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Protein Arginine Methylation in Parasitic Protozoa[▽]

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Protozoa constitute the earliest branch of the eukaryotic lineage, and several groups of protozoans are serious parasites of humans and other animals. Better understanding of biochemical pathways that are either in common with or divergent from those of higher eukaryotes is integral in the defense against these parasites. In yeast and humans, the posttranslational methylation of arginine residues in proteins affects myriad cellular processes, including transcription, RNA processing, DNA replication and repair, and signal transduction. The protein arginine methyltransferases (PRMTs) that catalyze these reactions, which are unique to the eukaryotic kingdom of organisms, first become evident in protozoa. In this review, we focus on the current understanding of arginine methylation in multiple species of parasitic protozoa, including *Trichomonas*, *Entamoeba*, *Toxoplasma*, *Plasmodium*, and *Trypanosoma* spp., and discuss how arginine methylation may play important and unique roles in each type of parasite. We mine available genomic and transcriptomic data to inventory the families of PRMTs in different parasites and the changes in their abundance during the life cycle. We further review the limited functional studies on the roles of arginine methylation in parasites, including epigenetic regulation in Apicomplexa and RNA processing in trypanosomes. Interestingly, each of the parasites considered herein has significantly differing sets of PRMTs, and we speculate on the importance of this diversity in aspects of parasite biology, such as differentiation and antigenic variation.

The posttranslational methylation of proteins was first demonstrated by Paik and Kim in the late 1960s (103–106). The field of protein methylation was underappreciated, however, until the mid-1990s, when more extensive investigations revealed the enzymes responsible for protein methylation (48, 84). The transfer of methyl groups from the methyl donor *S*-adenosylmethionine (AdoMet) to proteins has been documented on carboxyl groups (cysteine, leucine, glutamate, aspartate) and nitrogen groups (glutamine, histidine, lysine, arginine, and N-terminal alpha-amino). A major research focus to date has been the roles of histone lysine and arginine methylation, which function in regulating transcription activation or repression (73, 76, 127). However, arginine methylation is a widespread posttranslational modification, occurring on multiple classes of proteins in both the cytoplasm and nucleus (13), and many recent studies have addressed the functions of this modification on nonhistone proteins (reviewed in references 7 and 80). Protein arginine methylation appears to be an evolutionarily ancient modification. Although absent in prokaryotes, methylarginine proteins and enzymes responsible for their synthesis have been reported in earlier diverged organisms (reviewed in reference 4). Here, we provide an introduction to protein arginine methylation and discuss the current state of knowledge regarding the synthesis and functions of this modification in parasitic protozoa.

PROTEIN ARGININE METHYLATION: ENZYMES AND FUNCTIONS

The transfer of methyl groups from AdoMet to arginine residues in proteins is carried out by a family of seven beta fold (Rossmann fold) enzymes known as protein arginine methyltransferases (PRMTs) (4, 7, 49, 70). PRMTs are further classified into four types, designated I to IV, according to the terminal arginine modification produced by each type of enzyme. The first catalytic step of arginine methylation involves the transfer of a single methyl group from AdoMet to the terminal nitrogen group of arginine, yielding omega- N^G -monomethylarginine (MMA). The type I, type II, and type III PRMTs all catalyze this first methyl transfer reaction (Fig. 1), and type III PRMTs can catalyze only the production of MMA. Type I PRMTs subsequently transfer a second methyl group to the same terminal nitrogen, yielding omega N^G - N^G -asymmetric dimethylarginine (ADMA). Alternately, type II PRMTs transfer a second methyl group to the adjacent terminal nitrogen, yielding omega N^G - N^G -symmetric dimethylarginine (SDMA). Lastly, type IV PRMTs catalyze the transfer of only a single methyl group to the internal (guanidino) nitrogen of arginine (Fig. 1). To date, type IV PRMTs have been demonstrated only in the yeasts *Saccharomyces cerevisiae* and *Candida albicans* but may also be present in plants as well based on sequence similarities (90, 99) (Table 1). The vast majority of PRMT research has focused on three archetype organisms, the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* and humans (Table 1). Unicellular yeasts exhibit a trend apparent in most eukaryotes, which is the presence of a type I PRMT (Hmt1 in *S. cerevisiae*; Rmt1 in *S. pombe*) and a type II PRMT (Hsl7 in *S. cerevisiae*; Skb1 in *S. pombe*) (Table 1). Another type I PRMT, Rmt3, a homologue of the human PRMT3, has also been demonstrated in *S. pombe* (Table 1). In addition, *S. pombe* may also possess a type IV PRMT, but this has not been demonstrated. In humans, there are currently nine confirmed

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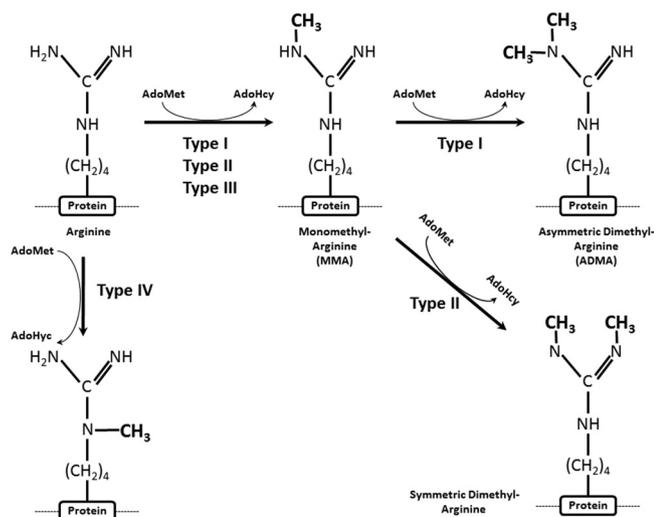


FIG. 1. Protein arginine methylation reactions. Using the methyl donor *S*-adenosyl methionine (AdoMet), protein arginine methyltransferases (PRMTs) catalyze the transfer to the terminal (ω) nitrogen, yielding monomethylarginine (MMA) and the by-product *S*-adenosyl homocysteine (AdoHcy). This initial reaction is carried out by PRMT types I, II, and III. Subsequently, type I PRMTs synthesize ADMA by catalyzing a second methyl group addition on the same terminal nitrogen, while type II PRMTs transfer a second methyl group to the adjacent terminal nitrogen, yielding SDMA. Type IV PRMTs catalyze the transfer of a single methyl group to the internal (δ) nitrogen, in a reaction that is currently thought to be confined to yeast.

PRMTs, the majority of which are type I enzymes (PRMT1, -2, -3, -4 [also known as CARM1], -6, and -8). Humans also possess a single type II enzyme (PRMT5) and a third enzyme whose designation as a type II or III PRMT is still in question (PRMT7). Finally, the enzymatic activity of PRMT9 has not been explored, but it may be a type II or III PRMT based on

sequence homology (7) (Table 1). The existence of multiple type I PRMTs in humans can be partially explained by enzyme redundancy (40, 68, 138). In addition, the substrate specificity of some human PRMTs is confined through subcellular localization or cell type (7, 49). For example, HsPRMT1 and HsPRMT8 are 72% identical; however, the N terminus of PRMT8 alters its enzymatic activity and confers localization to the cell membrane through posttranslational myristoylation (78, 122). Regardless, the human PRMT1 is the workhorse of the type I group, accounting for 85% of the methylation reactions in mammalian cells (137). Protein arginine methylation was long considered to be an irreversible modification, and this subject is still not resolved. An initial report of direct histone arginine demethylation (23) has not been reproduced (50, 145). It is clear, however, that the arginine methylation status of a protein can be modulated indirectly by protein arginine deiminases. This class of enzymes modulates protein function by catalyzing the deimination of arginine and monomethylarginine residues to citrulline, which are then unable to serve as substrates for PRMTs (29, 144, 148). Conversely, a dimethylated arginine cannot be citrullinated, so the two processes of arginine methylation and citrullination negatively regulate each other (115).

Protein arginine methylation modulates multiple cellular pathways in yeasts and humans. As stated above, much of the research in this area has focused on transcriptional regulation and the effects of arginine methylation on the exposed tails of histones H3 and H4. Methylation of arginine residues in histones has been linked to transcriptional activation or to indirect repression of transcription by subsequent activation of lysine methylation at adjacent sites (53, 54). For thorough reviews of histone methylation, refer to several existing reviews on this subject (76, 132, 148). In addition, several nonhistone substrates of arginine methylation have been reported to modulate transcription, either as transcriptional coactivators with

TABLE 1. Established PRMTs in yeasts and humans

Organism	PRMT name	Gene identifier	PRMT type	Size (no. of amino acids/mol mass [kDa])	Note	Reference(s)
<i>Saccharomyces cerevisiae</i>	Hmt1/Rmt1	NP_009590	I	348/40		42
	Hsl7	NP_009691	II	827/95		79, 121
	Rmt2	NP_010753	IV	412/47		99
<i>Schizosaccharomyces pombe</i>	Rmt1	NP_594825	I	340/39		146
	Rmt2	NP_594160	IV ^a	357/41		146
	Rmt3	NP_595572	I	543/62		5
	Skb1	NP_595936	II	645/73		113
<i>Homo sapiens</i>	HsPRMT1	NP_938074	I	353/41		84
	HsPRMT2	NP_001526	I	433/49		74
	HsPRMT3	NP_005779	I	531/60	Zn finger required for substrate recognition	138
	CARM1 (HsPRMT4)	NP_954592	I	608/66		24
	HsPRMT5	NP_006100	II	637/73		18
	HsPRMT6	NP_060607	I	375/42		40
	HsPRMT7	NP_061896	II/III ^a	692/78	Contains duplication of active domains	77, 93
	HsPRMT8	NP_062828	I	394/45		78
	HsPRMT9 (4q31)	NP_612373	^a	845/95	Contains duplication of active domains	6

^a Type of PRMT activity undetermined.

histones (1) or as DNA binding transcription factors (72, 95, 96, 151). The largest family of nonhistone proteins that are arginine methylated is RNA binding proteins (RBPs) (17, 85, 102, 110, 129). One reason for the abundance of arginine-methylated RBPs is the common occurrence in this class of proteins of glycine-arginine-rich (GAR) domains, which commonly serve as PRMT substrates (64, 85, 97). Arginine methylation of RBPs affects multiple pathways, including pre-mRNA splicing (12, 16, 25, 100), RNA stability (81), translation (5, 22, 116, 125), and small RNA pathways (65, 141). Other cellular pathways that are affected at several points by arginine methylation include DNA damage and repair (14, 15, 33, 47, 60), cell signaling (10, 11, 41, 57, 58, 71), and organelle biogenesis (107, 147, 152).

Protein arginine methylation generally alters protein function by influencing protein-protein interactions, protein-nucleic acid interactions, and protein trafficking. The addition of a methyl group to arginine, while not changing its charge, does alter the side chain shape, thereby increasing hydrophobicity and steric hindrance while also removing a potential hydrogen donor group (7). Therefore, arginine methyl proteins are often altered in their abilities to bind other protein partners or nucleic acids. For example, arginine methylation directly disrupts the binding of Sam68 to SH3 (8). Conversely, arginine methylation of several proteins is required for their binding to Tudor domain proteins (16, 27, 142). Direct effects on nucleic acid binding are less common. However, the arginine methylation of several RBPs, including the HIV Rev and nucleocapsid proteins, results in diminished RNA binding (55, 56, 117). Arginine methylation also greatly impacts the subcellular localization of proteins and RNA (3, 46, 52, 83, 124, 130). The first reported instance of this phenomenon was in yeast lacking Hmt1p, where the nuclear export of two RNA binding proteins, Npl3 and Hrp1, is blocked (124). Arginine methylation can also facilitate nuclear import of proteins such as hnRNPA2 and RNA helicase A (98, 130). Lastly, arginine methylation may impact subsequent posttranslational modification of a protein. For example, methylation of histone H3R2 antagonizes H3K4 methylation, and H4R3 methylation facilitates H4K9/K14 acetylation (53, 82). In summary, the effects of arginine methylation on target proteins can be profound, and many of these are still being elucidated.

CLASSES OF PRMTs IN PARASITIC PROTOZOA

In this review, we focus on several groups of parasitic protozoa of human health importance, including the amitochondrial metamonads (*Giardia*, *Trichomonas*) and archamoebae (*Entamoeba*), the Apicomplexa (*Plasmodium*, *Toxoplasma*), and the kinetoplastids (*Trypanosoma*, *Leishmania*). Overall, the current understanding of arginine methylation in these organisms is limited. Using several published reports and genome mining of parasite databases, we compiled a list of putative PRMT enzymes in each group (Table 2). Those parasites that contain PRMTs resemble yeasts in that they harbor at least one type I PRMT with homology to human (Hs) PRMT1 and at least one type II PRMT with homology to HsPRMT5 (Table 2). Outside this generalization, however, there are vast differences in the number and potential types of PRMTs in each parasitic group.

***Giardia*.** According to several genomic studies, *Giardia* and the related metamonads represent the most simple of eukaryotes, lacking the typical mitochondria and Golgi complexes found in other eukaryotes (30, 34, 126). Additionally, many genes and systems are believed to have been lost as the organisms transitioned to a parasitic lifestyle (94). In terms of protein methylation, at least six SET-like lysine methyltransferases can be identified in the *Giardia* genome database (131). In striking contrast, we were unable to identify any apparent PRMT homologues in the current annotation of the *Giardia* database (GiardiaDB) (Table 1), marking this as the only known eukaryotic group lacking PRMTs. Accordingly, to our knowledge, there are no reports of methylarginine proteins in this parasite. Barring the inclusion of a noncanonical PRMT in *Giardia*, it appears that the downstream effects of arginine methylation required for other eukaryotes are dispensable in *Giardia*.

***Trichomonas*.** The sexually transmitted parasite *Trichomonas vaginalis*, like *Giardia*, represents a group that underwent vast gene loss upon transition to a parasitic life cycle (20, 59). Also similar to *Giardia*, *Trichomonas* lacks a mitochondria or a true Golgi apparatus. However, *Trichomonas* differs substantially from *Giardia* in that it harbors at least eight putative PRMT enzymes in its genome (TrichDB) (Table 1). At least six of these enzymes appear to be homologues of the type I HsPRMT1, with various degrees of identity. TVAG_048280 and TVAG_225950 are 99% identical, indicating a true duplication event, which is expected considering the high number of transposable elements and repetitive genes evident in this organism (20). However, the remaining four type I PRMTs are dissimilar enough to raise the question of whether each serves a different function during the parasite life cycle. The *T. vaginalis* genome also contains two potential type II PRMTs, which are dissimilar enough to suggest that the enzymes play separate functional roles (Table 2). The putative *T. vaginalis* PRMTs have not been examined experimentally to date.

***Entamoeba*.** The intestinal parasite *Entamoeba histolytica* resembles *Giardia* and *Trichomonas* in its lack of a mitochondrion but differs in the presence of a rudimentary Golgi-like apparatus (19). In a trend similar to *Trichomonas*, *E. histolytica* also contains multiple homologues of HsPRMT1, two of which (EHI_202470 and EHI_152460) share more sequence similarity with the HsPRMT1 enzyme than does the annotated EhPRMT1 enzyme (EHI_105780) (Table 2). Although both *E. histolytica* and *T. vaginalis* have multiple type I PRMTs, none of these enzymes contain characteristics that would differentiate them from being purely a PRMT1 homologue. That is, all of the type I enzymes in these species display the highest homology to HsPRMT1, as opposed to other human type I enzymes, and they lack distinguishing features of some human type I PRMTs, such as the Zn finger present in HsPRMT3 or the N-terminal myristoylation signal present in HsPRMT8. In addition to its four putative type I PRMTs, the *E. histolytica* genome also encodes an apparent type II PRMT (EHI_158560), consistent with the common trend that all eukaryotes with PRMTs in their genomes have at least one type I and one type II enzyme. The activities of these putative PRMTs have not yet been demonstrated.

***Plasmodium*.** The genome of *Plasmodium falciparum* (PlasmoDB) encodes only three putative PRMTs, including ho-

TABLE 2. PRMTs in parasitic protozoa^a

Organism	Gene identifier	Published name	Inferred PRMT type	Activity type	Closest human homologue (% identity/similarity)	Size (no. of amino acids/mol mass [kDa])	Note	Reference
<i>Giardia lamblia</i>	NA	NA	NA	NA	NA	NA	No identifiable PRMT-encoding genes	
<i>Trichomonas vaginalis</i>	TVAG_048280	NA	I	ND	PRMT1 (22/28)	331/37	Duplication of TVAG_048280	
	TVAG_225950	NA	I	ND	PRMT1 (22/28)	331/37		
	TVAG_028100	NA	I	ND	PRMT1 (37/40)	320/37		
	TVAG_199700	NA	I	ND	PRMT1 (44/47)	327/37		
	TVAG_433490	NA	I	ND	PRMT1 (45/48)	327/37		
	TVAG_045760	NA	I	ND	PRMT1 (36/42)	320/37		
	TVAG_254540	NA	II	ND	PRMT5 (23/32)	436/50		
	TVAG_096150	NA	II	ND	PRMT5 (27/39)	528/60		
<i>Entamoeba histolytica</i>	EHI_202470	NA	I	ND	PRMT1 (41/48)	332/38		
	EHI_152460	NA	I	ND	PRMT1 (37/46)	328/38		
	EHI_105780	EhPRMT1	I	ND	PRMT1 (37/42)	319/37		
	EHI_159180	NA	I	ND	PRMT1 (25/32)	367/43		
	EHI_158560	NA	II	ND	PRMT5 (32/44)	586/69		
<i>Trypanosoma brucei</i>	Tb927.1.4690	TbPRMT1	I	I	PRMT1 (48/54)	345/39		109
	Tb927.10.3560	TbPRMT3	I	ND	PRMT3 (20/33)	389/42	Lacks Zn finger	108
	Tb927.10.640	TbPRMT5	II	II	PRMT5 (24/31)	784/87		108
	Tb927.5.3960	TbPRMT6	I	I	PRMT6 (28/31)	368/41		38
	Tb927.7.5490	TbPRMT7	III	III	PRMT7 (13/28)	390/44	Lacks C-terminal duplication	37
<i>Plasmodium falciparum</i>	PF14_0242	PfPRMT1	I	I	PRMT1 (37/51)	402/47		35
	PF08_0092	PfCARM1	I	ND	PRMT3 (17/27)	912/108	Lacks Zn finger	
	PF13_0323	PfPRMT5	II	ND	PRMT5 (30/44)	724/86		
<i>Toxoplasma gondii</i>	GT1_030400	TgPRMT1	I	ND	PRMT1 (46/57)	392/44		120
	GT1_074560	TgCARM1	I	I	CARM1 (28/48)	660/72		120
	GT1_001320	NA	I	ND	PRMT3 (24/29)	802/88	Weak Zn finger	
	GT1_126490	NA	II	ND	PRMT5 (24/39)	979/107		
	GT1_073730	NA	I ^b	ND	NA	1305/139	Unknown homologue, unusually large size	

^a NA, not applicable; ND, not determined.

^b Weak homology.

mologues of HsPRMT1 and HsPRMT5. This represents considerably fewer PRMTs than observed in the other parasitic protozoa presented here and is similar to the number of PRMTs found in the archetypical yeasts (Tables 1 and 2). Of the three putative *P. falciparum* PRMTs (PfPRMT), one has been experimentally confirmed to have PRMT activity, PfPRMT1 (35). Consistent with its sequence, PfPRMT1 is a type I enzyme. It contains an extended N terminus that is essential for enzyme activity and is present in both the cytoplasm and nucleus of the parasite. *In vitro*, PfPRMT1 is capable of methylating the transcriptionally activating histone H4R3, as well as histone H2A, although whether this enzyme can affect transcription *in vivo* is currently unknown. Several proteins involved in RNA metabolism are also *in vitro* substrates of PfPRMT1. The other two putative PfPRMTs were annotated PfPRMT5 and PfCARM1 due to sequence homologies, although the enzyme annotated PfCARM1 is actually quite diverged from other known CARM1 homologues, including that found in *Toxoplasma gondii*. In fact, this enzyme exhibits higher homology to HsPRMT3, although it lacks a zinc finger (Table 2) (120). Both PfPRMT5 and PfCARM1 contain stretches of asparagine residues, a common trend in the low-complexity, highly A/T-rich *P. falciparum* genome (112).

Toxoplasma. T. gondii contains five putative PRMTs in its genome (ToxoDB), two of which, TgPRMT1 and TgCARM1, have been experimentally shown to possess PRMT activity (120). These two enzymes exhibit substrate specificities similar to those of their human homologues in that TgPRMT1 methylates histone H4R3 and TgCARM1 methylates H3R17 using *in vitro* methylation assays. The addition of ATP and proteins with SWI/SNF activity significantly stimulated the ability of TgCARM1 to methylate nucleosomal histones *in vitro*, suggesting that this enzyme may act in combination with chromatin remodeling enzymes *in vivo*. TgCARM1 is likely homo-hexameric and localizes to the parasite nucleus. Functional studies implicate TgCARM1-mediated methylation in gene regulation and parasite development (see below). Of the three remaining putative TgPRMTs, one has significant homology to the type II HsPRMT5 (Table 2). Another gene (GT1_001320) has homology to HsPRMT3 and possesses a weak homology zinc finger, consistent with its being a PRMT3 homologue. The remaining TgPRMT (GT1_073730) is the most unique of this group due to its large size (139 kDa) and lack of significant homology to any other characterized PRMT in the literature, although it has very weak homology (<10%) in its conserved methyltransferase domain to PRMT6 and as such may be a type I PRMT

(Table 2). The activity of this noncanonical TgPRMT and its functions in the life cycle of *T. gondii* present a fascinating subject for future research.

Kinetoplastids. The kinetoplastid parasites (*Trypanosoma brucei*, *Trypanosoma cruzi*, and the *Leishmania* spp.) are the group of parasitic protozoa in which the most research on arginine methylation has been conducted to date. In this review, we will use the causative agent of African sleeping sickness, *T. brucei*, as the model for kinetoplastids, as the biochemical data are derived from this organism and the set of PRMT-encoding genes found in *T. brucei* is highly conserved in the other kinetoplastid parasite genomes (TriTrypDB). The *T. brucei* genome encodes five putative PRMTs, including obvious homologues of the type I HsPRMT1 and type II HsPRMT5 (Table 1). Like HsPRMT1, TbPRMT1 exhibits type I PRMT activity, and upon its knockdown through RNA interference (RNAi), a global reduction in ADMA was observed (109). Thus, TbPRMT1 is responsible for the vast majority of ADMA formation *in vivo*, similar to what is observed in mammalian cells (137). *In vitro*, TbPRMT1 appears to prefer GAR domain-containing substrates (109). *T. brucei* also contains a type II PRMT homologue, TbPRMT5, that displays a broad substrate range, including both GAR domain and non-GAR domain targets (108). TbPRMT5 associates *in vivo* with a DEAD-box RNA helicase (108) and is capable of methylating this enzyme *in vitro* (S. Menon and L. Read, unpublished data), suggesting that TbPRMT5 may affect RNA metabolism.

In addition to TbPRMT1, the *T. brucei* genome encodes two other type I PRMTs. However, in contrast to *Entamoeba* and *Trichomonas*, the three trypanosome type I enzymes do not constitute a family of PRMT1 homologues. Rather, each of the type I TbPRMTs displays clear homology to a different human type I enzyme (Table 2). One of these exhibits the highest homology to HsPRMT3. TbPRMT3 has not been characterized biochemically, although its sequence clearly positions it as a putative type I enzyme. In humans, PRMT3 interacts with its major known substrate, the ribosomal protein RPS2, through its N-terminal zinc finger, subsequently facilitating ribosome assembly (39, 135). Interestingly, TbPRMT3 lacks a zinc finger, as does the PRMT3 homologue from the fungus *Aspergillus* (140), suggesting that TbPRMT3 may exhibit a different substrate specificity than its human homologue. Yeast PRMT3 also displays methyltransferase-independent functions in ribosome assembly (111). The absence of the substrate binding zinc finger, in combination with nonconserved residues in the THW and double E loops in TbPRMT3, suggests that this enzyme might also have functions independent of methyltransferase activity.

The remaining two PRMTs in the kinetoplastids are intriguing, as they appear to be absent in other parasitic protozoa. We recently characterized the *T. brucei* homologues of HsPRMT6 and HsPRMT7 (37, 38). Like HsPRMT6, TbPRMT6 is a type I enzyme with an apparently narrow substrate specificity, and it undergoes automethylation, although the consequence of this is currently unknown (38). *In vitro*, TbPRMT6 apparently lacks the ability to methylate GAR domain-containing targets. Rather, it is capable of methylating bovine histones, raising the possibility that it might modify trypanosome histones *in vivo*. RNAi studies of TbPRMT6 indicate a vital role in the *T. brucei* life cycle (see below).

The trypanosome PRMT7 enzyme is the most unique of all kinetoplastid PRMTs (37). First, unlike HsPRMT7, which has a C-terminal duplication of the enzymatic domain that is essential for its activity, TbPRMT7 possesses only a single enzymatic cluster, making it roughly half the size of the human enzyme (Tables 1 and 2). Second, both GST-TbPRMT7 expressed in *E. coli* and TAP-TbPRMT7 expressed in *T. brucei* catalyze the formation of only MMA, marking this enzyme as the first exclusively type III PRMT demonstrated. Recently, a similar finding of a type III enzyme was also reported in the nematode *Caenorhabditis elegans* (136). In contrast, the type of activity catalyzed by HsPRMT7 is unresolved, having been reported as either type II or type III (77, 93). TbPRMT7 is also a very robust enzyme with a wide substrate range. It is estimated that TbPRMT7 is at least 30 times more active than its human homologue in terms of methyl groups transferred/hour/microgram of enzyme, making it one of the most active PRMTs described to date. *In vitro*, TbPRMT7 catalyzes methyltransfer to bovine histones, myelin basic protein, and multiple *T. brucei* RNA binding proteins, including the non-GAR domain protein MRP2 and the GAR domain proteins RBP16, TbRGG1, and TbRGG2, suggesting that it may be involved in numerous cellular processes *in vivo*. The presence in *T. brucei* of a very active PRMT that synthesizes only MMA is unique and suggests two potential functions of such an enzyme. First, MMA may be a more commonly used terminal methyl mark in kinetoplastids than other organisms. Although typically considered an intermediate in ADMA and SDMA synthesis, MMA has been reported in yeast to be an activating signal on histone H3(R2), compared to ADMA, which is commonly inhibitory at this position (66, 67). Likewise, MMA on the mammalian epidermal growth factor receptor was recently shown to affect downstream signaling (51). The second, but not mutually exclusive, possibility is the action of synergism between the type III TbPRMT7 with the other type I and type II trypanosome PRMTs. In this scenario, the highly active TbPRMT7 would deposit the initial MMA mark, which another type I or II PRMT could recognize as a substrate for catalysis of the subsequent ADMA or SDMA. Several human PRMTs have been demonstrated to have either processive or distributive enzymatic mechanisms (74, 75, 101), suggesting that different PRMTs have various abilities to recognize MMA in order to create ADMA or SDMA. Therefore, in the simplified trypanosomes, a distinct process may exist, involving a two-enzyme mechanism to facilitate the final dimethylarginine mark. Further kinetic studies as well as genetic knockout of the TbPRMT7 gene are necessary to finalize a definitive trypanosome model. Overall, trypanosomes may present a simplified model for studying the role(s) of MMA.

FUNCTIONS OF PROTEIN ARGININE METHYLATION IN PARASITE LIFE CYCLES

The presence of different sets of PRMT-encoding genes in the parasites discussed here suggests that protein arginine methylation makes distinct contributions during the life cycles of these organisms. Regulation of protein function through arginine methylation may be especially important during the dramatic life cycle transitions that these parasites undergo as they move through the environment, their vectors, and their

mammalian hosts. The complex lifestyles of parasitic organisms require massive changes in gene regulation and morphology, processes in which protein arginine methylation could feasibly play a role. Below, we summarize the currently limited information regarding developmental regulation of PRMTs and their functions in parasitic protozoa. We reference transcriptomic studies to address changing levels of PRMT-encoding RNAs during parasite life cycles. However, keep in mind that this may not necessarily reflect enzyme activity, since protein levels can differ from RNA levels and because PRMT activity can be modulated by association with regulatory proteins in the absence of changes in PRMT levels (62, 84, 118, 128).

Entamoeba. To our knowledge, no PRMT substrates have been identified, nor have any functional studies regarding protein arginine methylation been performed in *Entamoeba*. Transcriptomic data indicate that RNA levels of most *Entamoeba histolytica* PRMTs are unchanged during development. However, transcripts encoding the type I PRMT, EHI_202470, decrease substantially at early time points following intestinal *E. histolytica* infection in mice, suggesting a potential role in adaptation to different environmental niches (AmoebaDB). Histone lysine methylation is reportedly involved in regulation of the virulence factor amoebaphore A (Ehap-a), whose transcription is silenced following demethylation of H3K4, suggesting a role of methylation in pathogenesis (2). Because a typically repressive arginine at H3R2 is also conserved in *E. histolytica*, methylation at this arginine residue could feasibly alter the methylation at H3K4 and thus pathogenesis.

Plasmodium. The levels of RNAs encoding PfPRMTs are not substantially changed during development, with the exception of a slight (~30%) decrease for PfPRMT5 in the schizont stage (PlasmoDB). However, Western blot analysis of PfPRMT1 revealed developmentally regulated expression of this protein, which is present at very low levels in ring stages and highest levels in late trophozoites (35). Several methylarginine modifications on *P. falciparum* histones were detected in two mass spectrometry studies (92, 139). These include both mono- and dimethylated versions of H3R17, H4R3, and H3.3R17. As these same marks are often transcriptionally activating in other organisms, further research is needed to determine if these same sites are activating in the malaria parasite. H3R17 levels, which can potentially be attributed to PfCARM1 activity, were unchanged during development. In contrast, dynamic dimethylation of H4R3, which is mediated by PfPRMT1 *in vitro*, was observed during the parasite life cycle, with high levels of H4R3 observed in late trophozoites and schizonts (35). These data suggest a conserved role for histone arginine methylation in epigenetic gene regulation in *Plasmodium* along with other posttranslational modifications (28, 35, 91).

Toxoplasma. In the human host, *Toxoplasma gondii* exists in the rapidly growing tachyzoite form and the latent cyst form termed bradyzoites. Transcriptomic data are available for four of the five putative *Toxoplasma* PRMTs, all of which appeared unchanged at the RNA level during the life cycle. However, two functional studies have focused on the role of histone arginine methylation in *T. gondii* gene regulation and development (43, 120). Saksouk et al. (120) reported that several attempts to create null mutations of TgCARM1 were unsuccessful,

and even transient expression of enzyme-dead mutant TgCARM1 in tachyzoites resulted in parasites with decreased viability. These data strongly suggest that TgCARM1 is essential in tachyzoites, consistent with the perinatal lethality of CARM1 knockout in mice (120, 149). Treatment of extracellular tachyzoites with AMI-1, a PRMT inhibitor that affects TgCARM1 but not TgPRMT1, induced stage conversion to bradyzoite forms. Induced differentiation was correlated with a 50% decrease in global H3R17 methylation. Methylation of H3R17 *T. gondii* was also examined by comprehensive chromatin immunoprecipitation with microarray technology (ChIP-chip) studies of chromosome 1 (43). Gissot et al. detected dimethylated H3R17 at a specific subset of promoters, specifically at 4 of 52 genes exhibiting modified histone peaks (43). Interestingly, ESTs for all 4 of these H3R17-containing genes are present in both tachyzoites and bradyzoites, which is unusual since only 26 of 91 predicted genes on the chromosome 1 microarray have ESTs in both stages. This observation might suggest that H3R17 methylation is involved in tachyzoite to bradyzoite differentiation. While the specific role of TgCARM1-mediated H3R17 methylation still remains to be clarified, together these data point to this modification as an important regulator of signals for life cycle transitions in *T. gondii* (120). In addition, regulation of differentiation by TgCARM1-mediated methylation of nonhistone substrates remains to be explored.

Trypanosoma brucei. Three of the five *T. brucei* PRMTs (TbPRMTs 1, 5, and 6) are unchanged at the RNA level during development or transition from log to stationary phase (63, 114). In contrast, TbPRMT3 and TbPRMT7 RNA levels are decreased ~60% during the transition from log growth to stationary growth in the insect vector midgut procyclic form (PF), and TbPRMT7 RNA increases ~30% during differentiation from stumpy mammalian bloodstream forms (BF) to PF cells. To begin to define PRMT function in *T. brucei*, we knocked down each of the five individual PRMTs in the *T. brucei* genome using RNAi in the two major life cycle stages that can propagate in culture, the insect PF and the mammalian slender BF (37, 38, 108, 109) (J. Fisk, D. Tomasello, and L. Read, unpublished data). No growth defects were observed in TbPRMT3-, TbPRMT5-, or TbPRMT7-depleted cells or in TbPRMT1-depleted PF. The absence of a growth effect upon knockdown of these enzymes may reflect the partial depletion of protein levels by RNAi. Indeed, several attempts to knock out both alleles of TbPRMT7 in BF have been unsuccessful (J. Fisk and L. Read, unpublished data), suggesting that this enzyme may be essential for growth and that the reduced levels of this very robust enzyme that remain following RNAi are sufficient to maintain cell function *in vitro*. Redundant functions among some of the five TbPRMTs are also possible; several PRMTs are not essential in mammals and yeasts, indicating redundancy (89, 134, 150). In contrast to the studies described above, RNAi-mediated knockdown of TbPRMT1 in BF does result in a slow growth phenotype, consistent with the report of a growth defect and abnormal cell cycle progression noted for TbPRMT1 knockdowns in a chromosome 1-wide RNAi screen in BF (133). The essential function(s) of this enzyme in BF *T. brucei* awaits discovery.

In *T. brucei*, the most profound effect on growth and morphology was observed upon depletion of TbPRMT6 (38). Both

PF and BF cells ablated for TbPRMT6 grow slowly under normal culture conditions, and low serum stress in PF greatly exacerbates this effect. In addition to a growth defect, TbPRMT6 knockdown cells exhibit dramatic defects in cytokinesis with differential phenotypes in PF and BF (38). Thus, despite its apparently weak activity and narrow substrate range *in vitro*, TbPRMT6 appears to methylate an essential target *in vivo*. Western blot analysis of TbPRMT6 in subcellular fractions of PF *T. brucei* reveals a predominant cytoplasmic localization, with a small nuclear component. Interestingly, mass spectrometry of TbPRMT6-associated proteins in PF *T. brucei* revealed numerous histones and components of the nuclear pore complex, suggesting that these classes of proteins might serve as TbPRMT6 substrates *in vivo*. Whether TbPRMT6 modulates gene expression through histone methylation will be a particular area of future interest, and this putative function is consistent with our finding that bovine histones were the only identified TbPRMT6 substrate *in vitro*. To date, no arginine methylation has been detected on *T. brucei* histones, although a noncanonical methylated site was identified at H4R53 in the related *T. cruzi*, and this site is conserved in *T. brucei* (31, 61, 88). In addition, the major histone arginine residue that is methylated by PRMT6 in other organisms, namely, the H3R2, is conserved in *T. brucei* and other kinetoplastids (54, 61, 88). Analyses of histone modifications in *T. brucei* to date were performed using Edman degradation (61, 88), and recently developed mass spectrophotometric techniques for detection of methylarginines (143) may be useful for identification of previously overlooked methylarginine residues in histones of *T. brucei*. If so, it will be of interest to determine whether these sites are altered in TbPRMT6 knockdown cells.

In order to gain a broader understanding of the roles of arginine methylation in trypanosomes, we have used mass spectrometry to identify arginine methyl proteins in PF *T. brucei*. Early matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) experiments identified RBP16, a mitochondrial RNA binding protein that functions in RNA stabilization and editing, as an arginine methyl protein (44, 119). Methylation of RBP16 has also been detected in *Leishmania donovani* (44, 119). In *T. brucei*, RBP16 contains at least three arginine residues that can undergo methylation, and differentially methylated forms of the protein are present. Knockdown of TbPRMT1 abolished methylation at two of three sites examined, demonstrating that RBP16 is an *in vivo* TbPRMT1 substrate (44). Both TbPRMT1 knockdown and overexpression of non-methylatable RBP16 lead to destabilization of the mitochondrial ND4 RNA, which is also destabilized by knockdown of RBP16 itself. Thus, methylation of RBP16 is critical for some aspects of its function in RNA stabilization. Conversely, the role of RBP16 in RNA editing was unaffected by its methylation status, suggesting that arginine methylation can differentially affect RBP16 functions. Subsequent biochemical experiments demonstrated that methylation of RBP16 facilitates its association with macromolecular complexes within the trypanosome mitochondrion (45). RBP16 harboring arginine to lysine mutations at three methylatable positions fails to associate with these higher-ordered structures, an effect apparently due to perturbation of protein-protein interactions. The loss of these RBP16-containing protein complexes is correlated with the impaired ability of the protein to stabilize specific RNAs,

suggesting that methylation-specific protein-protein interactions may be important for this RBP16 function. The identities of methylarginine-specific protein binding partners of RBP16 are currently not known. However, it will be of great interest to identify proteins that bind in a methylarginine-sensitive manner since trypanosomes appear to contain very few of the Tudor domain proteins known to bind methylarginines in other species (27, 143). Arginine methylation also differentially affects the ability of RBP16 to bind to different classes of RNAs, increasing its association with mRNA but, conversely, decreasing its binding to guide RNAs. Therefore, limited studies of one trypanosome methyl protein reveal profound impacts of this modification on protein function.

More recently, we are utilizing a sensitive electron-transfer dissociation/collision-induced dissociation fragmentation method (32, 143) to examine the arginine methyl proteome of *T. brucei*. To date, we have identified over 850 arginine methyl proteins, originating from multiple cellular compartments and organelles. These include RNA binding proteins and modifying enzymes, DNA replication and repair proteins, signal transduction components, and several classes of metabolic enzymes. Approximately 200 of these proteins are mitochondrially localized and are predicted to affect many aspects of mitochondrial function (J. Fisk, J. Qu, and L. Read, unpublished data). Future efforts will be directed toward determining the cellular and biochemical consequences of arginine methylation in a subset of these proteins in *T. brucei*.

PERSPECTIVES

Multiple genes encoding putative PRMTs were readily identified in all of the species that we examined, with the exception of *Giardia* spp. Thus, arginine methylation is widespread throughout evolution. A common theme in parasitic protozoa, as in all PRMT-containing eukaryotes, is the presence of homologues of the type I HsPRMT1 and the type II HsPRMT5 in each species. The invariable presence of both of these enzyme classes indicates that ADMA and SDMA serve separate and essential purposes in these organisms. As PRMT1 accounts for the majority of PRMT activity in mammalian cells, it is not surprising that its archetype is found in each of these early branching eukaryotes. What is unusual is the propensity of some groups of parasitic protozoa to have multiple copies of a PRMT1-like enzyme (e.g., 6 in *T. vaginalis* and 4 in *E. histolytica*; Table 2). In human cells, there are six type I PRMTs, each with distinct sequence characteristics, substrates, and subcellular/tissue localizations that discriminate them from each other. In contrast, the PRMT1-like enzymes in *T. vaginalis* and *E. histolytica* lack distinct features enabling them to be differentiated from PRMT1 homologues. While some may simply have arisen through genetic duplication, the majority of PRMT1 homologues in *Trichomonas* and *Entamoeba* spp. have enough dissimilarity to suggest that they have distinct functions. The roles of these enzymes in parasite biology await discovery.

One potentially important role for arginine methylation in parasitic protozoa is in epigenetic control of gene expression through modification of histones. As noted above, histone arginine methylation has been reported in *Toxoplasma* and *Plasmodium* and likely affects life cycle transitions in the former.

Importantly, in the deadly human parasite *P. falciparum*, epigenetic regulation is also critical in modulating the expression of variant surface antigens (reviewed in references 28 and 91). *var* genes encode the variant PfEMP proteins that function in both immune evasion and in pathogenesis through their propensity to bind host cell surface receptors. Although epigenetic regulation of *var* gene expression has been attributed solely to changes in histone acetylation to date, histone arginine methylation could play an important role, potentially by modulating acetylation of nearby lysine residues. In the kinetoplastid parasite, *T. brucei*, epigenetic regulation also factors prominently in antigenic variation (36). Histone lysine acetylation and methylation are important in variant surface glycoprotein (VSG) transcription, and the latter affects VSG switching in BF *T. brucei*. While histone arginine methylation has not yet been reported in *T. brucei*, it is tempting to speculate that this modification could play a role in regulating VSG expression and/or switching by modulating the creation of adjacent lysine methyl and acetyl marks. Another potential role for arginine methylation in kinetoplastids is in cell cycle control. Histone acetylases and deacetylases, as well as lysine methyltransferases, have been shown to play a role in cell cycle progression in *T. brucei*. The cell cycle defects that we observed in TbPRMT6 knockdown cells (38) could result from secondary alterations in previously reported histone modifications and/or through direct effects of histone arginine methyl marks.

Posttranslational modifications of RBPs by arginine methylation may be especially important in kinetoplastids, as these organisms regulate gene expression primarily through post-transcriptional processes in which RBPs are prominent (26, 69, 86, 123). RBPs and RNA helicases have been reported to function in RNA decay, RNA editing, differentiation, and cell cycle control in *T. brucei*. As noted above, we have identified numerous RBPs and RNA helicases in the *T. brucei* arginine methylome, and several proteins that function in mitochondrial RNA editing and stability are PRMT substrates *in vitro* (37, 44, 108, 109). The ease with which *T. brucei* is genetically manipulated will allow us to define the roles of RBP arginine methylation in many critical aspects of the trypanosome life cycle.

Finally, the presence of multiple PRMTs in a wide range of parasitic protozoa raises the possibility of targeting these enzymes for chemotherapeutic attack. Anti-PRMT drugs are currently being developed for use as cancer therapeutics (9, 21, 87). Future studies on the functions of divergent PRMTs with unique features in parasitic protozoa could aid in drug development in addition to providing important insights into parasite biology.

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