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Novel insights into the functional role of three protein arginine methyltransferases in *Aspergillus nidulans*

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ABSTRACT

Protein arginine methylation has been implicated in different cellular processes including transcriptional regulation by the modification of histone proteins. Here we demonstrate significant *in vitro* activities and multifaceted specificities of *Aspergillus* protein arginine methyltransferases (PRMTs) and we provide evidence for a role of protein methylation in mechanisms of oxidative stress response. We have isolated all three *Aspergillus* PRMTs from fungal extracts and could assign significant histone specificity to RmtA and RmtC. In addition, both enzymes were able to methylate several non-histone proteins in chromatographic fractions. For endogenous RmtB a remarkable change in its substrate specificity compared to the recombinant enzyme form could be obtained. Phenotypic analysis of mutant strains revealed that growth of Δ*rmtA* and Δ*rmtC* strains was significantly reduced under conditions of oxidative stress. Moreover, mycelia of Δ*rmtC* mutants showed a significant retardation of growth under elevated temperatures.

1. Introduction

Protein arginine methylation is a posttranslational modification found on both nuclear and cytoplasmic proteins. According to the substrates identified, arginine methylation has been implicated in many cellular processes including transcriptional regulation, RNA processing and transport, signal transduction, and DNA repair (Bedford and Clarke, 2009). Methylation of histones and non-histone proteins is catalyzed by members of the protein arginine methyltransferase (PRMT) family (Gary and Clarke, 1998). PRMTs catalyze the transfer of methyl groups from S-Adenosyl-L-methionine (SAM) to the guanidino nitrogens of arginine. The PRMT family currently includes eleven mammalian enzymes which are distinguished by differences in domain structures, requirements of cofactors, molecular targeting, and substrate specificity (Krause et al., 2007). Moreover, these enzymes have been divided into two major classes, catalyzing the formation of either asymmetric dimethylarginine residues (type I PRMTs) or symmetric dimethylarginine residues (type II PRMTs). PRMT5, PRMT7, and PRMT9 are known to be the only type II enzymes. Recently, type I activity has been assigned to PRMT2 (Lakowski and Frankel, 2009), whereas methyltransferase activity of PRMT10, and PRMT11 has yet to be determined. Recent findings have indicated that arginine methylation is a dynamic process which can be reverted by protein demethylases (Klose and Zhang, 2007).

The sequencing and annotation of genomes from several eukaryotes revealed that PRMT1, PRMT3, and PRMT5 are the arginine methyltransferase-encoding genes that were most strictly conserved throughout eukaryotic evolution (Bachand, 2007; Krause et al., 2007). This conservation is also reflected in most of the genomes of fungi. For example, in *Schizosaccharomyces pombe* three proteins with homology to human PRMT1, PRMT3, or PRMT5, are present with proposed functional roles for the regulation of the nuclear poly(A)-binding protein PAB2 by RMT1-dependent methylation (Perreault et al., 2007), for ribosome function by methylation of RPS2 by RMT3 (Bachand et al., 2006; Bachand and Silver, 2004), and for the control of cell polarity for the PRMT5 homolog SKB1 (Wiley et al., 2003). In *Saccharomyces cerevisiae*, only two members of the PRMT family are present, type I HMT1, which is related to human PRMT1 (Gary et al., 1996; Henry and Silver, 1996; Sayegh and Clarke, 2008), and HSL7, a homolog of human PRMT5 with specificity for H2A, H4, and bovine myelin basic protein (Lee et al., 2000; Ma et al., 1996; Miranda et al., 2006). Whereas for HMT1 a role in mRNA biogenesis (e.g. Cote et al., 2003; Green et al., 2002; Shen et al., 1998; Yu et al., 2004) and in heterochromatin formation (Yu et al., 2006) has been suggested, HSL7 is involved in cell cycle control (McMillan et al., 1999; Ruault and Pillus, 2006). No PRMT3 homolog is present in the genome of *S. cerevisiae*. In contrast to *S. pombe* and *S. cerevisiae*, the genome of *Candida albicans* has only one PRMT gene (Arnaud et al., 2005; McBride et al., 2007). A role for Hmt1 in nuclear export of Npl3 was demonstrated (McBride et al., 2007). Interestingly, a recent BLAST search

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revealed a putative PRMT4/CARM1 homolog in the basidiomycete *Ustilago maydis*, a gene that cannot be found in ascomycete species (Bachand, 2007; Brosch et al., 2008).

In contrast to mammals and yeasts, only a few studies have dealt with the role of arginine methylation in filamentous fungi. Filamentous fungi represent a heterogeneous group of species which includes important model systems for genetics and cell biology (Casselton and Zolan, 2002) or organisms that have great importance as producers of useful antibiotic and pharmaceutical activities as well as less desirable toxic activities (Abarca et al., 2004). Therefore, the comparative understanding of molecular mechanisms in these organisms and other eukaryotes may lead to novel scientific but also clinical, biotechnological and agricultural insights.

Three genes encoding for PRMTs have been identified in the genomes of *Aspergillus* and *Neurospora* (Borkovich et al., 2004; Brosch et al., 2008), however, biochemical and genetic data for PRMTs have been provided only for *A. nidulans* (Trojer et al., 2004). In this study, sequence alignments of two of the three proteins, RmtA (arginine methyltransferase A) and RmtC, revealed homology to human PRMT1 and PRMT5, respectively. Analysis of RmtB, however, indicated sequence similarities to human PRMT3, but also exhibited structural differences and a different substrate specificity. Thus, it was concluded that fungal RmtB homologs appear as an outgroup of mammalian PRMT3 proteins (Brosch et al., 2008; Trojer et al., 2004). The study further demonstrated significant *in vitro* activities and specificities of all PRMTs when expressed as GST-fusion proteins. Whereas recombinant RmtA and RmtC proteins possessed H4-R3 specificity, RmtB, on the other hand, led to pronounced methylation of histones H4, H3, and H2A; site specific antibodies confirmed Arg 3 on H4 and Arg 26 on H3 as distinct methylation sites. The methylation pattern resembled that observed for *Aspergillus* core histones *in vivo*. Moreover, intense purification identified RmtA as the predominant *in vitro* activity when histones were used as substrates. However, endogenous RmtB and RmtC activities remained undefined in protein extracts. Finally, methylation of histone H4 by recombinant RmtA affected H4 acetylation by the histone acetyltransferase p300/CBP, supporting the interrelation of histone arginine methylation and lysine acetylation in transcriptional regulation (Trojer et al., 2004).

To further study the functional role of PRMTs in *A. nidulans,* we deleted the coding sequences of *rmtA*, *rmtB*, and *rmtC* and analyzed molecular and physiological consequences of the specific gene deletions. We present a detailed overview on the expression of the corresponding enzymes, their enzymatic activities and specificities after partial purification by ion exchange chromatography and gel filtration, and the phenotypic effects of the deletion strains when grown under different growth conditions. We demonstrate significant methyltransferase activities of endogenous RmtA, RmtB, and RmtC proteins with striking differences/changes in substrate specificities *in vitro*. Moreover, our finding that growth of Δ*rmtA* and Δ*rmtC* strains was significantly reduced under conditions of oxidative stress supports a role of protein methylation in mechanisms of oxidative stress response.

2. Materials and methods

2.1. Strains, growth conditions and media

A. nidulans strains used in this study for the deletion of *rmtA*, *rmtB*, and *rmtC* were derived from FGSC A89 (*biA1*; *argB2*; *veA1*) provided by the Fungal Genetics Stock Center (Kansas City, KS). Unless otherwise noted, strains were grown in shake culture in glucose minimal medium for 18 h at 37 °C and 180 rpm with an inoculum density of approximately 1×10^7 conidia per ml (Pontecorvo et al., 1953). Vectors and plasmids generally were propagated in *Escherichia coli* DH5α cells (Life Technologies).

2.2. Generation of rmtA, rmtB, and rmtC deletion mutants

A. nidulans rmtA, *rmtB*, and *rmtC* genes were deleted by targeted gene replacement. Transformation of the arginine auxotrophic strain FGSC A89 was performed with fragments containing the ornithine carbamoyltransferase selectable marker (*argB*) flanked by 1.1–1.5 kb of the respective 5′ and 3′ untranslated regions (UTRs) for homologous recombination. Deletion cassettes were constructed as follows: for *rmtA* a 4.4-kb fragment was amplified by PCR from *Aspergillus* genomic DNA using primers *rmtA*fwd (TAATGCGGCCGCGGTATTCTGAATCATGCCCAC) and *rmtA*rev (GCCG ATGCATGAACAAGAAGGTTACATACCGAG) containing *Not*I and *Nsi*I restriction sites (cleavage sites are underlined). The PCR product was ligated into a pGEM-T vector (Promega) and subsequently the *rmtA* coding sequence was excised with *Pst*I–*Eco*RI and was replaced by the *argB* gene from the plasmid pILJ16 (Johnstone, 1985). For transformation of the fungus, the cassette was cut out with *Not*I and *Nsi*I.

Construction of the *rmtB* deletion cassette was essentially as described for *rmtA*, using primers *rmtB*fwd (TGTCGCTTAGTGACAT ATGACTCAGACTGAACG) and *rmtB*rev (ATATGCGGCCGCTAGGACGG GGAGAAAGATGG) and *Xba*I–*Xho*I for exchange of the coding sequence with *argB*. The resulting plasmid was digested with *Not*I–*Nde*I and the resulting 5.5-kb fragment was used for transformation.

Due to the lack of appropriate restriction sites, the 5′- and 3′-UTRs of *rmtC* were amplified separately, using primer pairs *rmtC*f1 (ATTTCCATGGTTGACATTGTTCTTTCGTGGG)–*rmtC*r1 (TATAGGATCC AAAGAGAAGTCAGTAGTCAGAC) and *rmtC*f2 (ATTACGATCGGTAGC GGAGAAAGTCTGC)–*rmtC*r2 (ATATGCGGCCGCCAGTCACCCAAATCT AATCG), respectively. DNA fragments were cloned into pGEM-T vectors and 3′ and 5′ UTRs of *rmtC* were excised by digestion with *Cla*I and *Pst*I, and *Sac*II–*Bam*HI, respectively, and inserted into pBS-KS+(argB) (kind gift from H. Haas). *Sac*II and *Cla*I were used to obtain the fragment for transformation.

The gel-purified fragments were used for transformation of *A. nidulans* FGSC A89 as described previously (Tilburn et al., 1995). After transformation, arginine prototrophic transformants were screened for the homologous integration event by PCR and single-colony derived putative PRMT deletion strains were confirmed by Southern hybridization as described earlier (Graessle et al., 2000).

2.3. Northern blot analysis

For studies of transcription levels of different detoxifying enzymes of the cellular antioxidant response (SodA, SodB, CatA, CatB, CatC, CpeA) under oxidative stress conditions by Northern hybridization, the strains were grown in minimal medium for 12 h at 37 °C in shake culture and for an additional 3 h with various concentrations of H_2O_2. RNA preparation, blotting, and hybridization were done as described (Graessle et al., 2000). Results were corrected for loading and blotting of RNA by using the γ-actin gene of *A. nidulans* (Fidel et al., 1988) as an internal control.

2.4. Site-directed mutagenesis of rmtA

Site-directed mutagenesis of *rmtA* was done using the QuikChange kit from Stratagene using the primers *rmtA*mutf (GCTACTTCCTCCTTTATCAGA*GCAT G*CTGGACACCG) and *rmtA*mutr (CGGTGTCCA*GCATGC*TCTGATAAAGGAGGAAGTAGC). Exchanged nucleotides are underlined. Besides the codon exchange, leading to E144Q, a silent mutation, generating an *Sph*I restriction site

(italicized), was introduced for screening purposes. Positive candidates were then confirmed by sequence analysis.

2.5. Complementation of mutants

For the complementation of mutant phenotypes deletion strains (*ΔrmtA* and *ΔrmtC*) were co-transformed with plasmids harboring wild type or mutant genomic sequences of the deleted genes and pBC1003 carrying a phleomycin resistance marker (kind gift from H. Haas).

2.6. Protein extraction

The fungal mycelia were collected and filtered by gravity through one layer of gaze, thoroughly dried with filter paper, and immediately frozen in liquid nitrogen for subsequent lyophilization. Six gram of lyophilized mycelia were ground to powder in an IKA grinding machine and the powder was suspended in 10 ml of extraction buffer (15 mM Tris–HCl, pH 8.0, 600 mM NaCl, 5 mM $MgCl_2$, 0.25 mM EDTA, 5 mM ß-mercaptoethanol, 10% (v/v) glycerol) per g dry weight using a potter homogenizer. To clear the extract the mixture was centrifuged for 20 min at 38,000*g* at 4 °C.

2.7. Chromatographic methods

2.7.1. Desalting chromatography

For desalting and elimination of low molecular weight compounds, 45 ml of extracts were passed through a Sephadex G25 gel filtration column (500 ml; XK 50/30 column; GE Healthcare) equilibrated with buffer A (15 mM Tris–HCl, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 10% (v/v) glycerol) at a constant flow rate of 5 ml/min. The protein fraction was collected for further chromatographic separation.

2.7.2. Ion exchange chromatography

Desalted fungal extracts were applied onto a 20 ml Source Q (SQ) anion exchange FPLC column (HR 16/10 column; GE Healthcare), equilibrated with buffer A at a flow rate of 3 ml/min. Elution of proteins was performed with six column volumes of a linear gradient from 50 mM to 600 mM NaCl in buffer A at a flow rate of 3 ml/min. Fractions of 2.5 ml were collected and assayed for MT activity.

2.7.3. Size exclusion chromatography

Selected fractions of the SQ-chromatography runs were combined, concentrated and applied onto a Superdex 200 FPLC column (HiLoad 16/60 column; 120 ml; GE Healthcare), 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 methyltransferase activity.

2.8. Methyltransferase (MT) assay

MT activities were assayed with chicken erythrocyte core histones as substrate (Kolle et al., 1998). Seventy-five microlitre of protein fractions were incubated with 30 µg of histone substrate, 3 µl of 25× Complete® protease inhibitor cocktail (Roche®), and 9 kBq of S-Adenosyl-L-[methyl-^{3}H]-methionine (2.2 TBq/mmol to 3.1 TBq/mmol; GE Healthcare) for 45 min at 30 °C. After incubation, one part (50 µl) of the mixture was spotted onto a glass fiber filter (Whatman® GF/F; presoaked in 25% (w/v) TCA) under suction and washed three times with 3 ml of 25% (w/v) TCA and three times with 1 ml of ethanol. Air-dried filters (10 min, 70 °C) were mixed with 3 ml of scintillation cocktail (Rotiszint® eco plus, Roth) and radioactivity was measured by liquid scintillation spectrophotometry. Remaining aliquots of the reaction mix were used for subsequent immunoblotting and fluorography, respectively.

2.9. Fluorography

For determination of substrate specificity, MT assays were performed as described above, but Laemmli sample buffer was added directly to the reaction mixtures containing labeled histones. Histones were analyzed by 16% SDS–polyacrylamide gel electrophoresis as previously described (Laemmli, 1970), blotted onto nitrocellulose membrane and the incorporation of radiolabel into histones was detected by fluorography performed as described (Laskey, 1980; Laskey and Mills, 1975).

2.10. Immunoblotting

Sample aliquots were electrophoresed in 10% polyacrylamide gels (Novex, USA) at 150 V const. for 1.5 h at room temperature. Gels were blotted onto nitrocellulose membrane (Hybond ECL; GE Healthcare) at 25 V const. for 2 h in transfer buffer (96 mM glycine, 12.5 mM Tris base, 0.1%(w/v) SDS, 20% (v/v) methanol, pH 8.3) on ice 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 20) 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 IgG (GE Healthcare) and immunodetection was performed using a ready to use BCIP/NBT Color Development Substrate (Promega).

Specific antibodies used were: anti-RmtA (Trojer et al., 2004), anti-RmtB, and anti-RmtC for the identification and analysis of PRMTs in *A. nidulans*. For production of polyclonal antibodies against full length RmtB in rabbits, GST-tag of recombinant GST-RmtB (Trojer et al., 2004) was cleaved using PreScission Protease (GE Healthcare) according to the manufacturer's instructions. A HIS-tagged version of RmtC was used for the production of anti-RmtC antibodies. For generation of the recombinant protein, the coding sequence of RmtC was amplified from fungal cDNA. Recognition sites for restriction enzymes were fused to the PCR primers in order to clone the amplified products into a pQE9 expression vector (Qiagen). Subsequently, *E. coli* M15 cells were transformed with the expression construct. Induction with isopropyl-β-D-1-thiogalactopyranoside (IPTG) led to the recombinant protein with a 6x His affinity tag allowing purification of the fragments on an immobilized metal-ion affinity resin. IgG antibodies were purified by Protein-G immunoaffinity chromatography (GE Healthcare).

2.11. Immunoprecipitation

Fifty microlitre of partially purified PRMT activities of *A. nidulans* (S200) was mixed with 15 µg of affinity purified anti-RmtA or anti-RmtB antibodies and 30 µl of Protein-G Sepharose, equilibrated with IP buffer (15 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 10% (v/v) glycerol), and incubated for 1 h with permanent shaking at 4 °C. To avoid unspecific binding, the mixture was adjusted to 400 mM NaCl. After centrifugation for 20 s at 12,000*g*, the supernatant was saved for protein blotting. Pellets were washed three times with 500 µl of washing buffer (IP buffer adjusted to 400 mM NaCl and 0.5% Tween20), two times in IP buffer and finally resuspended in 65 µl of IP buffer. For immunoblotting, precipitates were mixed with SDS sample buffer, boiled, and centrifuged for 5 min at 12,000 *g*, and the resulting supernatant was used for SDS–PAGE.

3. Results

3.1. Deletion of Aspergillus rmtA, rmtB, and rmtC genes

We identified three PRMTs in the filamentous fungus *A. nidulans* (Trojer et al., 2004). Sequence comparison and expression as recombinant proteins revealed that two of the proteins, RmtA and RmtC, are homologous to human PRMT1 and PRMT5, respectively, whereas RmtB displays unique enzymatic and structural properties and therefore has a peculiar position within the PRMT family. To further study the functional role of PRMTs in *A. nidulans*, we constructed deletion strains (see Section 2 and Suppl. Fig. 1) and analyzed the biochemical properties and phenotypic effects in deletion strains. For all gene deletions (*rmtA, rmtB, rmtC*), several independent transformants exhibited the expected pattern of DNA hybridization for single gene replacements (Suppl. Fig. 2).

3.2. All Aspergillus PRMTs are expressed in wt strains and undergo oligomerization

Previous results of our laboratory have demonstrated that all three *A. nidulans* PRMTs have intrinsic MT activity when expressed as recombinant proteins in *E. coli* (Trojer et al., 2004). Moreover, the major activity in *Aspergillus* protein extracts could be assigned to the histone H4-R3 specific RmtA. After the successful disruption of PRMT genes in *A. nidulans* we set out to examine the effect of gene deletions on protein expression, enzyme activity, substrate specificity and complex formation *in vitro*.

In order to optimize the extraction of methyltransferase activities in *A. nidulans*, we developed a high salt extraction procedure, which was not applied in our previous study (Trojer et al., 2004). As a result, the methylation pattern differed significantly compared to our previous results where only a single enzymatic activity peak could be extracted (Trojer et al., 2004). PRMT activity of wild type mycelia was split into two enzyme activity peaks after SQ-chromatography, a major and a minor peak eluting at salt concentrations of 280 and 150 mM NaCl, respectively (Fig. 1A). Immunoblotting analysis of fractions revealed that all three *A. nidulans* PRMTs were expressed in wild type mycelia (Fig. 1A). In detail, RmtA and RmtB were found in the major activity peak (maximum in fraction 25 at 280 mM NaCl) whereas RmtC was present in fractions corresponding to a salt concentration of approximately 190 mM NaCl (maximum in fraction 19). In the minor enzyme peak (fraction 15 at 150 mM NaCl), no signal for either of the *Aspergillus* PRMTs could be detected, indicating the presence of other methyltransferase(s).

The presence of RmtA and RmtB within the same fractions after SQ-chromatography implies the possibility of physical interaction between the two enzymes as a possible means for regulation of their activities. To analyze complex formation and to study putative interactions of *Aspergillus* PRMTs we performed size exclusion chromatography and immunoprecipitation experiments. Chromatography fractions of wild type (Fig. 1A) were split into two parts, the first part corresponding to the elution of RmtC (fractions 17–21; termed P1; see Fig. 1A), and the second part corresponding to the elution of RmtA and RmtB (fractions 23–28; termed P2). Fractions were pooled, concentrated and subjected to a S200 chromatography. Fractions were then tested for MT activity and subjected to immunoblotting and fluorography.

Chromatographic analysis of fractions corresponding to RmtC (P1) by S200 revealed two enzyme activity peaks in the wild type strain (Fig. 1B), corresponding to molecular weights of 420,000 and 250,000. When chromatographic fractions were analyzed with anti-RmtC antibodies, RmtC protein matched with enzymatic activities in the 420 kDa fractions indicating significant endogenous MT-activity of RmtC (Fig. 1B) as part of a high molecular weight protein complex. Immunoblotting experiments further demonstrated that the 250 kDa peak was due to RmtA and RmtB activities which were present in the initially pooled SQ fractions (see Fig. 1A).

In parallel we also characterized RmtA/RmtB containing fractions of SQ-chromatographies (fractions 23–28; P2) by gel filtration. A single MT activity peak could be observed with a corresponding molecular weight of approximately 250,000 (Fig. 1C). Moreover, immunoblotting experiments again confirmed MT-activity of RmtA and RmtB within the same fractions as immunostained bands matched with the enzyme activity profile (Fig. 1C). Importantly, a physical interaction between these two enzymes could be ruled out as RmtB could not be co-immunoprecipitated with RmtA and *vice versa* (Fig. 2).

3.3. RmtA, RmtB, and RmtC possess significant in vitro activities and multifaceted specificities

As all *Aspergillus* PRMTs are present in wt protein extracts (see Fig. 1A) we investigated enzymatic activities and specificities of RmtA, RmtB, and RmtC in our mutant strains. When proteins of Δ*rmtA* or Δ*rmtB* extracts were separated by SQ-chromatography, the MT-activity in the major peak fractions was reduced to approximately 44% or 56% as compared to wild type (Fig. 3A and B). This finding on one side confirmed our recent observation of significant enzymatic activity in protein extracts due to RmtA (Trojer et al., 2004) and on the other side indicated that RmtB, which is also present in the major peak (compare immunoblots in Fig. 1A), also contributes considerably to MT activity. To confirm this assumption, chromatographic fractions were blotted and probed with antibodies against RmtA and RmtB. Both proteins were present in fractions of the major peaks matching with the MT activity patterns (maximum in fraction 25), providing evidence for endogenous RmtA and RmtB activities in the respective chromatographies (Fig. 3A and B). Moreover, an additional peak, not visible in wild type strains, appeared in the elution profile of the Δ*rmtA* SQ-chromatography (190 mM NaCl, fraction 19; Fig. 3A). Most probably this peak was masked by RmtA activity in wild type fractions. The activity of this peak matched with RmtC as detected with anti-RmtC antibodies. RmtA (Fig. 3A) and RmtB (Fig. 3B) could not be detected on immunoblots of the corresponding mutant strains, confirming their successful deletion.

When proteins of Δ*rmtC* extracts were separated by anion exchange chromatography, total MT-activity of the major peak was restored and activities were comparable to wild type (Fig. 3C). On a protein level, RmtA and RmtB could be visualized within the major peak.

We have previously demonstrated that core histones from chicken erythrocytes are substrates of recombinant RmtA, RmtB, and RmtC proteins (Trojer et al., 2004). Thus, to determine the substrate specificity and to investigate complex formation, we separated the endogenous enzymes by gel filtration as described for wild type (Section 3.2), and analyzed fractions of the S200 chromatography for MT activity with subsequent fluorography.

When analyzing the Δ*rmtB* strain (lacks endogenous RmtB) for the substrate specificity of RmtA, an intense label of histone H4 was visible in 250 kDa fractions containing RmtA protein (fractions 37–45; Fig. 3H). The pattern resembled that of wild type (Fig. 1C). In addition, several unknown non-histone proteins migrating between 30 and 200 kDa were also heavily labeled.

Surprisingly, no histone specificity could be assigned to RmtB since in the Δ*rmtA* strain only non-histone proteins were labeled (fractions 37–43; Fig. 3G). This is in contrast to recombinant RmtB, expressed as a GST-fusion protein in *E. coli*, which showed specificity for histones H3, H4 and H2A (Trojer et al., 2004). Thus, our data indicate an altered substrate specificity of endogenous RmtB.

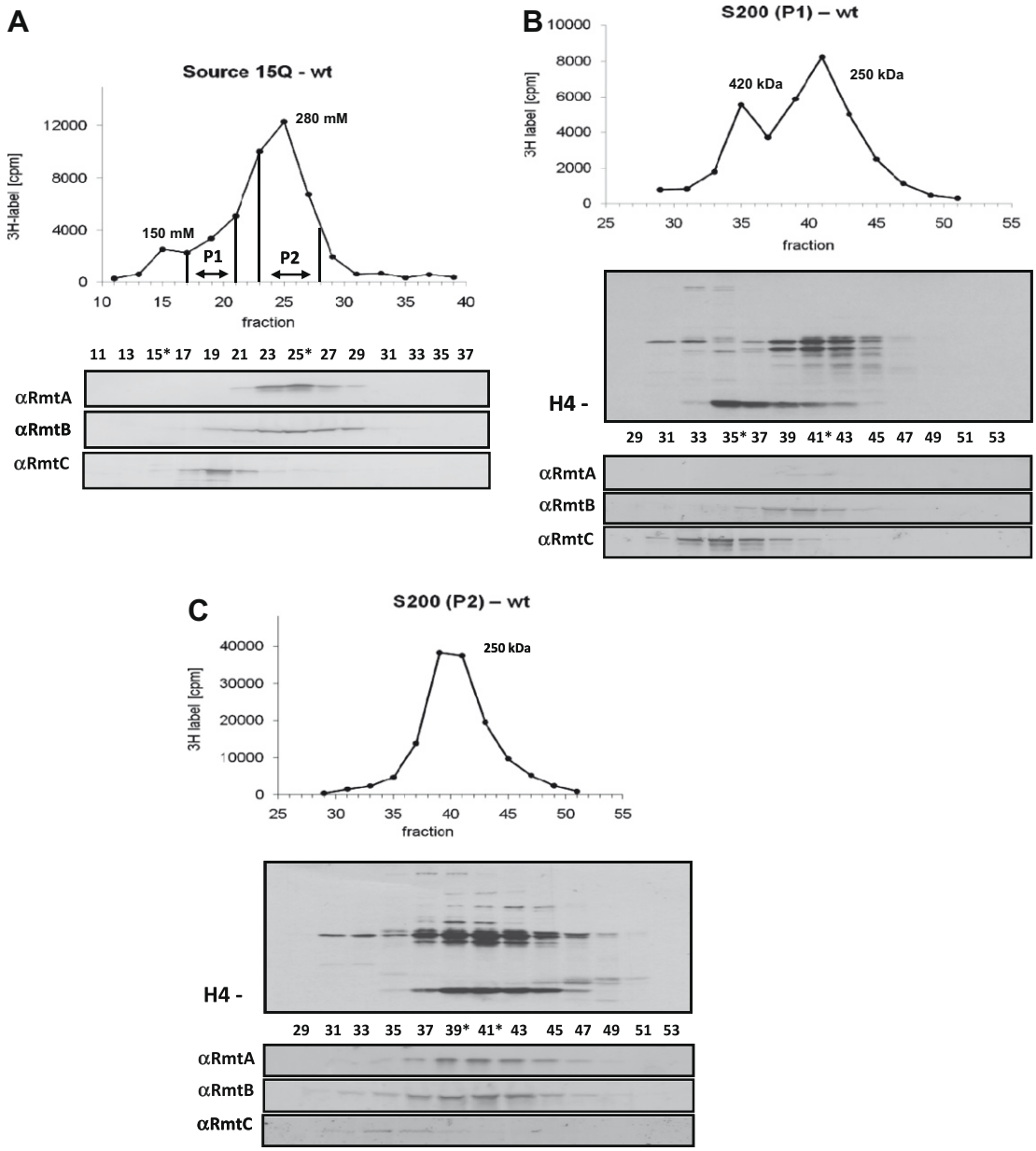

Fig. 1. Partial purification and characterization of *Aspergillus* PRMT protein complexes in wt strains. (A) Methyltransferase activity was determined in fractions after SQ-chromatography. Salt concentrations of eluting peak fractions are indicated. Aliquots of the activity assays were subjected to SDS–PAGE with subsequent immunoblotting. Peak fractions are indicated by asterisks. (B and C) Fractions 17–21 (termed P1) and fractions 23–28 (termed P2) of the SQ-chromatography were pooled, concentrated and subjected to two S200 chromatographies. Subsequently, chromatography fractions were tested for MT activity, fluorography, and immunoblotting using anti-RmtA, RmtB, and RmtC antibodies. Calculated molecular weight is given, peak fractions of S200 runs are indicated by asterisks and position of H4 is shown. As loading control of histones, gels were stained with Coomassie blue (not shown).

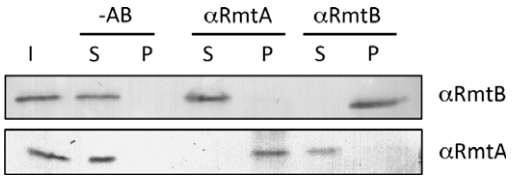

Fig. 2. Immunoprecipitation of RmtA and RmtB. Aliquots of fraction 41 from S200 chromatography (Fig. 1C) were mixed with or without (+/−AB) immunopurified anti-RmtA or RmtB antibodies and protein-A Sepharose. Equal amounts of supernatants and entire pellets were applied to SDS–PAGE with subsequent immunoblotting using anti-RmtA and anti-RmtB antibodies. An aliquot of the input was analyzed with the same antibodies (I). Data represent results of three independent experiments.

Our results also demonstrate histone specificity of endogenous RmtC *in vitro;* similar to RmtA, methylation of histone H4 (according to the recently observed recombinant activity) and of several non-histone proteins was visible in the 420 kDa fractions of mutants (Fig. 3D and E) and wild type (Fig. 1B), respectively.

3.4. Deletion of rmtC leads to increased sensitivity to heat stress

In order to analyze possible physiological consequences caused by the deletion of PRMT genes, mutant strains and corresponding controls were grown on media with different carbon sources (glucose, acetate, xylose, skim milk) or different nitrogen sources (ammonium tartrate, sodium nitrate, sodium nitrite) at

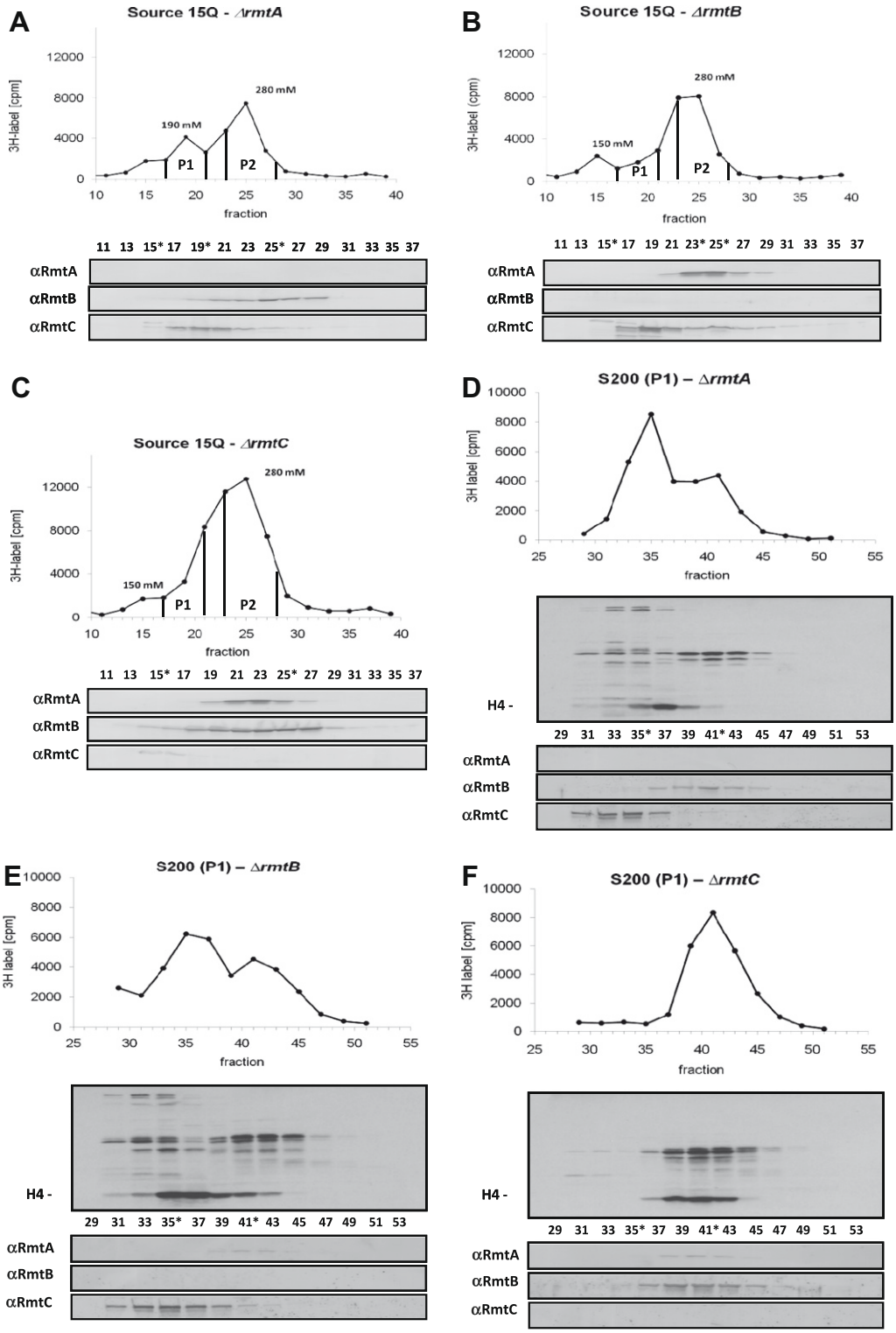

Fig. 3. Partial purification and characterization of *Aspergillus* PRMT protein complexes in mutant strains. (A–C) Protein extracts of the Δ*rmtA,* Δ*rmtB,* and Δ*rmtC* mutant strains were separated by SQ-chromatographies followed by methyltransferase assay. Salt concentrations of eluting peak fractions are indicated. Aliquots of the activity assays were subjected to SDS–PAGE with subsequent immunoblotting. Peak fractions are indicated by asterisks. Fractions used for subsequent S200 chromatographies are indicated (P1, P2). (D–I) Fractions corresponding to P1 and P2 of the SQ-chromatographies were pooled, concentrated and subjected to S200 chromatographies. Subsequently, chromatography fractions were tested for MT activity, fluorography, and immunoblotting using anti-RmtA, RmtB, and RmtC antibodies. Peak fractions of S200 runs are indicated by asterisks and position of H4 is shown. As loading control of histones, gels were stained with Coomassie blue (not shown).

temperatures of 25 °C, 37 °C, or 42 °C. Subsequently strains were compared with respect to growth rate and conidiation.

When glucose, xylose, or skim milk and ammonium tartrate, sodium nitrate, sodium nitrite, respectively, were used as substrates, no significant differences between controls and mutant strains were visible (Fig. 4 and data not shown), except for the Δ*rmtC* strain where a general reduction of radial growth could be observed at higher temperatures (42 °C; Fig. 4). This phenotypic effect was observed in all types of media (different carbon and nitrogen sources) indicating increased sensitivity of this mutant

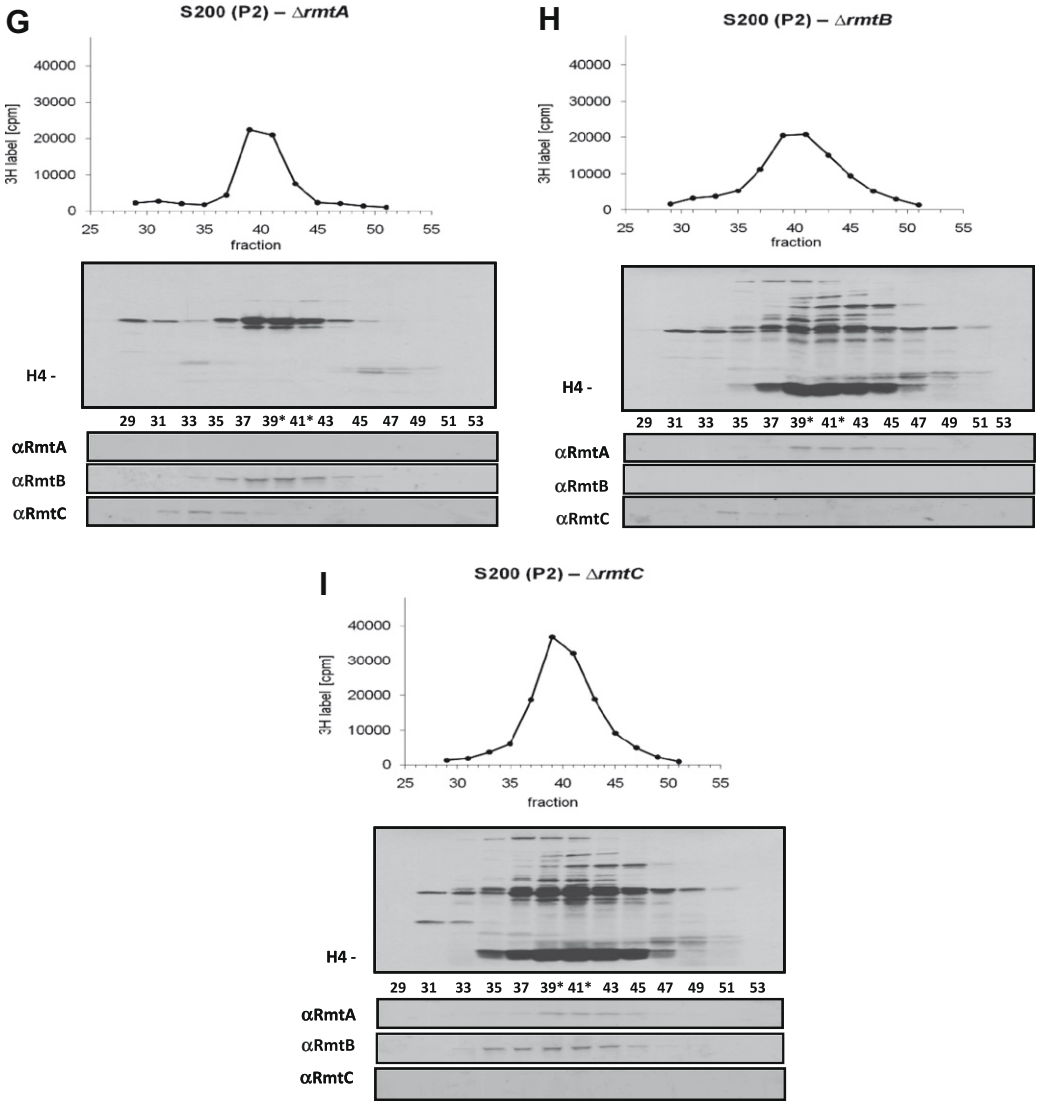

Fig. 3 (*continued*)

to heat stress conditions. However, in case of acetate as the sole carbon source, growth and conidiation of the Δ*rmtA* strain was significantly repressed at 37 °C with partial restoration at 42 °C, compared to wild type control. Conidiation of the Δ*rmtC* strain was largely abolished at all temperatures examined (Fig. 4B). No effect was observed for Δ*rmtB*.

As the life cycle of *A. nidulans* involves both sexual and asexual reproduction, we extended our phenotypic investigations also on the effects of mutants on sexual development. To test this, conidia of wild type and mutant strains were inoculated on GMM plates and incubated for 1 day. For induction of the sexual cycle, the plates were hermetically sealed and further incubated for 14 days in the dark. However, no significant differences in cleistothecia formation could be observed in the individual strains (Suppl. Fig. 3). Thus, our data indicate no direct effect of specific mutations on the sexual life cycle of *A. nidulans*.

3.5. Deletion of rmtA and rmtC results in growth reduction under conditions of oxidative stress

We next analyzed putative effects of *rmtA*, *rmtB*, and *rmtC* deletions under conditions of oxidative stress. For this purpose, deletion strains were exposed to different concentrations of hydrogen peroxide and strains were then compared with respect to growth rate and conidiation. As illustrated in Fig. 5A, increasing concentrations of hydrogen peroxide dramatically inhibited growth of Δ*rmtA* and Δ*rmtC* strains. In detail, a concentration of 3 mM H_2O_2 resulted in Δ*rmtA* growth retardation of more than 50%, and at 6 mM H_2O_2 conidiation was completely abolished. A similar, although slightly less pronounced effect was obtained for Δ*rmtC* (Fig. 5A).

The observed phenotype for RmtA prompted us to ask whether the lack of RmtA activity in the Δ*rmtA* mutant might be directly responsible for the reduced oxidative stress tolerance. Based on crystallographic studies of the mammalian ortholog PRMT1 (Zhang and Cheng, 2003), a catalytically inactive *rmtA* mutant was generated by site-directed mutagenesis. Mutation of an invariable active site glutamate (E153Q) impaired enzymatic activity of mammalian PRMT1 but did not influence oligomerization and thus the overall structural integrity of the protein. Therefore, the highly conserved glutamate E144 of RmtA (corresponding to E153Q of rat PRMT1) was mutated to glutamine as described in Section 2. Subsequently, complementation of the Δ*rmtA* strain was performed with either wild type or mutated *rmtA*. In order to prove that the mutation on RmtA indeed affected the activity of the enzyme, we expressed

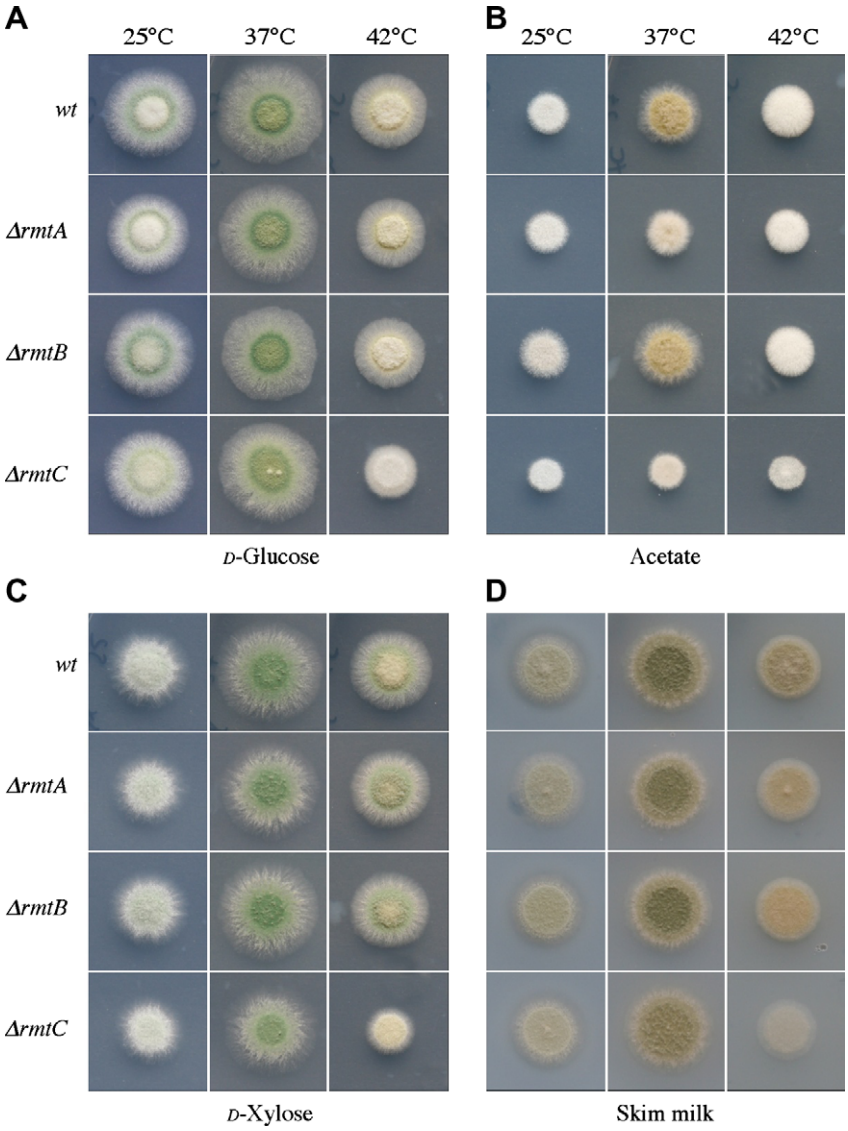

Fig. 4. Growth phenotypes of PRMT deletion strains on different carbon sources at different temperatures. Conidia (1×10^4) were point inoculated on minimal media plates, containing either: (A) D-glucose, (B) acetate, (C) D-xylose (50 mM each), or (D) skim milk (1%) as sole carbon sources. Plates were incubated at 25 °C for 72 h, and at 37 °C or 42 °C for 47 h. Ammonium chloride was used as nitrogen source in this experiment. Results represent phenotypic analyses derived from three to five independently isolated mutant strains.

both, wild type as well as the mutant protein as GST-fusion proteins in *E. coli* and analyzed them for MT-activity after affinity purification.

Our results demonstrate that only complementation of Δ*rmtA* with wild type *rmtA* (Δ*rmtA*^{c}) could restore hydrogen peroxide tolerance whereas transformation of Δ*rmtA* by a plasmid encoding catalytically inactive RmtA (Δ*rmtA*^{cE144Q}) had no restoration effect (Fig. 5A). Further, *in vitro* activity assays of wild type and mutant GST-RmtA fusion proteins revealed MT-activity for wild type GST-RmtA but not for the mutant enzyme (Fig. 5B). These data might indicate a direct link of RmtA enzymatic activity and oxidative stress response in *A. nidulans*.

4. Discussion

The recent sequencing and annotation of genomes from several eukaryotes identified putative PRMT encoding genes in various animals, fungi, plants, and protozoa (Bachand, 2007; Krause et al., 2007). However, analysis of arginine methyltransferase activities in these groups has been limited. For the understanding of the exact function of protein arginine methylation we need information (1) on the effects of the corresponding gene deletions (e.g. on viability of cells), and (2) on the features of the enzymes (e.g. specificity), which establish and maintain the methylation state.

The analysis of the genome of *A. nidulans* revealed the identity of three genes encoding for PRMTs. Subsequent expression and characterization of the corresponding recombinant proteins indicated that arginine methylation of histones plays a physiological role in this organism (Trojer et al., 2004). To further study the *in vivo* role of PRMTs in *A. nidulans,* we therefore deleted the three PRMT genes (*rmtA*, *rmtB*, *rmtC*) and analyzed molecular and physiological effects of deletion strains.

Given the important cellular roles played by PRMTs in different organisms and the conservation of at least PRMT1 and PRMT5 in many eukaryotes, it was surprising that none of the gene deletions in *A. nidulans* had significant effects on cell viability under standard growth conditions; hence, a late onset of conidiation and a decrease in radial growth was observed for the *rmtC* mutant (Fig. 4 and Suppl. Fig. 4). In contrast, when environmental conditions

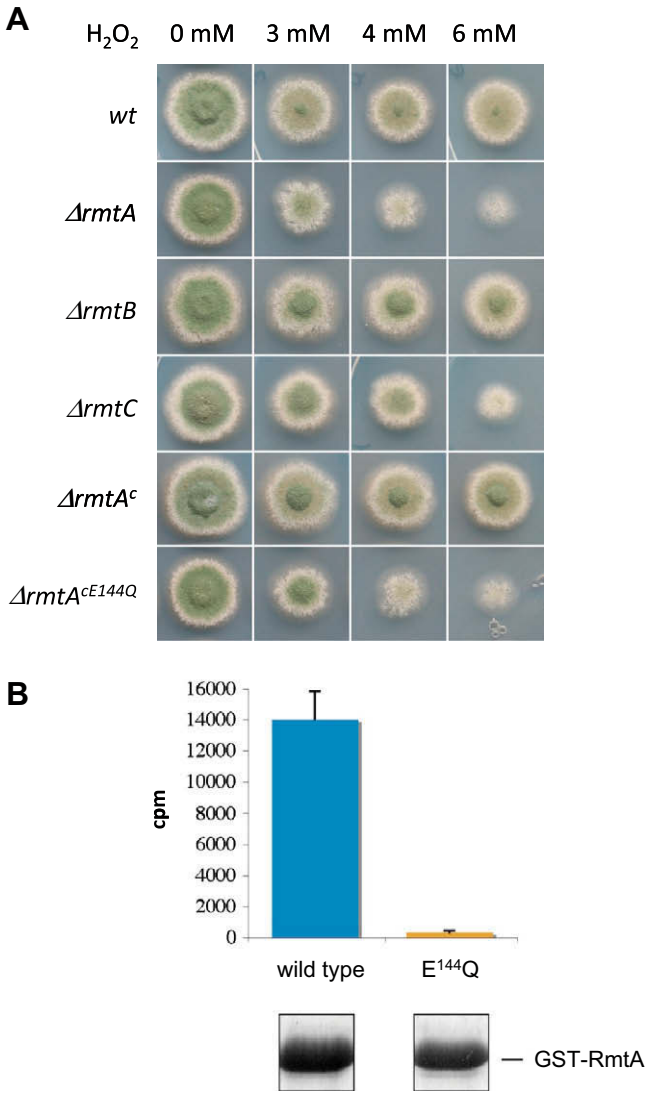

Fig. 5. Effect of hydrogen peroxide on growth of PRMT deletion strains, complementation of the oxidative stress phenotype, and *in vitro* activity of wild type and mutant GST-RmtA fusion proteins. (A) Conidia (1×10^4) were point inoculated on glucose minimal media plates and incubated at 37 °C. After 13 h, the agar was overlaid with top agar containing the indicated concentrations of hydrogen peroxide and strains were further grown at 37 °C for 24 h. For complementation, the *rmtA* mutant strain (*ΔrmtA*) was transformed with plasmids encoding either wild type *rmtA* (*ΔrmtA*^{C}) or a catalytically inactive *rmtA* mutant (*ΔrmtA*^{cE144Q}), which was generated by site-directed mutagenesis as described in Section 2. Analysis of the effect of hydrogen peroxide on growth of strains was performed as described in (A). Results represent phenotypic analyzes derived from three to five independently isolated mutant strains. (B) Wild type and mutant RmtA proteins were expressed in *E. coli* and equal amounts of affinity purified recombinant proteins were then assayed for MT activity. Experiments were done in triplicates. Standard deviation is indicated. Coomassie blue staining of purified proteins is shown as loading control.

were changed, e.g. when cells were exposed to oxidative stress conditions, growth of *rmtA* and *rmtC* mutant strains was negatively affected. These data might indicate that methylation of proteins by single *Aspergillus* PRMTs is associated with more specific functions within the cell, e.g. in stress response, rather than with general genome-wide methylation pathways.

Our observations that PRMT gene deletions had no or only subtle effects on growth, however, are in line with data obtained from other organisms. For example, in *S. cerevisiae* (Gary et al., 1996; Henry and Silver, 1996; McBride et al., 2000) and in *Candida albicans* (McBride et al., 2007), the *hmt1* mutant cells were viable, but a dramatic decrease in the levels of mono- and asymmetrically dimethylated arginine residues was observed. Mammalian PRMT1 is also not required for cell viability, however, is essential for embryonic development in mice (Pawlak et al., 2000). Mutants lacking RmtC homologous proteins were successfully generated in *S. cerevisiae* (Ma et al., 1996), *S. pombe* (Gilbreth et al., 1996), and *Arabidopsis thaliana* (Wang et al., 2007). For example, *S. cerevisiae* cells lacking Hsl7p have been shown to be delayed in G2/M transition resulting in a slow growth phenotype (Ma et al., 1996). Similarly, knockdown of PRMT5 led to reduced proliferation of transformed human cells (Pal et al., 2007).

The lack of pronounced phenotypes of PRMT mutants in *A. nidulans* under standard growth conditions, implies that these enzymes might play partially redundant functions. On the other hand, the classification of RmtA and RmtC into type I and type II PRMTs by their clear homology to PRMT1 and PRMT5 family proteins (Trojer et al., 2004) argues for more specific roles of *Aspergillus* PRMTs. Type I and type II PRMTs generate different states of methylarginine (asymmetric or symmetric dimethylation), which are regarded to have different functional consequences as well. For example, PRMT1 and PRMT5 can methylate H4R3, however, while asymmetric dimethylation of H4R3 by PRMT1 induces transcription (e.g. Wang et al., 2001), symmetric methylation of H3R3 and H4R8 by PRMT5 inhibits transcription (Pal et al., 2004). Since no enzymes that are able to catalyze both reactions have been identified yet, it seems unlikely that isolated RmtA and RmtC proteins in *Aspergillus* can substitute for each other.

The most pronounced phenotype we found for our PRMT mutants arose from exposure of mutant strains to oxidative stress conditions. Deletion of *rmtA* and *rmtC* resulted in significant growth reduction after treatment of cells with different concentrations of hydrogen peroxide. Hydroxyl radicals, reactive products of hydrogen peroxide, can cause damage to DNA, lipids peroxidation, and protein inactivation (Aguirre et al., 2006). Enzymatic detoxification, which is performed by superoxide dismutases, catalases, or peroxidases represents an important strategy to maintain low intracellular ROS levels (Aguirre et al., 2005). In a recent study, deletion of a class II histone deacetylase (*hdaA*) in *A. nidulans* displayed a similar phenotype as shown for *rmtA* and *rmtC* in this study (Tribus et al., 2005). Expression analysis of known detoxifying enzymes of *Aspergillus* demonstrated that CatB, one of three *Aspergillus* catalases (Kawasaki and Aguirre, 2001), was upregulated with increasing concentrations of ROS in wild type but not in Δ*hdaA* strains. Since CatB is known to be responsible for the detoxification of hydrogen peroxide in hyphae and during conidiation (Kawasaki et al., 1997), it was concluded that the failure to upregulate CatB expression was the major reason for the sensitivity of the Δ*hdaA* strains against ROS (Tribus et al., 2005). Because of the interactive role of histone modifying proteins in the regulation of chromatin structure and function and the observation of similar effects of mutations under induced growth conditions, we also considered an interrelation between histone deacetylation and arginine methylation in *A. nidulans*. Such crosstalk has been observed for example for the chromatin remodeling complexes NURD and hSWI/SNF, that harbor multiple chromatin-modifying enzymes with repressor activity such as HDACs (Sif et al., 2001; Zhang et al., 1999), PRMT5 (Pal et al., 2004; Pal et al., 2003), and MBD2/3 (Harikrishnan et al., 2005; Le Guezennec et al., 2006). We therefore analyzed expression of catB and other detoxifying enzymes, including the superoxide dismutases SodA and SodB (Oberegger et al., 2001; Oberegger et al., 2000), the three *Aspergillus* catalases CatA, CatB, and CatC (Kawasaki and Aguirre, 2001), and the catalase-peroxidase CpeA (Scherer et al., 2002) at increasing hydrogen peroxide concentrations in Δ*rmtA* and Δ*rmtC* strains, as well as wild type strains. However, in the PRMT mutants expression of none of the analyzed enzymes was significantly altered

compared to the wild type control (see Suppl. Fig. 5). Thus, other mechanisms that are involved in ROS resistance, for example non-enzyme mediated antioxidant mechanisms such as vitamins, melanin or mannitol or MAPK (mitogen-activated protein kinase) cascades (for review see Aguirre et al., 2006; Aguirre et al., 2005; Moye-Rowley, 2003) might be affected by the deletion of *rmtA* and *rmtC*, respectively.

We have recently demonstrated histone specificity for the recombinant RmtB protein. The affinity purified GST-fusion protein exhibited specificity for histones H4, H3, and H2A (Trojer et al., 2004). The analysis of endogenous RmtB activity in this study, however, revealed a dramatic change in its substrate specificity as the native form was unable to use histone proteins as substrate. Instead, some proteins, which eluted in the same fractions as the enzymatic activity served as substrates in our assay. Although the identity of these proteins is not clear yet, their binding to RmtB might regulate the specificity of the *Aspergillus* PRMT or, alternatively, RmtB might constitute a complex that alters substrate specificity. In this sense, RmtB might still have affinity for histones although not for its free form but rather for histones which are integrated into nucleosomes. A similar "switch" in substrate specificity of recombinant protein versus the complexed form was demonstrated for the PRMT4 enzyme CARM1 (Xu et al., 2004). Moreover, formation of homodimers or larger homooligomers has also been linked to enzyme activity e.g. for Hmt1 (Weiss et al., 2000), PRMT1 (Zhang and Cheng, 2003), and PRMT5 (Rho et al., 2001). All structurally characterized PRMTs (rat PRMT1, rat PRMT3, yeast RMT1/hmt1, and CARM1/PRMT4) form a ringlike dimer (Cheng et al., 2005), and dimer formation of PRMTs is thought to be necessary for methyltransferase activity (Troffer-Charlier et al., 2007; Weiss et al., 2000; Zhang and Cheng, 2003; Zhang et al., 2000). Thus, regulation of multimer formation could serve as a means of regulating PRMT enzymatic activity in *A. nidulans*. Finally, the specificity of RmtB might also be influenced by post-translational modifications (PM). Such putative modifications are not introduced in bacterially expressed proteins and their lack could be responsible for differences in activities and/or substrate specificities of the *Aspergillus* enzyme.

In this manuscript we demonstrate for the first time the complete biochemical analysis of enzymatic activities of PRMTs in a filamentous fungus and the phenotypic effects of the corresponding mutants. The intense investigation of substrate specificities, i.e. the identification of (novel) substrate proteins, the demonstration of the *in vivo* methylation along with further gene disruptions, including the generation of double and triple deletions will help to specify the roles of RmtA, RmtB, and RmtC in *A. nidulans*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2010.03.006.

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