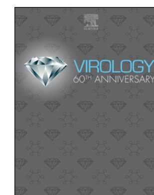
Virology 524 (2018) 18–31

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology



Mapping the domain of interaction of PVBV VPg with NIa-Pro: Role of N-terminal disordered region of VPg in the modulation of structure and function

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ARTICLE INFO

Keywords:
Potyviridae
Pepper vein banding virus (PVBV)
Viral protein genome linked (VPg)
Molecular recognition feature (MoRF)
Intrinsically disordered proteins (IDPs)
Nuclear inclusion a-protease (NIa-Pro)
Analytical ultracentrifugation (AUC)

ABSTRACT

VPg-Pro is involved in polyprotein processing, therefore its regulation is important for a successful potyviral infection. We report here that the N-terminal disordered region of VPg forms the domain of interaction with NIa-Pro. This region is also demonstrated to be responsible for modulating the protease activity of VPg-Pro, both in *cis* and *trans*. The disordered nature of VPg is elicited by the N-terminal 22 residues as removal of these residues (ΔN22 VPg) brought about gross structural and conformational changes in the protein. Interestingly, ΔN22 VPg gained ATPase activity which suggested the presence of autoinhibitory motif within the N-terminal region of VPg. The autoinhibition gets relieved upon interaction of VPg with NIa-Pro or removal of the inhibitory motif. Thus, the N-terminal 22 residues of VPg qualify as molecular recognition feature (MoRF), regulating both protease and ATPase activity of VPg-Pro as well as forming the domain of interaction with other viral/host proteins.

1. Introduction

Viruses code for only a few proteins due to their small genome size. They need to optimize the functions of these proteins such that they can utilize the host metabolism and replicate as well as shut off the host defence responses. This, they achieve by encoding intrinsically disordered proteins (IDPs) or domains that act as hubs for interaction with a variety of viral as well as host factors, thereby playing a key role in establishing infection. Invariably many of the viral encoded proteins are multifunctional and their functions are modulated by interaction with cognate partners via the IDPs. IDPs are abundant in nature (Dunker et al., 2008) and are encoded by all organisms starting from viruses to higher eukaryotes. They have higher proportion of hydrophilic and charged residues and are devoid of the hydrophobic core (Gsponer and Madan Babu, 2009; Cino et al., 2013). The IDPs fail to fold into well-defined three dimensional structures (Gsponer and Madan Babu, 2009), as the hydrophobic core is indispensable for the same. However, these proteins undergo a transition to form a more rigid structure (disorder-to-order transition) upon interaction with their cognate partners (Gsponer and Madan Babu, 2009; Cino et al., 2013; Eliezer and Palmer, 2007; Uversky, 2009). Therefore, the IDPs have a repertoire of distinct and dynamic conformations (Fink, 2005). Their higher structural flexibility (Mészáros et al., 2012) allows them to interact with several partners and regulate their functions (Chouard, 2011). These interactions are of higher specificity but low-affinity, thus being favourable for dynamic processes which require both reversibility and specificity within protein-protein interaction networks (Sun et al., 2013). IDPs thus highlight the importance of conformational flexibility and heterogeneity in protein function (Das et al., 2015). IDPs also render a larger intermolecular interface when compared to the folded proteins for interaction with other proteins/ligands and nucleic acids (Fink, 2005). A large number of these IDPs have been described from various proteomes with many being involved in cellular recognition and signalling pathways (Uversky, 2010). They are also abundant in various proteins/peptides causing diseases, that comprise the disease-related unfoldome (Uversky et al., 2009). Mutations that interfere with

Abbreviations: IDPs, Intrinsically disordered proteins; PVBV, *Pepper vein banding virus*; VPg, Viral protein genome linked; NIa-Pro, Nuclear inclusion a-protease; MoRF, Molecular Recognition Feature; SPR, Surface Plasmon Resonance; AUC, Analytical Ultracentrifugation

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https://doi.org/10.1016/j.virol.2018.08.002
Received 4 May 2018; Received in revised form 30 July 2018; Accepted 4 August 2018
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interfacial interactions of IDPs can result in disease conditions (Mittal et al., 2013). For instance, any mutation in p53 (a transcription factor with its intrinsically disordered C-terminal domain being involved in interaction with over a 100 proteins) which disrupts the interaction-network leads to cancer development (Uversky et al., 2009). IDPs enable many to one and one to many interactions (Wang et al., 2011) and such crucial interactions being weaker due to the entropic penalty, can be good drug targets (Dunker et al., 2008).

RNA viruses generally have smaller genomes. While this allows RNA viruses to mutate and evolve faster (Sanjuán and Domingo-Calap, 2016), it also confers a better adaptation to the environmental changes (Charon et al., 2018). Therefore in this context, encoding IDPs becomes essential for RNA viruses as intrinsically disordered regions of IDPs are permissive to faster mutation rates when compared to the structured regions (Charon et al., 2016). Therefore, studying IDPs from RNA virus is the focus of the present study.

Potyviridae is one of the largest family of plant viruses (Martínez et al., 2016) infecting the various economically important crops. Apart from infecting agriculture crops, metagenomics studies have pointed out that the potyviruses can infect wild plant hosts as well (Roossinck, 2012). *Pepper vein banding virus* (PVBV), a distinct member of the genus *Potyvirus* (Family: *Potyviridae*) is a flexuous, non-enveloped, rod-shaped RNA virus (Ravi et al., 1997). It has a 9.7 kb single-stranded positive sense RNA genome encoding a 340 kDa polyprotein (Anindya et al., 2004). The genome organization and expression strategies of potyviruses are similar to picornaviruses (Domier et al., 1987) and are therefore classified along with picorna-like superfamily of viruses. Potyviral polyprotein is proteolytically processed by three viral-encoded proteases viz., P1-Protease (P1-Pro), Helper component-protease (HC-Pro) and Nuclear Inclusion a-protease (NIa-Pro) to generate 11 mature proteins (Urcuqui-Inchima et al., 2001). P1-Pro and HC-Pro have *cis* cleavage activity and cleave themselves off at their C-terminus (Pasin et al., 2014), while NIa-Pro has both *cis* and *trans* cleavage activity (Runeberg-Roos et al., 2002). There is a sub-optimal cleavage site between VPg and NIa-Pro (Schaad et al., 1996) with a glutamate at P1 position (P1 position in the recognition sequence) instead of glutamine. Therefore, NIa-Pro exists mostly as VPg-Pro with N-terminal VPg domain and C-terminal NIa-Pro domain. It was shown that this suboptimal cleavage is essential for efficient virus multiplication (Carrington et al., 1993). Potyviral VPgs as well as those from other plant RNA viruses are intrinsically disordered with minimal residual structure (Grzela et al., 2008; Jiang and Laliberté, 2011; Mathur et al., 2012; Rantalainen et al., 2009; Satheshkumar et al., 2005). VPg is covalently linked to the 5′ terminus of RNA and acts as a primer for the initiation of negative and positive strand RNA synthesis during viral replication (Anindya et al., 2005). It interacts with various proteins at different stages of the viral life-cycle due to the presence of disordered domain and thus acts as a hub of the interaction-network (Chouard, 2011). In addition, VPg has a bipartite nuclear localization signal (NLS I and NLS II) at its N-terminus which localizes it to the nucleus and regulates the concentration of VPg-Pro present in the cytoplasm at a given time (Riechmann et al., 1992). Various partially processed intermediates are also generated during proteolytic processing of the polyprotein in potyviruses. Therefore, VPg is also present as 6K2–VPg–NIa-Pro where it is recruited to the viral replication complexes, thus playing a crucial role in viral RNA replication (Revers and García, 2015).

In an earlier study we have demonstrated that the interaction of VPg with NIa-Pro results in the activation of PVBV NIa-Pro (Mathur et al., 2012). Further, VPg by itself does not possess ATPase activity even though it contains the Walker A and Walker B motifs at the N-terminal region. However, upon interaction with NIa-Pro, VPg was shown to gain the ATPase activity (Mathur and Savithri, 2012). The present study aims to delineate the region of VPg responsible for interaction as well as modulation of the protease and ATPase activities of VPg-Pro. N-terminal deletion mutants of VPg and VPg-Pro were generated to monitor their interaction with NIa-Pro in *trans* and in *cis*, respectively. The results demonstrate that the disorder-to-order transition of VPg upon interaction with NIa-Pro is facilitated by the N-terminal 22 residues of VPg. There was a 3 fold decrease in the protease activity of ΔN22 VPg-Pro with a concomitant reduction in the interaction of ΔN22 VPg with NIa-Pro. Further, there is a gain of ATPase function in VPg when these residues are deleted indicating their autoinhibitory effect on the ATPase activity. Thus, the N-terminal 22 residues of VPg act as a positive regulator for the protease activity of VPg-Pro and an autoinhibitor for its own ATPase activity. The autoinhibition is alleviated upon interaction of these residues with NIa-Pro and finally these residues are also responsible for the disorder-to-order transition of VPg upon interaction with the cognate partners. Therefore, the N-terminal 22 residues are entitled to be termed as the molecular recognition feature (MoRF).

2. Materials and methods

Media and bacteriological reagents were primarily from Hi-Media, Mumbai, India. Other chemicals and reagents, including oligonucleotides, were purchased from Sigma-Aldrich. The custom peptides were from Genetix. DNA-modifying enzymes and restriction enzymes were obtained from Fermentas and New England Biolabs. [γ-^{32}P] ATP was obtained from Board of Radiation and Isotope Technology (BRIT), Unit of Atomic Energy, Hyderabad, India. The bacterial strains used in the study are *E.coli* DH5α, *E.coli* strain BL21(DE3) containing either pLysS or pSBET A.

2.1. Cloning, overexpression and purification of recombinant proteins

The pRVN clone (Mathur et al., 2012) was used as template to amplify various deletion mutants of VPg-Pro using primers as mentioned in Table S1. The amplified PCR products were cloned at *Nhe*I and *Bam*HI sites in pRSET C (Invitrogen). The recombinant clones (ΔN22 VPg-Pro, ΔN67 VPg-Pro and ΔN113 VPg-Pro) were confirmed by sequencing. Expression of hexa histidine tagged VPg-Pro in BL21 *E. coli* cells containing pSBET A, yielded hexa histidine tagged VPg and untagged NIa-Pro due to auto-catalytic cleavage between the domains by the protease. Therefore, to purify VPg, VPg-Pro was over-expressed in 1 l culture by induction with 0.3 mM IPTG and incubation for 12 h at 16 °C with constant shaking at 120 rpm. The cells were harvested at 6000 rpm for 12 min at 4 °C. The cell pellet was resuspended in 25 ml of 40 mM CAPS-NaOH buffer (pH 9.2) containing 200 mM NaCl, 10% glycerol and 1% triton X-100. Resuspended culture was treated with lysozyme and incubated on ice for 1 h. The cells were then lysed by sonication for 10 min. The crude lysate was then centrifuged at 12,000 rpm for 10 min to remove debris. The soluble fraction was allowed to bind to 2 ml of Ni-NTA resin using an end-to-end rotor for 2–4 h. The protein resin complex was packed into a column and washed with 20X bed volume with wash buffer (10 mM CAPS, 200 mM NaOH, 15 mM imidazole pH 9.2) to remove NIa-Pro and other contaminants. VPg was eluted with 10 mM CAPS-NaOH, pH 9.2, 200 mM NaCl and 250 mM imidazole and dialyzed against 10 mM CAPS-NaOH, pH 9.2, containing 200 mM NaCl and 10% glycerol to remove imidazole. The purity of the protein was checked by SDS-PAGE. Similarly, ΔN22 VPg, ΔN67 VPg and ΔN113 VPg mutants were also purified. Protein concentrations were determined using Bradford method for protein estimation (Hammond and Kruger, 1988). VPg (~ 1.4 mg), ΔN22 VPg (~ 4 mg), ΔN67 VPg (~ 2 mg) and 0.5 mg ΔN113 VPg were obtained from the respective one litre cultures. The pRVN E191A clone (Mathur et al., 2012) was used to generate the cleavage-site mutants [ΔN22 VPg-Pro (E191A), ΔN67 VPg-Pro (E191A) and ΔN113 VPg-Pro (E191A)] by PCR using the appropriate primers (Table S1). The PCR products were digested with *Nhe*I and *Bam*HI and cloned into pRSET C vect or double digested with the same enzymes. The clones were confirmed by restriction digestion and sequencing. VPg-Pro (E191A) cleavage site mutant and its deletion mutants were overexpressed and purified as described above. Approximately ~0.3–0.5 mg of purified VPg-Pro

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