ELSEVIER

Molecular & Biochemical Parasitology 114 (2001) 239–244

MOLECULAR & BIOCHEMICAL PARASITOLOGY

www.parasitology-online.com.

Short communication

A *Plasmodium falciparum* protein related to fungal RNA 5′-triphosphatases

Toshimitsu Takagi [1], Stephen Buratowski *

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA

Received 8 August 2000; received in revised form 21 February 2001; accepted 24 February 2001

Keywords: Nucleotide phosphohydrolase; *Plasmodium falciparum*; RNA 5′-triphosphatase

Messenger RNA of eukaryotic cells and most of their viruses carry a 'cap' structure at their 5′ end. RNA 5′-triphosphatase (RTPase) activity is required for the first step of cap biosynthesis. This activity removes the γ-phosphate from the 5′ end of a nascent mRNA to leave a diphosphate end. mRNA guanylyltransferase (GTase) subsequently adds GMP in a 5′–5′ orientation to form the structure, GpppN1-. These two activities are tightly associated and co-purify as 'mRNA capping enzyme' [1]. One or more methyltransferase completes the capping modifications.

At least two groups of cellular RTPases have been identified. Metazoan capping enzymes consist of a single polypeptide with two separable domains; a GTase domain and a RTPase domain that resembles the protein tyrosine phosphatases (PTPs) [2–4]. This type of RTPase domain appears to use a mechanism similar to that of PTPs, as a conserved nucleophilic cysteine is required for activity [2,5–8]. The capping enzymes of the yeasts *Saccharomyces cerevisiae* and *Candida albicans* define a second class of RTPases. In these organisms, GTase and RTPase are carried by distinct proteins that form a heterodimer complex. The amino acid sequence of the catalytic region of yeast RTPase subunit has no obvious similarity to PTPs [9,10]. Unlike metazoan RTPases, the yeast proteins require divalent cations for activity [11,12]. It has been suggested that fungal RTPases comprise a new family of metal-dependent nucleotide phosphohydrolase (NTPase)/RTPase. This family may also include capping RTPases encoded by some DNA viruses. Although there is no extensive sequence similarity between the viral and yeast RTPases, several short sequence elements found in all of these proteins have been shown to be essential for the activity [11–14].

To identify other possible members of the yeast RTPase family, we used the BLAST algorithm [15] to search GenBank for proteins with similarity to Cet1, the *S. cerevisiae* capping enzyme RTPase subunit [9]. Known relatives of Cet1 include CaCet1, the *C. albicans* RTPase subunit [10] and Ctl1 (also known as Cth1), a second RTPase from *S. cerevisiae* whose function is unknown but nonessential for viability [16,17]. In addition to these fungal proteins, an open reading frame from human parasite *Plasmodium falciparum* (PFC0985c, accession number: CAB39040) was found to have significant similarity to Cet1 (P = 3e-5). No related proteins were found in the complete Drosophila, C. elegans, or human genomes.

PFC0985c is a hypothetical protein with 591 amino acids whose predicted size is 69 kDa. This protein has 109 asparagine residues and 89 acidic residues that are largely clustered in the N-terminal (amino acids 100–250) and the C-terminal (aa 405–591) regions (Fig.

Abbreviations: CIP, calf intestine alkaline phopshatase; GTase, mRNA guanylyltransferase; IPTG, isopropyl β-D-thiogalactopyranoside; NTPase, nucleotide phosphohydrolase; PCR, polymerase chain reaction; PEI, polyethyleneimine; PTP, protein tyrosine phosphatase; RTPase, RNA 5′-triphosphatase; TLC, thin-layer chromatography.

* Corresponding author. Tel.: +1-617-4320696; fax: +1-617-7380516.

E-mail address: steveb@hms.harvard.edu (S. Buratowski).

[1] Present address: Shionogi BioResearch Corporation, 45 Hartwell Avenue, Lexington, MA 02421, USA.

PII: S0166-6851(01)00254-7

1A). The region containing residues 280–400 of PFC0985c has significant similarity to the C-terminal half of the Cet1 catalytic domain (aa 417–549), as well as the homologous regions from CaCet1, Ctl1/Cth1, and pct1 (an RTPase from *Schizosaccharomyces pombe*, GenBank accession number: CAB90131 [18]).

Particularly well conserved are motifs B and C [11,13], two consensus sequences identified in the fungal

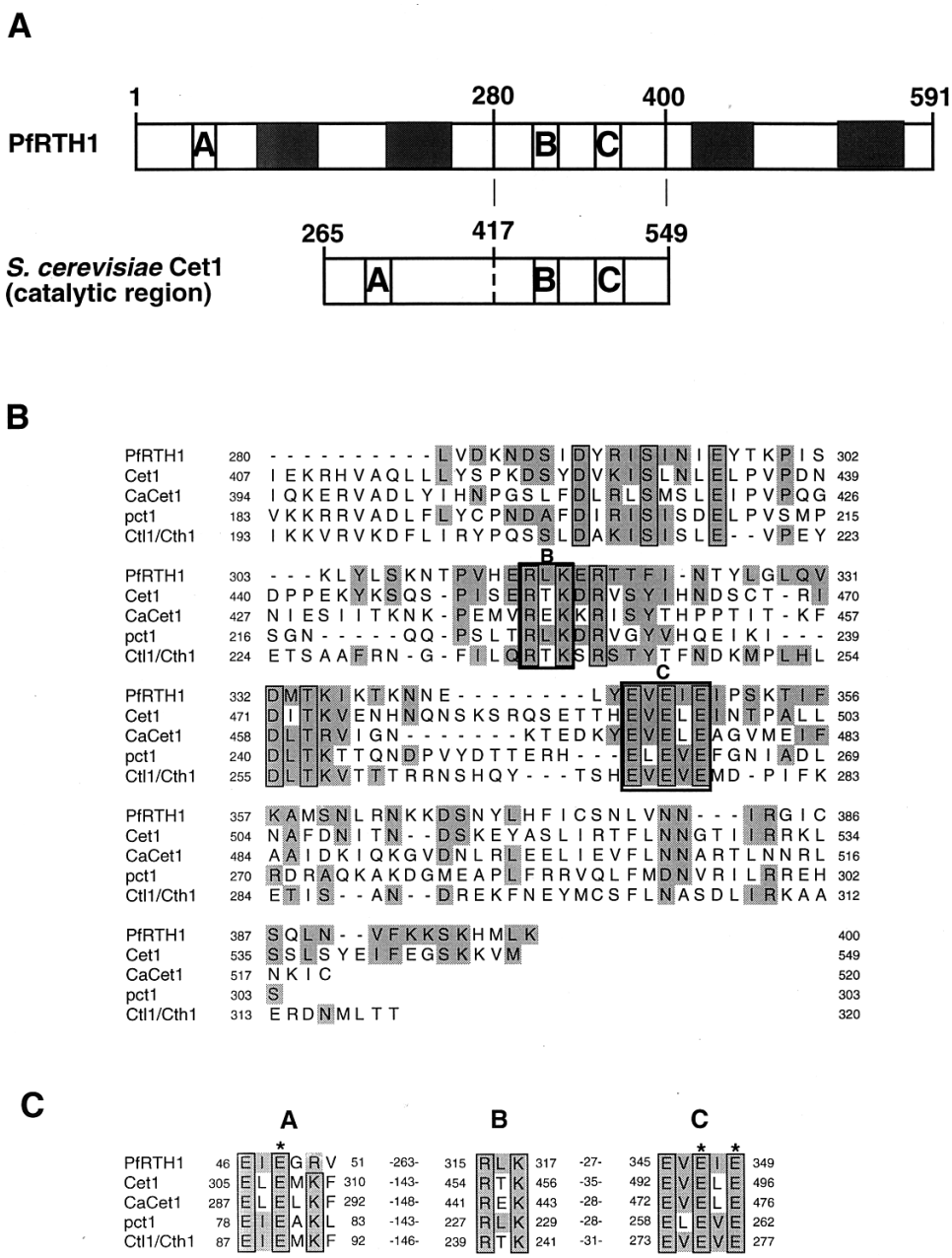

Fig. 1. *PfRTH1* encodes a protein similar to the fungal RTPases. (A) Schematic representation of the structure of PfRTH1 aligned with the catalytic domain of Cet1, the RTPase subunit of mRNA capping enzyme from *S. cerevisiae*. A, B, and C denote three consensus elements conserved in fungal and viral RTPases [11–14,17,18]. Shaded regions contains multiple repeats of aspargine and acidic amino acid residues. (B) Sequence alignment of PfRTH1 with fungal RTPases. The following sequences are shown: PfRTH1 (PFC0985, GenBank accession number CAB39040, residues 280–400); Cet1, (accession number O13297, residues 407–549 [9]); CaCet1, a subunit of *C. albicans* mRNA capping enzyme (accession number O93813, residues 394–520 [10]); pct1, an RTPase from *S. pombe* (accession number CAB90131, residues 183–303 [18]); Ctl1/Cth1, a second RTPase from *S. cerevisiae* (accession number NP013905, residues 193–320 [16,17]). Alignments were carried out using the BLAST algorithm [15]. Letters represent the single-letter amino acid code, and numbers represent the positions of the amino acid residues. Boxed residues denote identities, and shaded residues indicate similar amino acids. Motifs B and C are marked by lines. (C) The sequence of three conserved motifs found in PfRTH1 and fungal RTPase. The numbers of residues separating the motifs are also indicated. The asterisks indicate the PfRTH1 residues mutated in Fig. 2E.

and viral RTPases (Fig. 1B and C). A possible match for a third conserved element (motif A) was found in the N-terminal region of PFC0985c where no other similarity was apparent (Fig. 1C). The crystal structure of Cet1 shows that motifs A and C are involved in metal-binding [19]. Based on the sequence similarity as well as biochemical results presented below, it appears that the Plasmodium protein represents the first eukaryotic member of the metal-dependent NTPase/RTPase family found outside yeast. Accordingly, we have designated the gene *PfRTH1*, for *P. falciparum* RNA triphosphatase homologue.

Since there were no predicted introns in the genomic sequence, the *PfRTH1* gene was amplified by PCR using genomic DNA from *P. falciparum*. Histidine-tagged protein (his_7-PfRTH1) was expressed in *Escherichia coli* strain BL21 (DE3) using a bacteriophage T7 promoter/polymerase system and purified using Ni^{2+}-NTA agarose column chromatography. Although the protein was not detectable using SDS-PAGE and Coomassie staining (data not shown), immunoblotting with anti-polyhistidine antibody revealed a protein of about 70 kDa as well as multiple bands of smaller sizes (data not shown). It was unclear if these are proteolytic products of a full-length protein or the result of premature translation terminations that may be caused by unfavorable codon usage. We could not obtain complete purification of intact protein away from these smaller products (data not shown). For biochemical analysis, an immunoprecipitate was prepared from the Ni^{2+}-NTA agarose fraction using the anti-polyhistidine antibody. A control precipitate from *E. coli* containing only T7 expression vector was prepared in parallel. The immunoprecipitates were tested for RTPase activity (Fig. 2A) using an RNA trinucleotides labeled at the γ position of its 5′-triphosphate end (**p**ppACC; bold denotes position of radioactive phosphate). Phosphate was released in the presence of either magnesium (lane 4) or manganese (lane 6), but not when EDTA was added to chelate divalent cations (lane 2). The fraction prepared from cells transformed with vector did not release phosphate (lane 1, 3, and 5). Activity was also not detected when the antibody was omitted from the immunoprecipitation (data not shown). Based on these negative controls, we concluded that PfRTH1 protein has a metal-dependent RTPase activity.

Fungal RTPases also exhibit NTPase activity in the presence of manganese [11,12,16,17]. Therefore, we tested the substrate specificity of PfRTH1 at different concentrations of magnesium and manganese (Fig. 2B). Release of phosphate from **p**ppACC was detectable at 0.1 mM magnesium, but not at 0.01 mM (left panel,

Fig. 2. Recombinant PfRTH1 protein has metal dependent RTPase and NTPase activities. (A) RTPase assay. Ni^{2+}-NTA agarose fractions were prepared from cells containing empty vector (lanes 1, 3, and 5), or the expression plasmid for PfRTH1 (lanes 2, 4, and 6). Polyhistidine-tagged PfRTH1 protein was then immunoprecipitated and was incubated with [γ-^{32}P]ATP-terminated trinucleotide RNA (**p**ppACC) (see below for methods). In addition, reactions contained 2 mM EDTA (lanes 1 and 2), 0.5 mM $MgCl_2$ (lanes 3 and 4), or 0.5 mM $MnCl_2$ (lanes 5 and 6). Products were analyzed by polyethyleneimine (PEI)-cellulose thin layer chromatography (TLC) and visualized by Fuji Bas X Phosphor-Imager. The position of phosphate was determined by treating the substrate with CIP (data not shown). Asterisks indicate location of ^{32}P label. (B) Effect of concentration of divalent cation on NTPase and RTPase activities. Immunoprecipitated PfRTH1 protein was incubated with: left panel, [γ-^{32}P]ATP-terminated trimer RNA (**p**ppACC); right panel, [γ-^{32}P]ATP (**p**ppA). Reactions contained 2 mM EDTA (lane 1) or the indicated concentration of $MgCl_2$ (lanes 2–4) or $MnCl_2$ (lanes 5–7). (C) PfRTH1 leaves a diphosphate end. Immunopreceipitate fraction prepared from cells containing PfRTH1 plasmid was assayed for ATPase with [α-^{32}P]ATP (pp**p**A) in the presence of either 2 mM EDTA (lane 1) or indicated concentration of $MnCl_2$ (lanes 2–4). The positions of authentic ADP and AMP markers were detected by UV light, and the position of phosphate was determined by treating the substrate with CIP (data not shown). (D) Substrate specificity. PfRTH1 protein immunoprecitated was incubated with **p**ppA, [γ-^{32}P]ATP-terminated dimer RNA (**p**ppAC), or **p**ppACC with the indicated concentration of divalent cation. Released ^{32}Pi separated by TLC was visualized by autoradiography and radioactive spots were cut out and counted by liquid scintillation. (E) Mutation analysis. Ni^{2+}-NTA-agarose/immunoprecipitate fractions were prepared from cells containing empty vector, PfRTH1 plasmid, mutant E48Q plasmid, mutant E347D plasmid, or mutant E349D plasmid. The pellets were used for the NTPase assay with **p**ppA or the RTPase assay with **p**ppACC in the presence of the indicated concentration of divalent cation. Quantitative analysis was carried out as described in Fig. 2D, and values are expressed relative to 1.0 for released ^{32}Pi by wild-type PfRTH1. Methods; The *PfRTH1* open reading frame was amplified from genomic DNA (*P. falciparum* 3D7 strain) and restriction sites were introduced by PCR using Vent DNA polymerase (New England BioLabs) and oligonucleotide primers, Plasmodium-5′ (5′-GGA TCC **ATG G**TA AGA GAA GCA CAT GAA TTA TTA GAT-3′); Plasmodium-3′ (5′-GGA TCC TTA TGT ATC ATC ATA AAA GTC CTT ATA ATT-3′) (*Nco* I site bolded, *Bam*HI site underlined). Through a set of intermediate cloning steps (details available upon request) the *PfRTH1* open reading frame was subcloned into the T7 expression vector $pSBEThis_7$ [16]. Site-directed mutagenesis was performed using a PCR-mediated method [23] and three mutagenic oligonucleotides, RTP1-E48Q (5′-CCC CAC CCT TCC TT**G** AAT TTC AAT TTG GAT ATC TTT ATT ATT TA-3′), RTP1-E347D (5′-GAG TTA TAT GAA GTA GAT ATC GAA ATA CCA TCA AAA AC-3′), and RTP1-E349D (5′-TTA TAT GAA GTA GAA ATA GAT ATC CCA TCA AAA ACT ATT-3′) (mutations bolded, introduced *Eco* RV site underlined). Wild-type and mutant proteins were expressed in *E. coli* BL21 (DE3) by inducing with 50 μM IPTG. Soluble fraction preparation and column chromatography with Ni^{2+}-NTA-agarose resin (Qiagen) were as described [16]. Immunoprecipitation from 10 μg protein of Ni^{2+}-NTA-agarose fraction was performed as described [24] using monoclonal anti-polyhistidine antibody (anti 6× His, Clontech). The precipitated pellets contained equivalent levels of protein. They were tested for RTPase with 0.1 μM of termini of [γ-^{32}P]ATP-terminated dimeric (**p**ppAC) or trimeric (**p**ppACC) RNA [Takagi et al., in prep.] or ATPase with 0.1 μM of either [γ-^{32}] (**p**ppA) or [α-^{32}P] (pp**p**A) ATP. Reactions were incubated at 37°C for 15 min in 20 μl reaction mixture containing 50 mM Tris–HCl (pH 7.9), 5 mM DTT, 50 μg ml^{-1}, BSA, the indicated RNA or NTP substrate, and the indicated amount of divalent cation or EDTA. Control experiments showed that these conditions were within the linear range of the reaction.

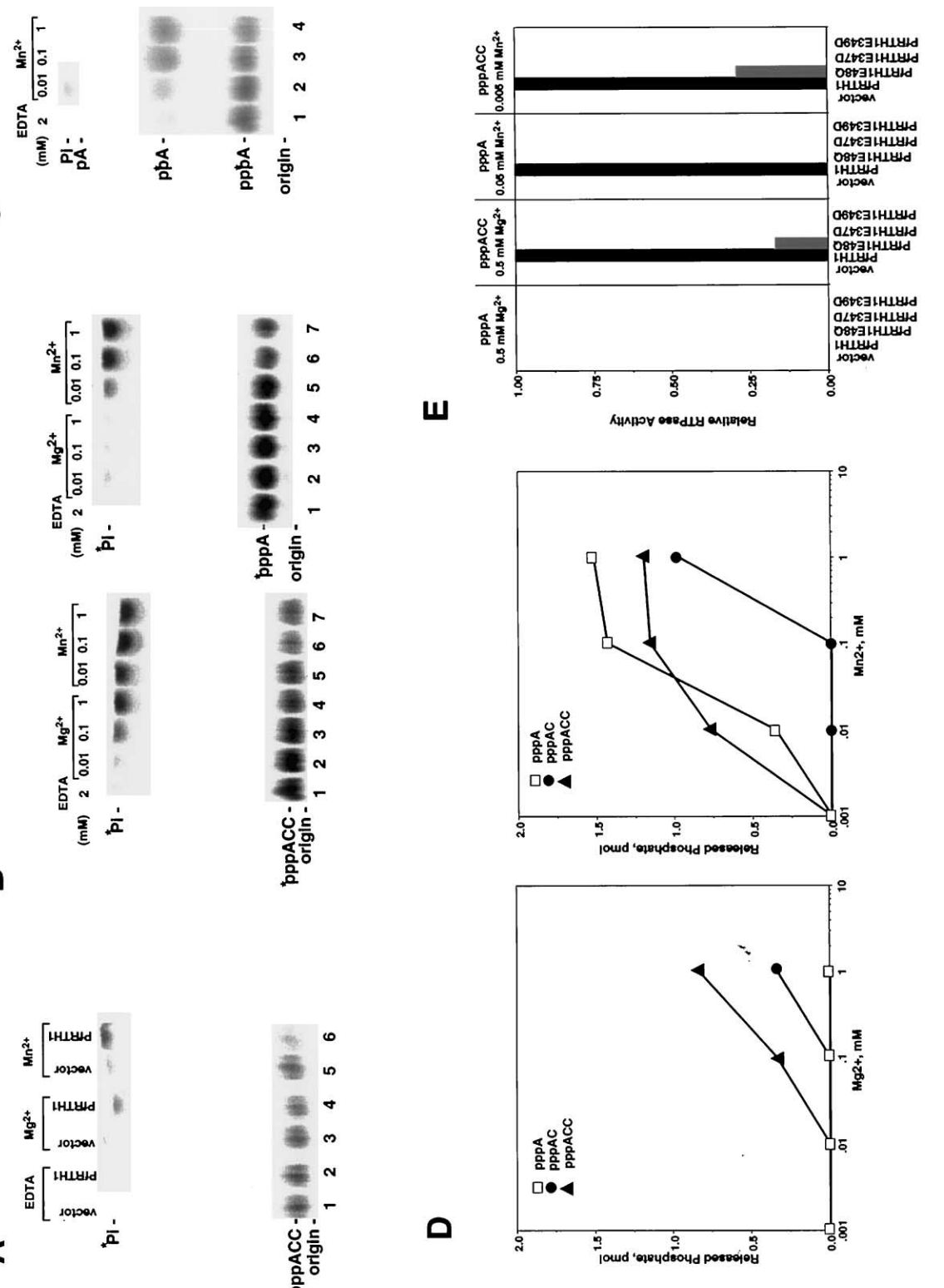

Fig. 2. (*Continued*)

lanes 2 and 3). Manganese significantly enhanced RTPase activity compared with the same concentration of magnesium (Fig. 2B left panel, compare lanes, 2 and 3 with 5 and 6). To assay NTPase activity, we used [γ-^{32}P]ATP (**p**ppA) as substrate (Fig. 2B, right panel). Only very weak ATPase activity was observed up to 15 mM magnesium (lanes 2–4 and data not shown). In contrast, manganese strongly enhanced the release of phosphate from ATP (lane 5–7). When [α-^{32}P]ATP (pp**p**A) was used as substrate, label was recovered in [α-^{32}P]ADP (p**p**A) (Fig. 2C). Therefore, PfRTH1 releases only the γ-phosphate and is not a nonspecific phosphatase. PfRTH1 also catalyzes hydrolysis of [α-^{32}P]UTP to [α-^{32}P]UDP and [α-^{32}P]dATP to [α-^{32}P]dADP in the presence of manganese (data not shown). Fig. 2D quantitates RTPase and NTPase activities at different divalent cation concentrations. In the presence of magnesium, there is a strong preference for a polynucleotide substrate. In the presence of manganese, ATPase activity is comparable to RTPase activity. In contrast to fungal RTPase activity, which is more active with magnesium [11,12,16], the RTPase of PfRTH1 was more active in the presence of manganese.

Site-directed mutagenesis of other metal-dependent RTPases reveals that conserved glutamate residues in motifs A and C are essential for activity [11–14,17,18]. Structural studies of the *S. cerevisiae* Cet1 protein indicate that motifs A and C together comprise the metal-binding site [19]. Therefore, PfRTH1 glutamate 48 in motif A was mutated to glutamine and glutamates 347 and 349 in motif C were individually changed to aspartates. The proteins were expressed in *E. coli* and RTPases and NTPase assays were carried out with immunoprecipitated proteins from Ni^{2+}-NTA fraction (Fig. 2E). Supporting the hypothesis that PfRTH1 uses a mechanism similar to the yeast RTPases, both E347D and E349D mutants were inactive. The RTPase activity of the E48Q mutant was reduced to about 20% of the wild-type protein. Motif A of PfRTH1 may be less important than that of yeast RTPases. This region of PfRTH1 has very limited similarity with fungal RTPases and it remains to be seen if it plays the same structural role.

In conclusion, we find that PfRTH1 is a metal-dependent RTPase/NTPase. Like Cet1 and its relatives, PfRTH1 specifically hydrolyzes the β–γ phosphate bond to leave a 5′ diphosphate end. Like several other RTPases recently discovered, [5,16,17,20,21], the physiological role of PfRTH1 is unknown. To our knowledge, PfRTH1 represents the first Cet1-like protein found outside of yeast. No proteins resembling Cet1 and PfRTH1 are found by searches of the completed Arabidopsis, C. elegans, Drosophila, and human genomes (data not shown). The capping enzymes of these higher eukaryotes resemble protein tyrosine phosphatases and are fused to the guanylytransferase. Recently, capping enzyme genes from *Trypanosoma* were described, and their presumed RTPase regions do not resemble either the yeast of metazoan RTPases. Rather, they share some sequence similarity with adenylate kinases [22]. Unlike *Trypanosoma*, little is known about mechanism of gene expression and RNA processing of *Plasmodium*. There is currently no evidence indicating whether PfRTH1 is involved in mRNA capping but it would be quite surprising if it was. The biochemical data presented here provide a starting point for future physiological experiments including the analysis of 5′ mRNA ends. The unexpected appearance of a Cet1-like protein in Plasmodium raises interesting questions about the evolution and functions of this class of enzymes.

Acknowledgements

We thank Dr Dyann Wirth and Dr Connie Chow (Harvard School of Public Health, USA) for *P. falciparum* genomic DNA, Dr Yasutaka Takase (Eisai Tsukuba Research Laboratories, Japan) for pointing out the Plasmodium protein and making the original version of the alignments shown in Fig. 1. This work was supported by NIH Grant GM56663 to S. Buratowski. T. Takagi was a senior postdoctoral fellow of the American Cancer Society, Massachusetts Divison, Inc., S. Buratowski is a scholar of the Leukemia and Lymphoma Society.

References

[1] Shuman S. Structure, mechanism, and evolution of the mRNA capping apparatus. Prog Nucl Acids Res Mol Biol 2000;66:1–40.
[2] Takagi T, Moore C, Diehn F, Buratowski S. An RNA 5′-triphosphatase related to the protein tyrosine phosphatases. Cell 1997;89:867–73.
[3] Yue Z, Maldonado E, Pillutla R, Cho H, Reinberg D, Shatkin AJ. Mammalian capping enzyme complements mutant *Saccharomyces cerevisiave* lacking mRNA guanylytransferase and selectively binds to the elongation form of RNA polymerase II. Proc Natl Acad Sci USA 1997;94:12898–903.
[4] McCracken S, Fong N, Rosonina E, Yankulov K, Brothers G, Siderovski D, Hessel A, Foster S, Amgen EST Program, Shuman S, Bentley DL. 5′-Capping enzymes are targeted to pre-mRNA by binding to the phosphorylated carboxy-terminal domain of RNA polymerase II, Genes Dev. 1997;11:3306–3318.
[5] Takagi T, Taylor GS, Kusakabe T, Charbonneau H, Buratowski S. A protein tyrosine phosphatase-like protein from baculovirus has RNA 5′-triphosphatase and diphosphatase activities. Proc Natl Acad Sci USA 1998;95:9808–12.
[6] Ho CK, Sriskanda V, McCracken S, Bentley D, Schwer B, Shuman S. The guanylyltransferase domain of mammalian mRNA capping enzyme binds to the phosphorylated carboxyl-terminal domain of RNA polymerase II. J Biol Chem 1998;273:9577–85.
[7] Ho CK, Schwer B, Shuman S. Genetic, physical, and functional interactions between the triphosphatase and guanylyltransferase

components of the yeast mRNA capping apparatus. Mol Cell Biol 1998;18:5189–98.
[8] Wen Y, Yue Z, Shatkin AJ. Mammalian capping enzyme binds RNA and uses a protein tyrosine phosphatase mechanism. Proc Natl Acad Sci USA 1998;95:12226–31.
[9] Tsukamoto T, Shibagaki Y, Imajoh-Ohmi S, Murakoshi T, Suzuki M, Nakamura A, Gotoh H, Mizumoto K. Isolation and characterization of the yeast mRNA capping enzyme β subunit gene encoding RNA 5′-triphosphatase, which is essential for cell viability. Biochem Biophys Res Commun 1997;239:116–22.
[10] Yamada-Oakabe T, Mio T, Matsui M, Kashima Y, Arisawa M, Yamada-Okabe H. Isolation and characterization of the *Candida albicans* gene from mRNA 5′-triphosphatase: association of mRNA 5′-triphosphatase and mRNA 5′-guanylyltransferase activities is essential for the function of mRNA 5′-capping enzyme in vivo. FEBS Lett 1998;435:49–54.
[11] Ho CK, Pei Y, Shuman S. Yeast and viral RNA 5′ triphosphatases comprise a new nucleoside triphosphatase family. J Biol Chem 1998;273:34151–6.
[12] Pei Y, Lehman K, Tian L, Shuman S. Characterization of *Candida albicans* RNA triphosphatase and mutational analysis of its active site. Nucl Acid Res 2000;28:1885–92.
[13] Ho CK, Martins A, Shuman S. A yeast-based genetic system for functional analysis of viral mRNA capping enzymes. J Virol 2000;74:5486–94.
[14] Ho CK, Gong C, Shuman S. RNA triphosphatase component of the mRNA capping apparatus of *Paramecium bursaria* Chlorella virus 1, J. Virol. 2001;75:1744–1750.
[15] Altschul SF, Thomas LM, Alejandro AS, Jinghui Z, Zheng Z, Webb M, Lipman DJ. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl Acids Res 1997;25:3389–402.
[16] Rodriguez CR, Takagi T, Cho E-J, Buratowski S. A *Saccharomyces crevisiae* RNA 5′-triphosphatase related to mRNA capping enzyme. Nucl Acid Res 1999;27:2181–8.
[17] Pei Y, Ho CK, Schwer B, Shuman S. Mutational analyses of yeast RNA triphosphatases highlight a common mechanism of metal-dependent NTP hydrolysis and a means of targeting enzymes to pre-mRNA in vivo by fusion to the guanylyltransferase component of the capping apparatus. J Biol Chem 1999;274:28865–74.
[18] Pei Y, Schwer B, Hausmann S, Shuman S. Characterization of *Schizosaccharomyces pombe* RNA triphosphatase. Nucl Acid Res 2001;291:121–5.
[19] Lima CD, Wang LK, Shuman S. Structure and mechanism of yeast RNA triphosphatase: an essential component of the mRNA capping apparatus. Cell 1999;99:533–43.
[20] Deshpande T, Takagi T, Hao L, Buratowski S, Charbonneau H. Human PIR1 of the protein tyrosine phosphatase subfamily has RNA 5′-triphosphatase and diphosphatase activities. J Biol Chem 1999;274:16590–4.
[21] Martins A, Shuman S. Mechanism of phosphoanhydride cleavage by Baculovirus phosphatase. J Biol Chem 2000;275:35070–6.
[22] Silva E, Ullu E, Kobayashi R, Tschudi C. Trypanosome capping enzymes display a novel two-domain structure. Mol Cell Biol 1998;18:4612–9.
[23] Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 1989;77:51–9.
[24] Takase T, Takagi T, Komarnitsky PB, Buratowski S. The essential interaction between yeast mRNA capping enzyme subunits is not required for triphosphatase function in vivo. Mol Cell Biol 2000;24:9307–16.