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A carboxy terminal domain of the L protein of rinderpest virus possesses RNA triphosphatase activity – The first enzyme in the viral mRNA capping pathway☆

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ABSTRACT

The large protein L of negative-sense RNA viruses is a multifunctional protein involved in transcription and replication of genomic RNA. It also possesses enzymatic activities involved in capping and methylation of viral mRNAs. The pathway for mRNA capping followed by the L protein of the viruses in the *Morbillivirus* genus has not been established, although it has been speculated that these viruses may follow the unconventional capping pathway as has been shown for some viruses of *Rhabdoviridae* family.

We had earlier shown that the large protein L of *Rinderpest virus* expressed as recombinant L–P complex in insect cells as well as the ribonucleoprotein complex from purified virus possesses RNA triphosphatase (RTPase) and guanylyltransferase activities, in addition to RNA dependent RNA polymerase activity. In the present work, we demonstrate that RTPase as well as nucleoside triphosphatase (NTPase) activities are exhibited by a subdomain of the L protein in the C terminal region (a.a. 1640–1840). The RTPase activity depends absolutely on a divalent cation, either magnesium or manganese. Both the RTPase and NTPase activities of the protein show dual metal specificity. Two mutant proteins having alanine mutations in the glutamic acid residues in motif-A of the RTPase domain did not show RTPase activity, while exhibiting reduced NTPase activity suggesting overlapping active sites for the two enzymatic functions. The RTPase and NTPase activities of the L subdomain resemble those of the Vaccinia capping enzyme D1 and the baculovirus LEF4 proteins.

1. Introduction

Mononegavirales order consists of viruses with negative sense single-stranded RNA genome which replicate in the cytoplasm employing three virus-coded proteins in association with the genomic RNA present as RNP. The L (large) protein in this complex performs transcription as well as replication, and post-transcriptional modification of mRNA such as capping, methylation and polyadenylation [1]. The viruses of the *Rhabdoviridae* family of *Mononegavirales* order follow a distinct unconventional mRNA capping pathway unique to this class of viruses [2]. *Rinderpest virus* (RPV) belonging to *Paramyxoviridae* family has been shown to possess RNA triphosphatase activity, a component of conventional eukaryotic capping cascade. The L protein of RPV has been shown to exhibit RTPase activity [3].

In the present work, a putative RTPase domain of the L protein of *R. virus* has been identified through homology analysis using ClustalW with *Vaccinia virus* D1 protein and *AcNPV* LEF4 protein which are known to possess RTPase and NTPase activities [4,5]. This putative RTPase domain of L protein (1640–1840 a.a.) was cloned and expressed in bacteria and the recombinant protein was purified. The recombinant domain was shown to possess RTPase as well as NTPase activities. Sequence homology analysis showed three motifs for RTPase subdomain similar to D1 and LEF4 protein with conserved acidic amino acid residues, present in three sequence

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motifs separated by closely matching number of amino acid residues. Two point mutants, E1645A and E1647A in motif-A of this subdomain were generated and the mutant proteins were expressed. Abrogation of activity of these mutants further demonstrated the similarity of RPV RTPase with D1 and LEF4 proteins.

2. Materials and methods

Materials – *Escherichia coli* DH5α and BL21 (DE3) used for the propagation of plasmid DNA and expression, respectively, and the cloning vector pRSET-A were procured from Invitrogen, USA and a recombinant plasmid having full length L gene of RPV (GenBank ID: Z30698.1) cloned in a yeast vector, pSR 782 was earlier generated in the laboratory. All the restriction enzymes were purchased from New England Biolabs, USA. T7 RNA polymerase, NTPs and dNTPs were obtained from Fermentas, USA. An antibody made in rabbit against recombinant L protein domain III (1717–2183 amino acids) expressed in *E. coli* was generated earlier [3]. Ni-NTA beads and plasmid midi-prep kit were procured from Qiagen, USA. Superdex-75 HR 10/30 FPLC column was purchased from Amersham Bioscience, USA. Sodium phosphate, Sodium pyrophosphate, Sodium tripolyphosphate, Lysozyme, Trizol and DAB (3,3′-diaminobenzidine), anti-his antibody (monoclonal) and plasmid miniprep kit were purchased from Sigma Chemicals, USA. IPTG (Isopropyl β-D-1-thiogalactopyranoside) was obtained from GIBCO-URL, USA. The following primers synthesized and supplied by Sigma Chemical Co., India, were used to generate *in vitro* transcribed RNA substrate for RTPase -

T7 promoter primer:

5′-TAATACGACTCACTATA-3′
N-5′ T7 oligonucleotide:
5′-TCCAGTCGATAGGATCTTGAATCCTTAT
AGTGAGTCGTATTA-3′

The N-5′ T7 oligonucleotide consists of 25 nucleotides (underlined) corresponding to the complement of first 25 nucleotides of N protein mRNA of RPV.

Cloning of the RTPase domain – The putative region on L protein for RTPase was amplified from pSR782 (603 bp) using sequence specific forward primer having BamHI restriction site and reverse primer having SalI site and cloned into another yeast vector, to enable subsequent subcloning in pRSET-A vector for bacterial expression (pHK845). The RTPase region was mobilized from pHK845 by digestion with BamHI and SalI and cloned at BamHI/XhoI site of pRSET-A vector, giving a recombinant plasmid pHK846. E1645A and E1647A mutant RTPases were generated through site-directed mutagenesis employing mutant primers and sequence specific primers on pHK845 template DNA. The clones were confirmed by sequencing.

Expression of wild-type and mutant RTPase domain in E. coli and purification – *E. coli* BL21 (DE3) cells were transformed with plasmids expressing the wild-type or mutants and cultures were grown till the OD_{600nm} reached 0.8 after which the cells were incubated on ice for half-an-hour and incubated at 18 °C for 24 h without IPTG induction. The cells were harvested by centrifugation and the cell pellet was resuspended in equilibration buffer (50 mM Tris, 0.1 M NaCl, 5 mM β-mercaptoethanol, 0.01% Triton X-100 and 10% glycerol, pH 8.0) containing protease inhibitor cocktail (Sigma–Aldrich, USA) and lysozyme (0.1 mg/ml), and incubated at 4 °C for 45 min. Subsequently, the cells were lysed by sonication and supernatant collected by centrifugation at 13,000 rpm at 4 °C. The soluble supernatant was mixed with 1 ml of Ni-NTA agarose for 1.5 h at 4 °C. The resin was washed with 5 volumes of equilibration buffer and the protein (29 kDa) was eluted with 5 volumes of equilibration buffer containing 100 mM Imidazole and 0.5 M NaCl. The elution fractions containing the recombinant protein were pooled and concentrated using 10 kDa cut-off centrifugal filter tubes. A second-step purification was performed by employing gel permeation chromatography using Superdex-75 FPLC column (3–70 kDa separation range) with 25 mM Tris-Cl (pH 8.0) as running buffer at room temperature. The column was washed with 2 volumes of double distilled degassed water and equilibrated with running buffer. Ni-NTA purified protein was loaded onto the column and eluted. The fractions containing the protein with the desired molecular mass (as determined by SDS-PAGE) were pooled and dialyzed against buffer containing 5% glycerol, 1 mM DTT, 1 mM EDTA and 25 mM Tris (pH 8.0). Protein concentration was estimated by Bradford method and samples were stored at −80 °C.

Preparation of substrate *RNA* – Substrate RNA was prepared by *in vitro* transcription of a synthesised DNA template corresponding to the 5′ end of N mRNA as described earlier [3]. 500 pmol of T7 promoter primer was annealed with 1.5 nmol of N-5′ T7 oligonucleotide primer. 1 mM of each NTP was added and transcription reaction was performed with 1U T7 RNA polymerase. Further, the DNA was cleaved using 0.1U of DNaseI. The RNA was purified with Trizol reagent, washed with 70% ethanol and stored in DEPC treated double-distilled water.

RTPase assay – RTPase reaction mixtures (50 μl) containing 25 mM HEPES-K^+ (pH 7.5), 1 mM $MgCl_2$ and 15 μM *in vitro* generated RNA (AGGAUUCAAGAUCCUAUCGACUGGA) or as indicated were incubated for 30 min (unless otherwise indicated) at 37 °C [3]. Reactions were stopped by addition of 25% TCA. A freshly prepared chromogenic solution containing a 6:1 ratio of 0.42% ammonium molybdate in 1 N H_2SO_4 and 10% ascorbic acid was added and incubated for 20 min at 42 °C. The quantity of Pi was then determined from a calibration curve derived from solutions of known Pi concentration (KH_2PO_4). Release of phosphate was determined by measuring A_{780} [6].

NTPase assay – NTPase reaction mixtures (200 μl) which contained 50 mM Tris-MES (Tris, 4-Morpholineethanesulfonic acid) pH 8.15, 7.5 mM NaCl, 5 mM $MgCl_2$ (unless specified), 2 mM of ATP (unless specified) and enzyme concentration as indicated were incubated at 30 °C for 15 min or as indicated. Reactions were terminated by the addition of 10% TCA. Chromogenic substrate was made fresh by adding 1 volume of 10% ascorbic acid in 6 volumes of 0.42% ammonium molybdate in 1 N H_2SO_4 and added to the reaction mix. The quantity of Pi was then determined from a calibration curve derived from solutions of known Pi concentration (KH_2PO_4). Release of phosphate was determined by measuring A_{655} [7].

3. Results

Identification of R. virus L Protein region 1640–1840 a.a. as a candidate RTPase – The yeast/viral RTPases have been shown to possess three motifs/sequence elements, two of which are glutamate rich motifs (motif A and C) essential for catalytic activity and comprise of the metal binding site and one basic peptide motif (motif B) which has been implicated in binding the 5′ triphosphate moeity of the substrate [8,9]. We had earlier demonstrated that the recombinant L protein of *R. virus* exhibits RTPase activity which is metal dependent [3]. In order to define the region on L protein having RTPase activity, we performed multiple alignment of RTPase sequences with the sequence of L Protein and manually identified the presence of three motif sequences in the region 1640–1840 a.a. of L protein (Fig. 1A). The two alternate glutamate residues in motif A are conserved in the L protein implying that these residues may play similar roles in modulating enzyme function.

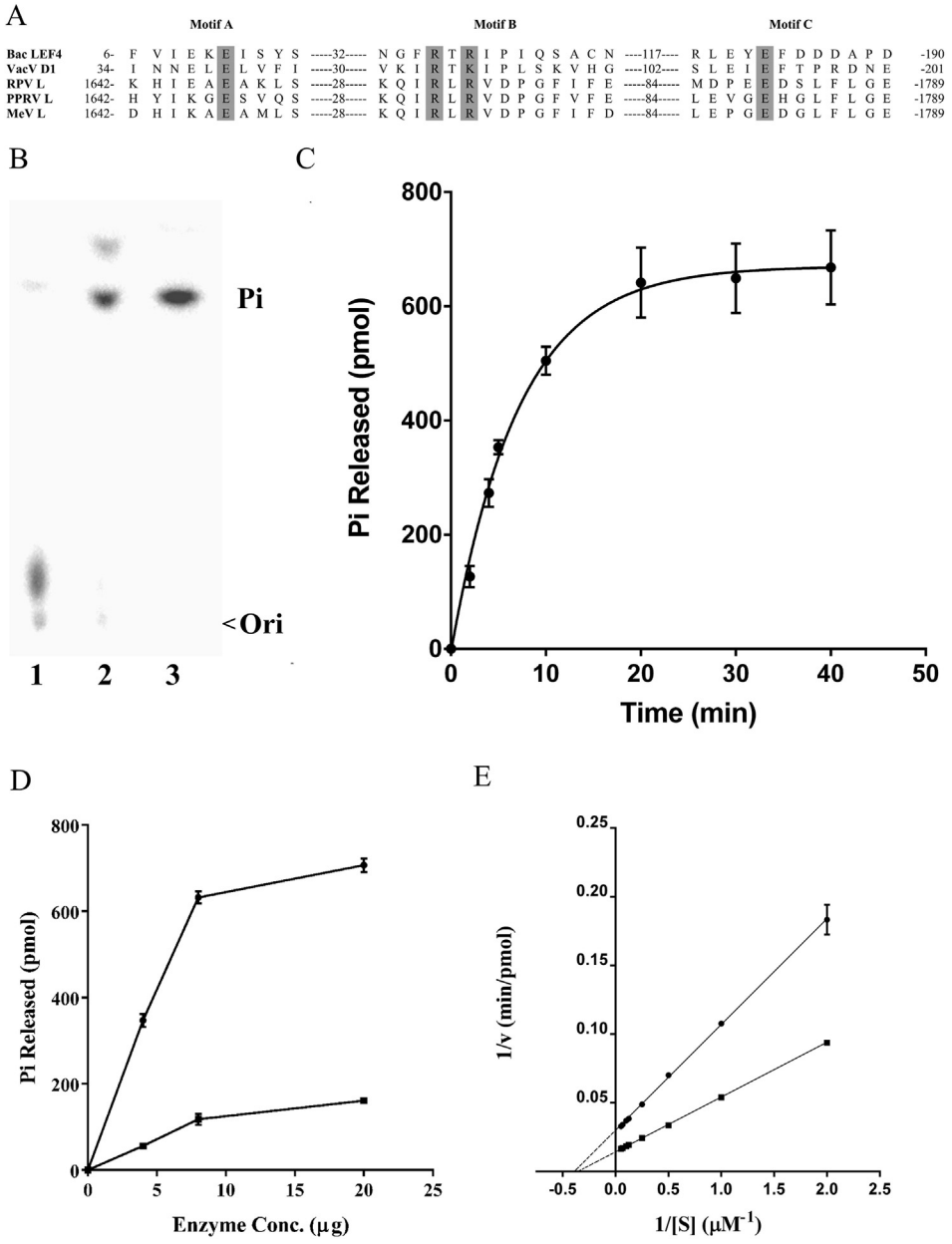

Fig. 1. RNA Triphosphatase activity of the domain. A) Identification of the RTPase domain of L protein of RPV. Partial sequence alignment showing motif structure of RTPase domains of *Autographa californica Nuclear Polyhedrosis Virus* Late Expression Factor-4 (Bac LEF4), *Vaccinia Virus* D1 (VacV D1), *Rinderpest Virus* Large protein (RPV L), *Peste-des-petits-ruminants Virus* Large protein (PPRV L) and *Measles Virus* Large protein (MeV L). The numbers above the dotted lines represent the number of residues between these motifs and the numbers at the left and right end of the domains represent the position of the first and the last amino acid residues respectively, of the domain in the respective protein. **(B) RTPase activity of recombinant protein**. 15 pmol γ-P^{32}-ATP terminated RNA was incubated with 1 µg of heat-inactivated purified recombinant protein (Lane 1), purified recombinant protein (Lane 2) and calf intestinal alkaline phosphatase (Lane 3) for 30 min at 37 °C. The reaction mixtures were analyzed by TLC and Pi was detected by phosphorimaging. Ori – origin of spotting. **(C) Time course of RTPase activity**. RTPase assay was performed at pH 7.5, 37 °C with 15 µM of RNA as substrate and 10 µg of protein. The reaction mixtures were incubated for various time periods. **(D) Substrate specificity of RTPase activity**. Non-viral RNA was synthesized in-vitro using T7 promoter primer and empty pRSET-A vector DNA as template. Assay was performed with 5 µg of protein at 37 °C for 30 min with 15 µM of viral (●) and non-viral RNA (■) as substrate. **(E) Determination of K_m and k_{cat} of RTPase activity**. The double-reciprocal plot was generated using 10 µg (●) and 20 µg (■) of protein at 0–20 µM of RNA as substrate.

Expression and purification of C-terminal 1640–1840 amino acids region (RTPase domain) of L protein in bacteria - *E. coli* BL21(DE3) were transformed with pHK845, encoding the RTPase domain tagged with hexa-histidine. The protein was expressed in the absence of IPTG and inclusion of IPTG did not enhance the level of expression. SDS-PAGE and immunoblot analysis showed high amount of leaky expression of the protein without IPTG induction (data not shown). It was observed that the recombinant protein showed electrophoretic anomaly (with apparent molecular weight of ~29–~35 kDa), an illustration of its acidic nature [10]. It was seen that the protein in the soluble fraction of cell lysate got enriched on purification by Ni-NTA affinity chromatography. The resulting protein still had contaminating high molecular weight proteins and thus a size exclusion chromatographic step by FPLC was carried out (Figure S1A). The authenticity of the recombinant protein was verified by immunoblotting analysis after SDS-PAGE using poly-clonal antibody specific for 1717–2183 a.a. region on L protein (Conserved region 3) (Figure S1B).

Recombinant protein possesses RTPase activity – To test for the RTPase activity in the L-RTPase domain, a 25 nt 5′-triphosphate-ended viral RNA was synthesized with 5′-γP^{32} label. The release of radioactive phosphate by recombinant RTPase protein was demonstrated by comparing its mobility with the Pi released by calf intestinal alkaline phosphatase after separation by thin-layer chromatography. Recombinant protein denatured by heat did not show release of phosphate (Fig. 1B). Further assays were performed employing colorimetric estimation of non-radioactive phosphate. Kinetic properties of the enzyme were also investigated. The velocity of the reaction increases linearly with time upto 7 min and reaches saturation by 20 min (Fig. 1C). In order to test the specificity of the RTPase activity, non-viral RNA was provided as a substrate. It was seen that viral RNA released five times more Pi than the non-viral RNA (Fig. 1D). The kinetic parameters (K_m, k_{cat}) were determined by measuring the velocity of reaction at different concentrations (0–20 μM) of substrate viral RNA. K_m of RTPase activity was found to be 2.72 μM and k_{cat} to be 99.8 mmol/min/mol of protein (Fig. 1E). RTPase activity of the domain depended on the presence of divalent cations: Mg^{+2} (Fig. 2A) and Mn^{+2} (Fig. 2B) thereby showing that the L-RTPase of RPV belongs to metal-dependent phosphohydrolase family. It was observed that at higher concentration of the metal ions, the release of Pi started diminishing indicating that the interaction of metal-ion with RNA might have a substrate limiting effect on the RTPase activity [11], the same phenomenon is exhibited by *V. virus* RTPase [5]. Synergistic activation of RTPase activity was observed when combination of sub-optimal concentrations of Mg^{+2} and Mn^{+2} were used, indicating two-metal mechanism (Fig. 2C).

Metal dependent NTPase activity of RTPase domain – Many viral RTPases are known to possess NTPase activity [5,12,13]. The fungal and viral triphosphatase family of proteins show NTPase activity which are also metal ion dependent. The L-RTPase also exhibited ATPase activity. Kinetic analysis showed that the ATPase reaction attained saturation by around 10 min (Fig. 3A) faster than the RTPase activity (Figs. 1C and 3A). NTPase activity, alike RTPase activity, was also found to be metal-dependent (Fig. 4A and B). The Pi release maxima was observed at 5 mM of Mg^{+2} and at 1 mM of Mn^{+2}. Similar to the results of RTPase activity, it was found that NTPase activity also proceeds through two-metal mechanism (Fig. 4C). The enzyme acted on all NTP substrates as well as dNTPs, although the activity was higher with purines than with pyrimidines (Fig. 3B). K_m of ATPase activity of the protein was determined to be 391 μM and the k_{cat} 87.29 mol/min/mol of the protein (Fig. 3C).

Glu1645 and Glu1647 are essential for the enzymatic activities of the RTPase domain – The mutants E1645A and E1647A were generated by site-directed mutagenesis. These residues are similar to the residues of *V. virus* D1 and Baculoviral LEF4 proteins which have been shown to be critical for RTPase activity in these viruses [14–16]. The mutant proteins were expressed and purified in the same manner as the wild-type and tested for RTPase and ATPase activities. The results shown in Table S1 clearly demonstrate the involvement of the alternate glutamic acid residues in catalysis.

4. Discussion

Since the L protein of rhabdoviruses possesses polyribonucleotidyltransferase activity that converts 5′-triphosphorylated RNA to 5′-monophosphorylated RNA and transfers it to GDP to form the cap via enzyme-RNA covalent intermediate [17], it is believed that the members of *Paramyxoviridae* too follow this novel unconventional capping of the *Rhabdoviridae* family members which has been widely studied [2,18,19]. The L protein of RPV has earlier been shown to exhibit RTPase activity which is the first enzyme associated with the conventional eukaryotic capping pathway indicating that the viruses of *Morbillivirus* genus may follow the conventional eukaryotic capping pathway [3]. Search for the minimal domain for RTPase activity led to the sequence comparisons of L proteins of morbilliviruses with the sequences of baculoviral LEF4 and *V. virus* D1 proteins, both of which have been established as RTPase enzymes [4,5]. A domain extending from 1640 to 1840 amino acids was predicted to have RTPase activity and it was observed that the RTPase domain of L protein of RPV shared similar three motif structure as that of *Baculovirus* LEF4 and *V. virus* D1 proteins.

The RTPase domain exhibited both RTPase and NTPase activities as is known for many RTPases [5,8,12,13,20]. The RTPase domain exhibited 145 times higher affinity for RNA substrate than ATP as substrate. Similar observations have been made for *V. virus* RTPase [5]. The RTPase domain of RPV L protein shows high specificity towards its own mRNA.

In the present work, the RTPase domain of L Protein has been shown to belong to metal-dependant class of enzymes which reveal a two-metal mechanism similar to the RTPase domain of other viruses [14,21,22]. The L-RTPase has associated NTPase activity, and also pyrophosphatase and tripolyphosphate activities (data not shown). NTPase activity was found to be non-specific. The protein has a much higher affinity towards RNA than the NTPs, however, the product release rate is 12 times more for ATPase

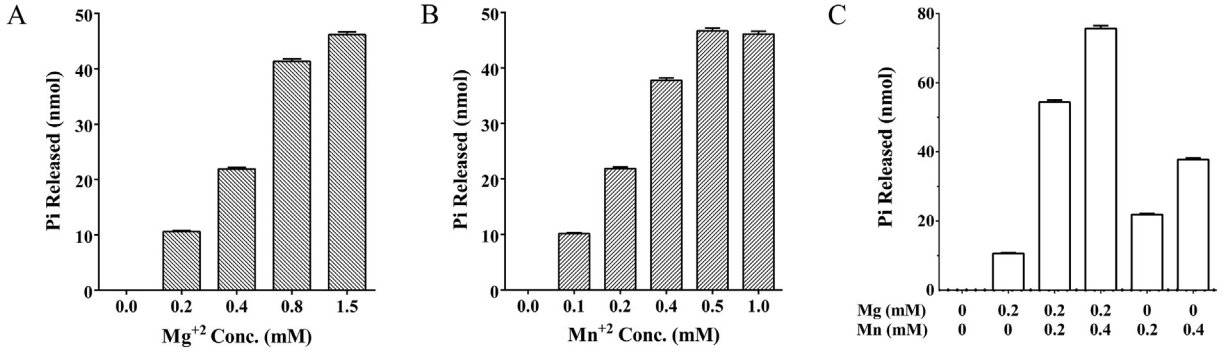

Fig. 2. Effect of divalent cations on RTPase activity of the domain. (A) Dependence of the enzyme on Mg^{+2} was shown using 0–1.5 mM of $MgCl_2$ and 10 μg of protein with 15 μM viral RNA as substrate. (B) Dependence of the enzyme on Mn^{+2} was shown using 0–1 mM of $MnCl_2$ and 10 μg of protein with 15 μM viral RNA as substrate. (C) The synergistic effect of dicationic metals: Mg^{+2} and Mn^{+2} was shown using varying concentrations of metals with 10 μg of enzyme and 15 μM viral RNA as substrate. Reaction was performed at 37 °C for 30 min.

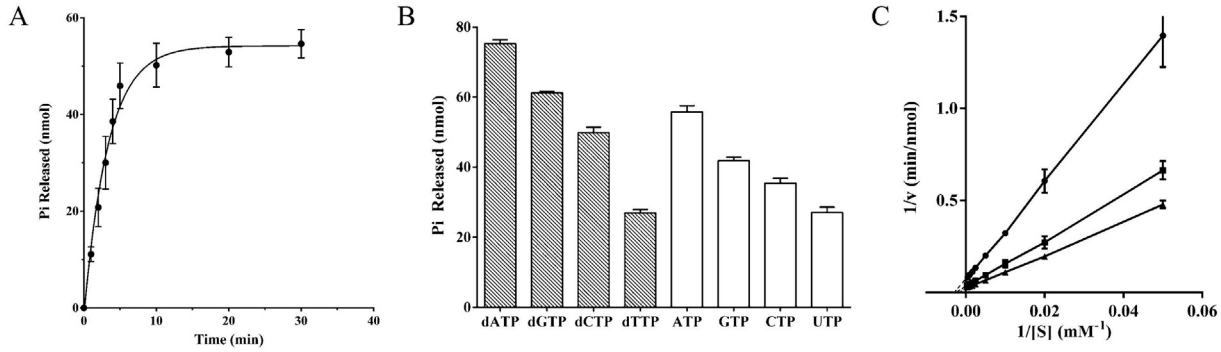

Fig. 3. The RTPase domain possesses NTPase activity. (A) Time course of ATPase activity. The ATPase assay was performed at 30 °C for various time periods with 2 mM of ATP as substrate and 5 μg of protein. **(B) Substrate specificity of NTPase activity.** NTPase assay was performed with 2 mM NTPs and dNTPs with 5 μg of protein. Reaction was performed at 30 °C for 10 min. **(C) Determination of K_m and k_{cat} of ATPase activity.** The double-reciprocal plot was generated using 5 (●), 10 (■) and 15 (▲) μg of protein at different ATP concentrations (0–3 mM).

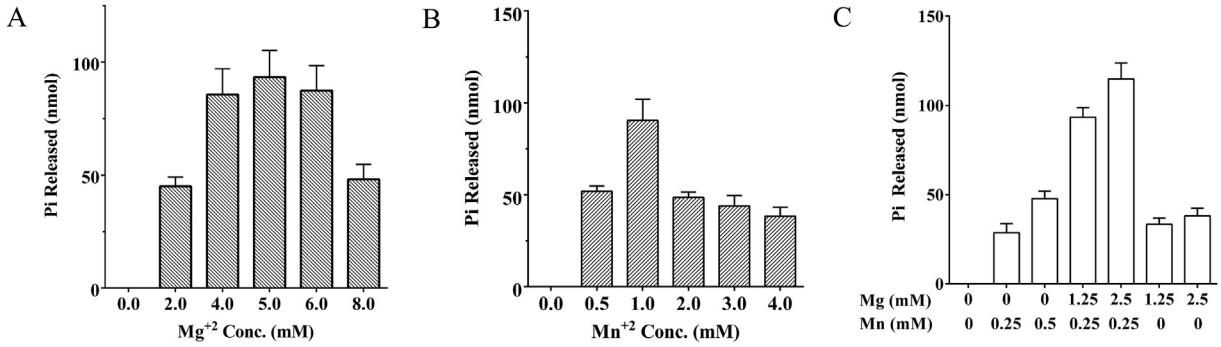

Fig. 4. Effect of divalent cations on ATPase activity. (A) Dependence of the enzyme on Mg^{+2} was shown using 0–8 mM of $MgCl_2$ and 10 μg of protein with 2 mM ATP as substrate. (B) Dependence of the enzyme on Mn^{+2} was shown using 0–4 mM of $MnCl_2$ and 10 μg of protein with 2 mM ATP as substrate. (C) The synergistic effect of dicationic metals: Mg^{+2} and Mn^{+2} was shown using varying concentrations of metals with 10 μg of enzyme and 2 mM ATP as substrate. Reaction was performed at 30 °C for 15 min.

activity. Dependence of NTPase activity on divalent metals conformed with the nature of other RTPase associated NTPases [8,14,21]. A two-metal mechanism for NTPase has also been demonstrated for LEF4 protein [14]. The two-metal mechanism followed by both RTPase and NTPase activities indicate that the RTPase and NTPase active sites on the domain might be overlapping.

Mutating the E1645 and E1647 to Alanine abrogated the NTPase activity completely and RTPase activity by ~80%. The residual RTPase activity of the mutants indicate the possibility of the enzyme having partial overlap of active sites for RTPase and NTPase.

The motif-A of the RTPase domain of the L protein overlaps with the Conserved Region (Domain) II (650–1696 a.a.). The motifs B and C of RTPase domain exist in the Conserved Region III (1717–2183 a.a.). Domain III which does not have motif-A does not possess RTPase activity but retains 10% of ATPase activity as compared to the RTPase domain (unpublished data). This shows the importance of motif-A in the functions of the RTPase domain. Domain II was found to have no ATPase activity (unpublished data). Earlier work on other RTPases show the importance of motifs B and C for RTPase and NTPase activities [14–16]. Since the same motif structure is maintained in the L protein RTPase, it is construed that these motifs (B and C) would play important role in the RTPase and NTPase activities of the RTPase domain of L protein.

Demonstration and characterisation of RTPase activity of a 200 aa region of the L protein of RPV and the earlier demonstration of guanylyltransferase activity associated with recombinant L protein [3] indicates that the *Morbillivirus* genus in the *Paramyxoviridae* family may follow conventional eukaryotic mRNA capping pathway and not the unconventional capping as was earlier believed [2,18,19,23].

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.07.026.

Transparency document

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