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Novel ATPase activity of the polyprotein intermediate, Viral Protein genome-linked-Nuclear Inclusion-a protease, of *Pepper vein banding potyvirus*

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ABSTRACT

Potyviruses temporally regulate their protein function by polyprotein processing. Previous studies have shown that VPg (Viral Protein genome-linked) of *Pepper vein banding virus* interacts with the NIa-Pro (Nuclear Inclusion-a protease) domain, and modulates the kinetics of the protease. In the present study, we report for the first time that VPg harbors the Walker motifs A and B, and the presence of NIa-Pro, especially *in cis* (cleavage site (E191A) VPg-Pro mutant), is essential for manifestation of the ATPase activity. Mutation of Lys47 (Walker motif A) and Asp88:Glu89 (Walker motif B) to alanine in E191A VPg-Pro lead to reduced ATPase activity, confirming that this activity was inherent to VPg. We propose that potyviral VPg, established as an intrinsically disordered domain, undergoes plausible structural alterations upon interaction with globular NIa-Pro which induces the ATPase activity.

1. Introduction

Pepper vein banding virus (PVBV) is a positive sense single-stranded RNA virus (Supplementary Fig. S1) that belongs to the genus *Potyvirus*, family *Potyviridae* [1]. Ten potyviral proteins are encoded as a 340–360 kDa polyprotein and one more protein is translated by ribosomal frameshifting [2]. The polyprotein undergoes proteolytic processing by three virus-encoded proteases – P1 protease, helper component-protease (HC-Pro) and nuclear inclusion-a protease (NIa-Pro) – to give rise to intermediate and mature proteins. NIa-Pro, a serine-like cysteine protease, cleaves at seven sites within the polyprotein (Supplementary Fig. S1). It recognizes a heptapeptide sequence and cleaves between a glutamine and a serine/threonine/alanine residue [3,4]. PVBV NIa-Pro has been shown to exhibit a protease as well as a DNase activity [5–7]. The cleavage site between VPg (Viral Protein genome-linked) and NIa-Pro contains a glutamate residue instead of a glutamine (Fig. 1A), thus making it a suboptimal cleavage site, which results in prolonged presence of VPg-Pro intermediate in the cell milieu [3,4]. Infact, mutations that either enhance or abolish cleavage at this site have debilitating effects on viral replication [3]. In an earlier study, we have demonstrated that PVBV VPg interacts with NIa-Pro *in cis* and *in trans* [5]. This interaction can alter the orientation of an exposed Trp143 residue on NIa-Pro. Such structural perturbation gets transmitted *via* an interaction network, to a Cys151 residue present within the active site, thus modulating the kinetics of the protease activity.

VPg is known to be involved in various other viral processes [8]. The N-terminal region of VPg harbors the bipartite nuclear localization signal (NLS I: 4–9, NLS II: 41–50; *Potato virus A* (PVA) numbering) [3,9] and an NTP binding site (38–44; PVA numbering) [10]. This region is also known to be involved in interaction with host proteins [11], RNA-binding, uridylylation, viral translation and suppression of RNA silencing [7,9,12–15].

In the present study, we identified for the first time that VPg domain harbors the Walker motifs commonly found in proteins involved in nucleotide binding. Walker motif A forms a "P-loop" [16] that binds β- and γ-phosphates of NTP [17,18]. Such proteins often display NTPase activity and contain a conserved Walker motif B (typically represented by DEAD sequence), which chelates Mg^{2+} of the Mg^{2+}-NTP complex [17,18]. However, several NTP-binding proteins contain a modified Walker motif B such as the DEAH-box proteins or the Ski2 family (DEXH motif) of proteins [19]. VPg-Pro, a polyprotein processing intermediate, was found to exhibit ATPase activity, by virtue of the Walker motifs present in the VPg amino acid sequence.

2. Materials and methods

2.1. Protein expression and purification

Histidine-tagged (His)-NIa-Pro (~29 kDa) was over-expressed in BL21 pLysS *Escherichia coli* (*E. coli*) cells and, His-VPg-Pro and

Abbreviations: PVBV, *Pepper vein banding virus*; GdnHCl, guanidine hydrochloride; IDP, intrinsically disordered protein; TLC, thin layer chromatography.

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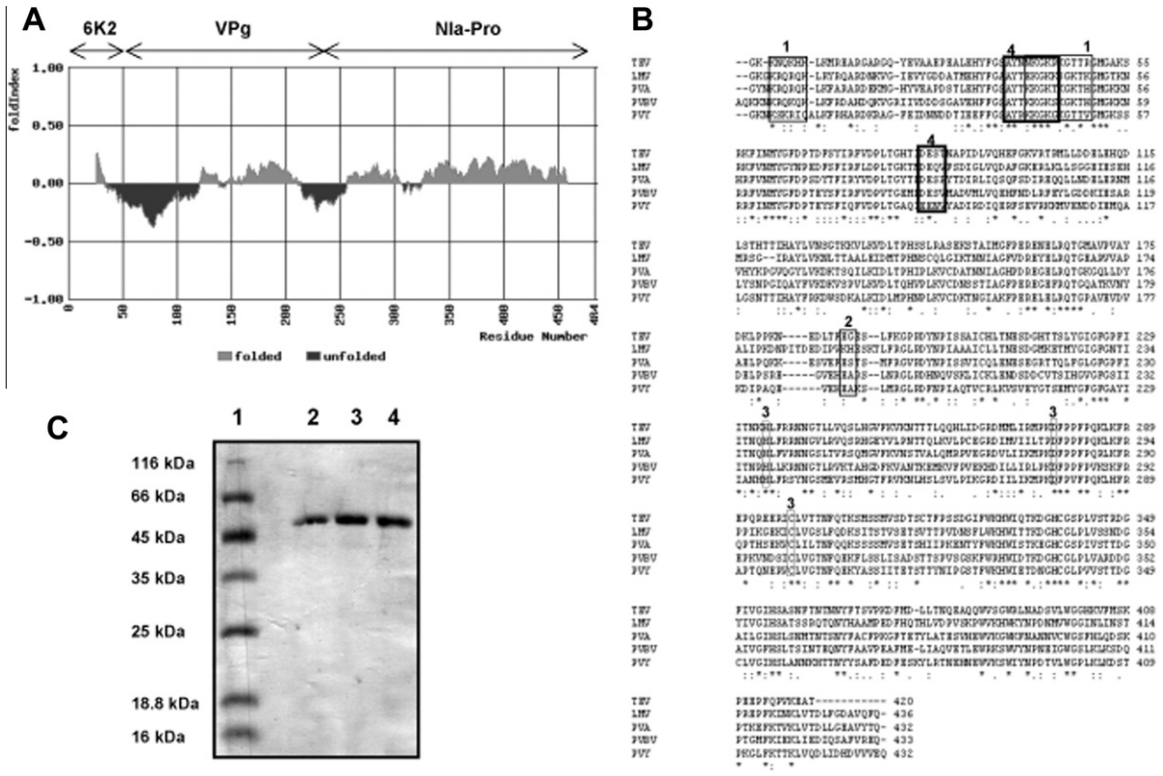

Fig. 1. (A) Prediction of disordered regions in PVBV 6K2-VPg-Pro using Foldindex©. (B) Multiple sequence alignment (MSA) of VPg-Pro from *Lettuce mosaic virus* (LMV), *Potato virus A* (PVA), *Pepper vein banding virus* (PVBV), and *Potato virus Y* (PVY) and *Tobacco etch virus* (TEV) using ClustalW program. (This is representative of MSA of a set of potyviruses for which the sequence similarity of VPg ranged from 43% to 55%.) Box 1: NLS I and II; box 2: cleavage site between VPg and Nla-Pro; box 3: Nla-Pro active site residues; box 4: Walker motifs A and B. (C) Purified VPg-Pro mutants. Lane1: molecular weight markers, lane 2: E191A VPg-Pro, lane 3: K47A:E191A VPg-Pro, lane 4: D88A:E89A:E191A VPg-Pro.

His-E191A VPg-Pro (~51 kDa) were over-expressed in BL21 *E. coli* cells containing pSBET A. His-VPg (~26.5 kDa) was obtained upon *in vivo* cleavage of VPg-Pro during expression. All proteins were purified by Ni^{2+}-NTA affinity chromatography and stored in buffer containing 10 mM CAPS-NaOH pH 9.2, 200 mM NaCl and 10% glycerol, as described earlier [5].

Walker motif mutants (K47A and D88A:E89A) of E191A VPg-Pro were generated using oligonucleotide primers (Sigma): VNK47A sense – 5′-ACACGAAGAAA***GGCGCC***TCAAAAGGGAAG-3′, VNK47A antisense – 5′-GTGTCTTCCCTTTTGA***GGCGCC***TTTCTTCG-3′; VND88A:E89A sense –5′-GGTGAAATGCTT*GCT****GCTAGC***GTCAT GGCACATGTAATG-3′, VND88A:E89A antisense –5′-CATCTGCTACG AC***GCTAGC****AGC*AAGCATTTCACCTGTAAC-3′ (mutated codon in italics, restriction enzyme site (*Ehe*I or *Nhe*I) in bold), screened by restriction digestion and confirmed by DNA sequencing. These mutant proteins were purified under conditions identical to those used for E191A VPg-Pro purification.

2.2. ATPase assay

ATPase assay was carried out (30 min at 25 °C) in 50 mM MOPS buffer, pH 7.0, 0.2 mM $MgCl_2$, 2 mM DTT and 0.5 mg of BSA per ml of reaction mixture (henceforth called as ATPase assay buffer). A standard reaction mixture contained 0.2 μM protein, 20 μM unlabeled NTP, and 0.008 mCi [γ-^{32}P]ATP (or [α-^{32}P]ATP or [γ-^{32}P]GTP) as the tracer. For kinetic studies, unlabeled NTP substrate was varied from 1–20 μM. Reactions were stopped by adding 10 mM EDTA (final concentration), and analyzed by thin layer chromatography (TLC; [20]) using PEI-cellulose F TLC sheets (Merck). Calf intestinal alkaline phosphatase (CIAP) was used as the positive control (Supplementary Fig. S2A) to identify the position of released orthophosphate (Pi) on a TLC sheet. Empty vector control, wherein the eluant from purification, carried out using bacterial cells transformed with unmodified pRSET C vector, was used as the negative control for the enzyme source (Supplementary Fig. S2A). The intensity of the spots was quantified using the Multigauge software (Fujifilm) for estimating fractional cleavage, and subsequently, the amount of product released. Kinetic constants, K_m and V_{max}, were determined using Lineweaver–Burk plots. Experiments were performed in triplicates, and standard deviations are indicated.

3. Results and discussion

3.1. Sequence and structural analysis

In recent reports, VPg from potyviruses such as *Potato virus Y* (PVY), *Lettuce mosaic virus* (LMV) and PVA have been shown to contain a flexible hydrophobic core, along with distinct regions of disorder [13,21–23], and are categorized under the intrinsically disordered proteins (IDPs) [8,24,25]. The amino acid sequence of PVBV VPg shows that it has a lower hydrophobic amino acid content and a higher proportion of polar and charged residues. When the theoretical propensity of protein folding was determined for VPg-Pro using Foldindex© [26], the N- and C- termini of VPg were predicted to be largely disordered (Fig. 1A). Furthermore, PONDR® analysis categorized VPg under the "twilight zone", in between ordered and disordered proteins (data not shown). The disordered regions of potyviral VPgs have been earlier reported to adopt more structured conformations in the presence of trifluoroethanol [22,23], anionic phospholipids and SDS [27]. Thus, it is likely that

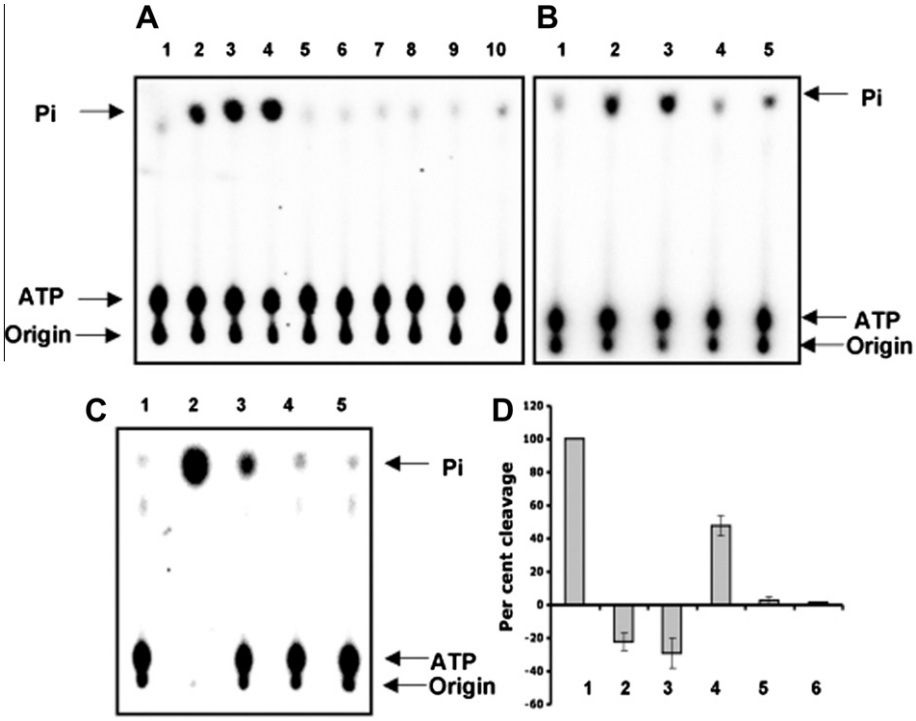

Fig. 2. Comparative and mutational analysis of ATPase activity. ATPase assay was carried out in a reaction mixture containing ATPase assay buffer, 20 μM ATP and 0.008 mCi [γ-^{32}P]ATP. 1 μl reaction mixture was spotted on PEI-cellulose TLC sheets, developed in a chromatographic chamber and exposed to phosphorimager. Unhydrolyzed [γ-^{32}P]ATP and released radiolabeled Pi are marked. (A) Lane 1: negative control for enzyme, lanes 2–4: reaction carried out with 0.2 μM, 0.4 μM, 0.6 μM E191A VPg-Pro, respectively, lanes 5–7: reaction carried out with 0.2 μM, 0.4 μM, 0.6 μM NIa-Pro, respectively, lanes 8–10: reaction carried out with 0.2 μM, 0.4 μM, 0.6 μM VPg, respectively. (B) Lane 1: negative control for enzyme, lane 2: 0.2 μM VPg-Pro, lane 3: 0.3 μM E191A VPg-Pro, lane 4: 0.2 μM (VPg + Pro), lane 5: 0.3 μM (VPg + Pro). (C) Lane 1: negative control for enzyme, lane 2: positive control with 0.28 U CIAP, lane 3: 0.2 μM E191A VPg-Pro, lane 4: 0.2 μM K47A:E191A VPg-Pro, lane 5: 0.2 μM D88A:E89A:E191A VPg-Pro. (D) The fractional cleavages were used to quantitate extent of hydrolysis and normalized as 100% for E191A VPg-Pro. The comparative percent cleavage for all proteins was plotted (bar 1: E191A VPg-Pro, bar 2: NIa-Pro, bar 3: VPg, bar 4: equimolar VPg + Pro *in trans*, bar 5: K47A:E191A VPg-Pro, bar 6: D88A:E89A:E191A VPg-Pro).

PVBV VPg also has disordered regions which may fold into distinct secondary and possibly tertiary structures under suitable conditions.

On similar lines, while over-expressed PVBV His-VPg has been observed to form inclusion bodies, the His-VPg expressed at the N-terminus of NIa-Pro could be purified from the soluble fraction upon *in vivo* cleavage by the protease [5]. This was the first indication that the presence of NIa-Pro could play a role in folding and expression of VPg. The circular dichroism (CD) profile of purified His-VPg (Supplementary Fig. S3A), its GdnHCl denaturation (Supplementary Fig. S3B), non-cooperative thermal melting, ANS binding (data not shown), aberrant behavior on SDS–PAGE [5] and sequence analyses, altogether indicated that PVBV VPg, like other potyviral VPgs, exists as a molten-globule [21,28,29]. Moreover, when PVBV His-VPg was present along with the globular NIa-Pro *in cis* (E191A VPg-Pro; Fig. 1C, lane 2) or *in trans* (VPg + Pro), both the domains were found to influence each other's structural features (Supplementary Fig. S3A). Noticeably, E191A VPg-Pro was seen to adopt a folded structure which followed an unfolding profile (Supplementary Fig. S3B) similar to that observed for human retinol-binding protein and carbonic anhydrases ([30] and references therein).

A multiple sequence alignment of PVBV VPg-Pro, carried out using ClustalW with sequences from PVA, PVY, *Tobacco etch virus* (TEV) and LMV (Fig. 1B), showed the conserved residues forming the catalytic triad of NIa-Pro (His46, Asp81 and Cys151), the suboptimal cleavage site between the two domains, the NLS I and II. Interestingly, a conserved Lys47 was found to lie within the VPg domain in the context of the consensus sequence corresponding to Walker motif A (G/AX$_4$GKS/T), and was followed by another downstream sequence (^{88}DESV^{91}) which was found to resemble the Walker motif B (Fig. 1B).

3.2. NIa-Pro-induced ATPase activity of VPg

In order to examine the role of Walker motifs in the VPg domain and the influence of NIa-Pro on VPg function, ATPase assays (refer section 2.2) were carried out using equimolar concentrations of either His-NIa-Pro (Fig. 2A, lanes 5–7), His-VPg (Fig. 2A, lanes 8–10) or His-E191A VPg-Pro (Fig. 2A, lanes 2–4), as the enzyme, along with radiolabeled [γ-^{32}P]ATP as the tracer. Interestingly, only E191A VPg-Pro was seen to exhibit an ATPase activity (Fig. 2A and D).

Optimum ATPase activity was exhibited at pH 7.0 and 37 °C, and the reaction was linear up to 50 min (Supplementary Fig. S2B–D). Further, the heat-denatured E191A VPg-Pro did not exhibit any activity (Supplementary Fig. S2A). The presence of Mg^{2+} or Mn^{2+} ions (optimally 0.2 mM) was found to enhance the ATPase activity of E191A VPg-Pro (Fig. 3A), whereas the Co^{2+} ions had no influence, and the Zn^{2+} ions inhibited the ATPase reaction (data not shown). Presence of 5 mM EDTA in the reaction mixture was found to completely abolish ATP hydrolysis (Fig. 3B), further confirming that E191A VPg-Pro exhibits a metal-dependent ATPase activity. Fig. 3C depicts that the addition of low concentration (50 mM) of NaCl further enhanced the reaction rate. A modest two fold increase in ATPase activity was also observed in the presence of poly(A) single-stranded RNA (Fig. 3D). However, the activity remained unaltered when single-stranded M13 DNA was added (data not shown). Moreover, inability of E191A VPg-Pro to hydrolyze *p*-nitrophenyl phosphate, unlike CIAP, implied that E191A

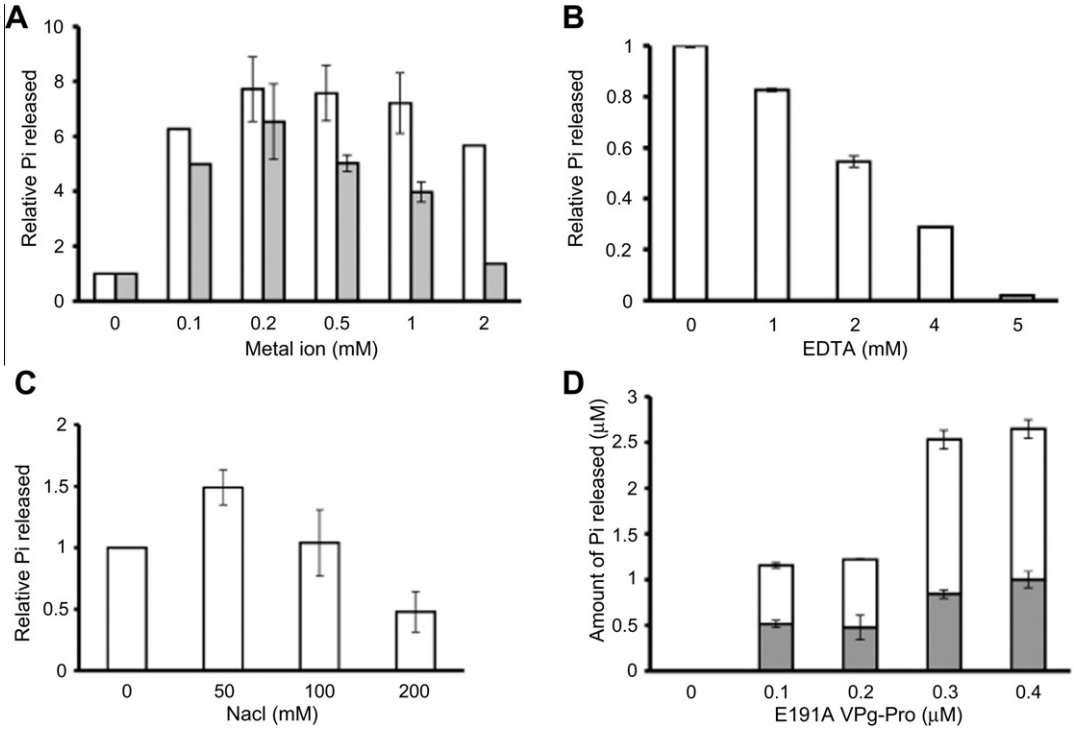

Fig. 3. Metal ion and poly(A) dependence of ATPase activity of E191A VPg-Pro. (A) ATPase assay was carried out at 25 °C in a reaction mixture containing ATPase assay buffer (without $MgCl_2$), 20 μM ATP and 0.008 mCi [γ-^{32}P]ATP and varying concentrations of $MgCl_2$ (white bars) or $MnCl_2$ (gray bars). The Pi released in the absence of $MgCl_2$ was taken as 1. (B) ATPase reaction was carried out with additional 0.2 mM $MgCl_2$ at 25 °C for 30 min with varying concentrations of EDTA. (C) ATPase reaction was carried out with additional 0.2 mM $MgCl_2$ and with varying concentrations of NaCl. The Pi released with 0.2 mM $MgCl_2$ and 0 mM EDTA, was taken as 1 and plotted against the varying parameter in Fig. 3B and C. (D) ATPase reaction was carried out with additional 0.2 mM $MgCl_2$ with (white bars) or without (gray bars) 1 μg poly(A) RNA. The amount of product released was plotted against E191A VPg-Pro concentration.

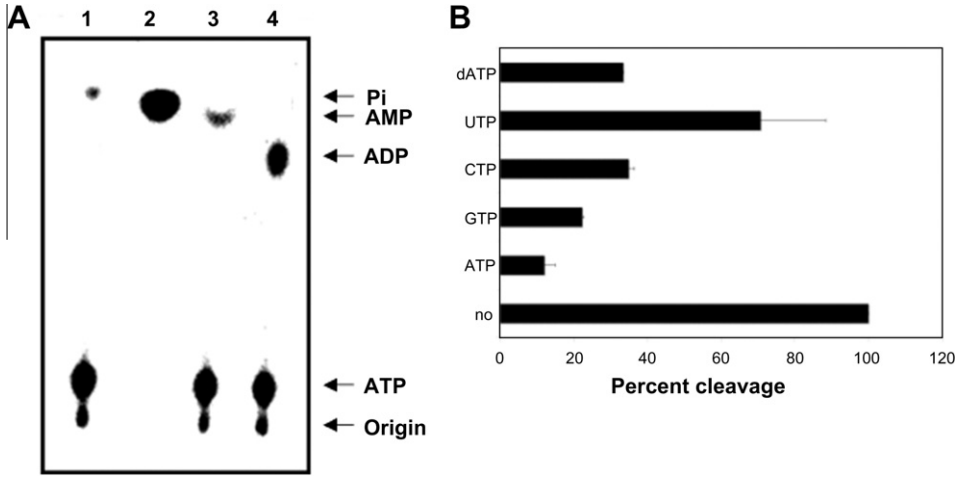

Fig. 4. Nucleotide specificity. (A) ATPase assay was carried out in a reaction mixture containing ATPase assay buffer, 20 μM ATP and 0.008 mCi [α-^{32}P]ATP. Lane 1: negative control for enzyme, lane 2: positive control using 0.28 U CIAP, lane 3: using 1 U yeast pyrophosphatase to monitor [α-^{32}P]AMP, lane 4: using 0.2 μM E191A VPg-Pro. Reaction mixture was spotted on TLC sheets, developed in a chromatographic chamber, exposed to phosphorimager. Arrows indicate the position of ATP, ADP, AMP and Pi. (B) NTPase assay was carried out in a reaction mixture containing 0.2 μM E191A VPg-Pro in ATPase assay buffer, 0.008 mCi [γ-^{32}P]ATP, and 20 μM (d)NTPs. Per cent cleavage with respect to a control reaction with only [γ-^{32}P]ATP was plotted.

VPg-Pro was not a general phosphatase (data not shown). Subsequently, when purified His-NIa-Pro (equimolar concentration) was added *in trans* with His-VPg, Pi release could be detected (Fig. 2B, lanes 4–5). However, the ATPase activity observed upon such *trans* interaction was lesser as compared to that reported for E191A VPg-Pro (*cis* interaction; Fig. 2B, lanes 2–3).

Overall, a comparative analysis (Fig. 2D) confirmed that when the partially disordered VPg and globular NIa-Pro were present together, a gain of ATPase function could be observed. This gain was more apparent when the two domains were present *in cis* as compared to when they were present *in trans*. It is possible that the interaction with NIa-Pro assists the flexible VPg domain to attain

a conformation which might optimally orient the Walker motif residues, thus resulting in viable ATPase activity. It is interesting to note that this is the first report demonstrating that a viral VPg domain can possess an enzymatic activity.

3.3. Substrate specificity and product characterization

The nature of products formed by E191A VPg-Pro was analyzed by conducting the ATPase assay using [α-^{32}P]-labeled ATP as the tracer. The phosphorimager result demonstrated that the radiolabeled product was observed at a position corresponding to ADP (Fig. 4A). Therefore, E191A VPg-Pro catalyzed the removal of a single phosphate moiety from [α-^{32}P]ATP, to release [α-^{32}P]ADP and Pi as the products.

Competition assays were performed using [γ-^{32}P]ATP as tracer along with unlabeled nucleotides (20 μM). When only radiolabeled ATP was used, nearly the entire substrate was found to undergo hydrolysis catalyzed by E191A VPg-Pro. However, unlabeled ATP was observed to compete with [γ-^{32}P]ATP for binding to E191A VPg-Pro, leading to reduced hydrolysis of the labeled ATP (<10%; Fig. 4B). GTP was seen to compete with [γ-^{32}P]ATP more efficiently as compared to dATP, UTP and CTP. The preference of E191A VPg-Pro to bind to the tested nucleotides was found to vary in the order of ATP > GTP > dATP ≈ CTP > UTP. Using [γ-^{32}P]GTP, it was determined that the rate of hydrolysis of GTP was slower (eight fold) as compared to ATP, and ⩾15 μM GTP concentration was found to inhibit GTPase activity (data not shown). Therefore, ATP was found to be the most optimal nucleotide substrate for nucleotide hydrolytic activity of E191A VPg-Pro.

3.4. Mutational analysis of VPg-Pro

In order to assess the importance of residues present in the putative Walker motifs of VPg, Lys47 of Walker motif A and, Asp88 and Glu89 of Walker motif B were mutated to Ala in E191A VPg-Pro (Fig. 1C, lanes 3–4; refer section 2.1). These proteins were purified and used at equivalent molar concentrations (Fig. 2C, lanes 4–5) as E191A VPg-Pro (Fig. 2C, lane 3) in the ATPase assays. Both mutants showed reduced activities (Fig. 2D, bars 5–6) as compared to either *cis*-interacting E191A VPg-Pro, or *trans*-interacting VPg + Pro (Fig. 2D, bars 1 and 4). Kinetic constants for the ATPase activity of VPg-Pro mutants were determined (Supplementary Fig. S4). The K_m for E191A VPg-Pro was 19.9 ± 2.0 μM and the V_{max} was 0.77 ± 0.17 nmoles/mg/min. This activity was inhibited by higher concentration (50–100 μM) of ATP. The kinetic parameters of ATPase activity for E191A VPg-Pro were found to be comparable to those of other potyviral ATPases [31,32]. The K_m and V_{max} for K47A:E191A VPg-Pro were found to be 9.8 ± 2.6 μM and 0.13 ± 0.03 nmoles/mg/min, respectively. Thus, while the K_m values for both the proteins were comparable, the V_{max} for the Walker motif A mutant (K47A:E191A) was much lower (seven times) as compared to the E191A VPg-Pro. The far-UV CD profiles of E191A VPg-Pro and K47A:E191A VPg-Pro were found to be similar (data not shown), which confirmed that the loss in activity was not due to alterations in secondary structure of the Walker motif A mutant protein. The ATPase activity exhibited by D88A:E89A:E191A VPg-Pro was highly reduced and accurate determination of kinetic parameters was beyond the scope of sensitivity of the assay used. Additionally, the yield and stability of D88A:E89A:E191A VPg-Pro varied from batch-to-batch.

Overall, these results confirmed that ATPase activity is an inherent property of the flexible VPg domain within VPg-Pro and, Lys47 of Walker motif A and Asp88 and Glu89 of Walker motif B are crucial for optimal function. However, the presence of a globular NIa-Pro domain, *in cis* or *in trans*, is essential for the manifestation of this activity. Interestingly, the residue corresponding to Lys47 (Lys44, PVA numbering) has been shown to be crucial for multiple potyviral functions, such as RNA binding, uridylylation, interaction with host proteins, balance between viral and host translation, viral RNA stability, suppression of RNA silencing and the overall virus viability [8,9,12–14]. Thus, it is likely that the ATPase activity of VPg-Pro intermediate, exhibited by virtue of Lys47, is utilized during viral translation initiation, replication initiation, VPg uridylylation or polyprotein processing [3,8].

In addition, potyviral VPg has also been demonstrated to play crucial roles in cell-to-cell and long distance movement of the virus. In fact when PVBV virions, purified from infected plants, were analyzed by Western analysis using antibodies raised against NIa-Pro, a band corresponding to VPg-Pro was observed (Supplementary Fig. S5B). Two-dimensional electrophoresis of virions demonstrated that this VPg-Pro was linked to an oligonucleotide (Supplementary Fig. S5C). These results implied that the VPg-Pro obtained from PVBV virions was covalently linked to the genomic RNA of the virus. Similar observation has been made in virions purified from TEV- and BtMV-infected plants [33,34]. The ATPase activity of the VPg-Pro present in the virions could thus be utilized for fulfilling the energy requirements for viral movement. The natively unfolded ~8 kDa protein (P8) of *Sesbania mosaic virus* (SeMV) has been demonstrated earlier to enhance the ATPase activity of the adjacent P10 domain which has been suggested to fuel the movement process of this virus [35].

Other potyviral proteins, such as the cylindrical inclusion (CI) protein, HC-Pro and the coat protein, have been reported to exhibit ATPase activities in earlier studies [31,36]. It is notable that VPg-Pro would be the only potyviral ATPase that is targeted to the nucleus. In fact, nuclear fractions from PVBV-infected plants have been shown to contain the VPg-Pro intermediate [7]. Therefore, it is possible that the ATPase activity could assist VPg-Pro during suppression of host functions [7,9]. The ATPase activity has indeed been observed to be modulated by poly(A) RNA (Fig. 3), as is true for other helicases [20]. However, whether this protein harbors a helicase activity remains to be determined.

Taken together, the observations of the present study and those presented earlier [5] provide a biochemical insight into the importance of prolonged retention of VPg-Pro intermediate, as well as its subsequent cleavage (into individual VPg and NIa-Pro domains), in modulating both the ATPase (VPg) and protease activities (NIa-Pro) at different stages of the potyviral life cycle.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.020.

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