3

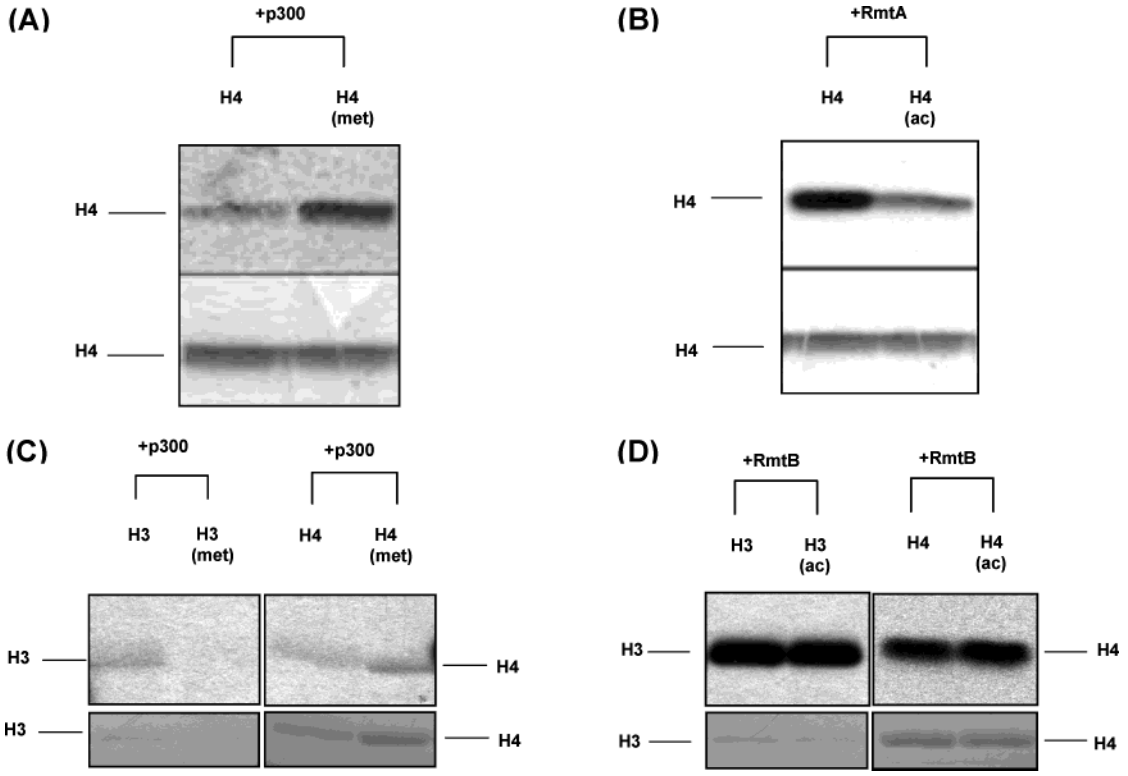

FIGURE 5: H3 and H4 methylation affects acetylation by the HAT p300. (A) Recombinant H4 was incubated with recombinant RmtA and *S*-adenosyl-L-methionine for 45 min. Subsequently, recombinant p300 and [^{14}C]acetyl-CoA were added to the reaction mixture, and the HAT assay was performed for 45 min [H4(met) + p300]. The HAT assay was also performed with nonmethylated H4 (H4 + p300). (B) Recombinant H4 was acetylated in vitro with nonlabeled acetyl-CoA by p300; recombinant RmtA and [^{3}H]-*S*-adenosyl-L-methionine were added to the reaction mixture [H4(ac) + RmtA]. The HMT assay with nonacetylated H4 is also shown (H4 + RmtA). (C) Recombinant H3 and H4 were incubated with recombinant RmtB and *S*-adenosyl-L-methionine with subsequent acetylation by recombinant p300, adding [^{14}C]acetyl-CoA to the reaction mixture [H3/H4(met) + p300]. As control, the HAT assay was performed with nonmethylated H4 (H3/H4 + p300). (D) Recombinant H3 and H4 were acetylated in vitro with nonlabeled acetyl-CoA by p300; recombinant RmtB and [^{3}H]-*S*-adenosyl-L-methionine were added to the reaction mixture [H3/H4(ac) + RmtB]. HMT assays with nonacetylated H3 and H4 are shown (H3/H4 + RmtB). Sample aliquots of enzyme activity assays of (A), (B), (C), and (D) were electrophoresed and analyzed by fluorography. The position of H3 and H4 and the Coomassie blue stained gel (bottom) are shown. Fluorographies represent typical results obtained for three independent experiments.

homologues whereas RmtB and its fungal homologues in *N. crassa* and *A. fumigatus* lack this domain, and therefore this structural difference might also be reflected in a different substrate specificity. Analysis of the domain structure of the RmtB amino acid sequence revealed a coiled-coil motif as the only significant structural motif. Although coiled-coil domains are abundant protein interaction domains (*37*, *38*), this domain was not identified in other *Aspergillus* PRMT sequences (Figure 1). Since purified recombinant RmtB, lacking the GST tag, elutes as an enzymatically active 250 kDa protein complex after gel filtration chromatography (data not shown), it is conceivable that this domain allows the formation of RmtB as an enzymatically active tetramer with a calculated molecular mass of 61 kDa for the subunit polypeptide (Table 1). Endogenous PRMT3, however, was assumed to be present as a monomer in RAT1 cells after size exclusion chromatography (*39*).

It is remarkable that RmtB exhibits specificity for H3 besides other histones, a substrate specificity that has only been demonstrated for CARM1/PRMT4 until now (*14*, *22*); PRMT1 was shown to be specific for H4 (*15*) in vivo, and PRMT5 in addition to H4 can methylate H2A in vitro (*18*, *40*). As shown in this paper, human PRMT1 and PRMT5 homologous proteins were identified in *Aspergillus* (RmtA and RmtC). However, when we have searched the *Aspergillus* genome database, no match for a CARM1/PRMT4 homologous gene was found. It is therefore feasible that RmtB might be implicated in chromatin modulating processes that require the specific methylation of H3, eventually in cooperation with other proteins. Indeed, methylation of recombinant H3 in our in vitro assay system had a negative effect on subsequent acetylation by p300 whereas prior acetylation did not affect methylation (Figure 5); this indicates that an interplay between H3 methylation and acetylation and eventually other modifications seems likely.

It is of great interest to know if selective arginine methylation of histones occurs in vivo. Most of arginine methylation in nature has been assigned to methylation of non-histone proteins (*6*, *41*), and the observation that only low levels of methylarginine were found in eukaryotic histones (*22*) might give rise to the question if PRMTs carry out meaningful methylation on histones H3 and H4, respectively. However, the use of antibodies that selectively discriminate between methylated and unmethylated forms of H3 and H4 clearly has demonstrated that H3 and H4 in *A. nidulans* are methylated in vivo (Figure 4) and provides evidence that this type of modification is physiologically significant in *A. nidulans*. Intriguingly, the in vivo methylation pattern of extracted *Aspergillus* H3 (Arg 26 is methylated, Arg 17 is not) is identical to the site specificity of recombinant RmtB (Figure 2C). This is a strong evidence that RmtB might be responsible for in vivo methylation of

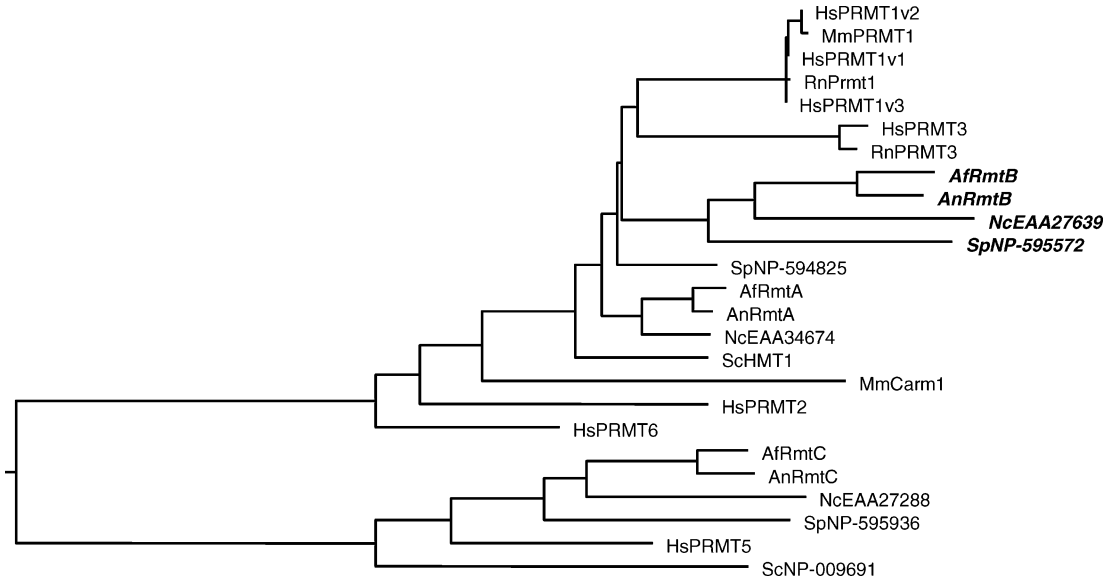

FIGURE 6: Unrooted phylogenetic tree of mammalian and fungal PRMTs. Sequences were aligned using CLUSTAL W (*46*), and trees were calculated using the Lasergene V5 program. AnRmtA, AnRmtB, and AnRmtC are from *A. nidulans* (this paper). Sequences of *A. fumigatus* (Af) and *N. crassa* (Nc) were derived by searches of the corresponding genome databases (http://www.sanger.ac.uk/Projects/A_fumigatus/; http://www.genome.wi.mit.edu/annotation/fungi/neurospora/). *A. fumigatus* sequences were termed according to *A. nidulans* orthologues. Sequences from human (Hs), mouse (Mm), rat (Rn), *S. cerevisiae* (Sc), and *S. pombe* (Sp), respectively, were obtained from GenBank. Fungal RmtB orthologues are highlighted in bold.

H3. This view is further supported by the lack of CARM1 homologous proteins in *Aspergillus*, the only histone H3-specific PRMT reported so far (*42*).

The identification and characterization of several histone methyltransferases have provided evidence for the involvment of histone methylation in transcriptional regulation. PRMT1 and CARM1/PRMT4 have been shown to positively affect gene activity (*14*, *15*), and for PRMT5 a specific role in the control of transcription and proliferation was demonstrated (*24*). Wang and co-workers (*15*) furthermore demonstrated an interplay between Arg 3 methylation of PRMT1 and lysine acetylation linking methylation of Arg 3 to transcription. Our data also provide evidence for a relationship between different modifications occurring on histones. Whereas methylation of H4 by RmtA and RmtB enhanced subsequent acetylation, supporting the role of this modification for gene activation, methylation of H3 by RmtB had a negative effect on acetylation in vitro. Since RmtB specifically methylates R26 on H3, this site might represent a mark for transcriptional repression in contrast to methylation of H3−R17: CARM1 has been shown to preferentially methylate R17 on H3, and this process has been linked to gene activation (*43*, *44*). Moreover, the recruitment of CBP has been demonstrated to be a prerequisite for the subsequent tethering of CARM1 to chromatin, thereby stimulating R17 methylation (*45*). Although our data are derived from in vitro experiments, they suggest that recruitment of *Aspergillus* RmtB and the subsequent methylation of H3/R26 might also inhibit the tethering of p300 in vivo. Alternatively, recruitment of RmtB might occur downstream of HAT recruitment since acetylation of H3 had no inhibitory effect on subsequent RmtB methylation (Figure 5D).

Finally, it is important to note that all PRMTs of *Aspergillus* exhibit histone-specific activity in vitro, indicating an important role of this modification for chromatin structure and function. Further genetic experiments performed in this fungus will hopefully help us to substantiate this view.

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