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The VPgPro protein of *Turnip mosaic virus*: In vitro inhibition of translation from a ribonuclease activity

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Abstract

A role for viral encoded genome-linked (VPg) proteins in translation has often been suggested because of their covalent attachment to the 5′ end of the viral RNA, reminiscent of the cap structure normally present on most eukaryotic mRNAs. We tested the effect of *Turnip mosaic virus* (TuMV) VPgPro on translation of reporter RNAs in in vitro translation systems. The presence of VPgPro in either wheat germ extract or rabbit reticulocyte lysate systems lead to inhibition of translation. The inhibition did not appear to be mediated by the interaction of VPg with the eIF(iso)4E translation initiation factor since a VPg mutant that does not interact with eIF(iso)4E still inhibited translation. Monitoring the fate of RNAs revealed that they were degraded as a result of addition of TuMV VPgPro or of *Norwalk virus* (NV) VPg protein. The RNA degradation was not the result of translation being arrested and was heat labile and partially EDTA sensitive. The capacity of TuMV VPgPro and of (NV) VPg to degrade RNA suggests that these proteins have a ribonucleolytic activity which may contribute to the host RNA translation shutoff associated with many virus infections.

Keywords: Inhibition of translation; Virus infection; Potyviruses; Picornaviruses; RNA degradation

Introduction

Turnip mosaic virus (TuMV; *Potyviridae*) is a member of the picorna-like supergroup of positive-sense RNA viruses. For many plant and animal RNA viruses, the virus-encoded VPg protein (*V*iral *P*rotein *g*enome-linked) is covalently attached to the 5′ terminus of their genomic RNA (van Regenmortel et al., 2000). This protein is positioned on the viral RNA where the m^7G cap structure is normally found on cellular mRNAs; it is not clear whether the VPg plays the same functional role as the cap structure in translation initiation for viral RNA.

The VPg protein, and its VPgPro precursor form, are multi-functional proteins that play important roles in the replication cycle of potyviruses (Urcuqui-Inchima et al., 2001). The VPg was shown to interact with translation initiation factors eIF(iso)4E (eukaryotic initiation factor (iso)4E) and PABP (poly(A)-binding protein) (Léonard et al., 2004; Wittmann et al., 1997). These interactions suggest a role for the VPg in the recruitment of translation initiation factors for viral RNA translation. Mutations in either VPg or eIF(iso)4E result in reduced ability of the virus to infect its host. Mutations in the eIF(iso)4E-interacting domain of VPg lead to loss of virus infectivity (Léonard et al., 2000), and disruption of plant eIF(iso)4E gene prevented TuMV infection (Duprat et al., 2002; Lellis et al., 2002). The involvement of VPg in facilitating viral RNA translation was shown for *Feline calicivirus* (FCV) (Goodfellow et al., 2005) where FCV translation is dependent on the presence of VPg at the 5′ end of the viral genome. The VPg–eIF4E interaction is required for virus RNA translation since sequestration of eIF4E by 4E-BP1 inhibited translation. It was suggested that FCV VPg acts as a ‘cap substitute’ during translation initiation of virus mRNA.

Viruses that infect eukaryotic cells use a variety of mechanisms for subverting the functions of the host cell. Several viruses alter the translation machinery such that they effectively block translation of host mRNAs. Viruses often target translation initiation factors as a mean to increase their own translation at the expense of that of their host. The main strategies are either to compromise eIF4G or PABP functions by proteolytic cleavage, to sequester eIF4E or to alter the

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phosphorylation state of host translation factors (reviewed by Gale et al., 2000). Another key aspect of virus infection in plants and animals is the associated host transcription shut-down (Aranda and Maule, 1998; Jen et al., 1980). For instance, infection of plant cells with a potyvirus (carrying a 5′-bound VPg) leads to transient disappearance of most cellular mRNAs in cells supporting active viral replication (Aranda et al., 1996). It is not clear whether the disappearance of RNAs is the consequence of mRNA destabilization resulting from a stress response, of host transcription inhibition, or of targeted degradation resulting from a viral ribonuclease activity.

A more direct line of evidence implicating VPg in cellular translation inhibition was found for *Norwalk virus* (NV). NV VPg was shown to inhibit translation of a reporter RNA in rabbit reticulocyte lysate (RRL; Daughenbaugh et al., 2003). VPg added to cell-free translation reactions that contained either capped RNA or RNA with an internal ribosomal entry site (IRES) inhibited translation of these reporter RNAs in a dose-dependent manner. Although potyviral VPgPro protein was found to have non-specific RNA-binding properties (Daròs and Carrington, 1997), as well as a deoxyribonuclease activity (Anindya and Savithri, 2004), no link has been made between those activities and an inhibition of translation or the disappearance of mRNAs in plant cells. The mechanism by which this occurs remains unclear.

In this report, we investigated the effect of addition of TuMV VPgPro on the translation of reporter RNA in in vitro translation systems. Purified TuMV VPgPro inhibited translation of capped reporter RNA, as was observed when VPg of NV was added to an in vitro translation system. The VPgPro-eIF(iso)4E interaction was likely not involved in the inhibition of translation since a VPg mutant that does not interact with eIF(iso)4E did inhibit translation. We observed that this inhibition of translation was concurrent with degradation of reporter RNA in a rabbit reticulocyte lysate. Purified total plant RNA was also degraded when VPgPro was added; the same effect was observed with the VPg domain alone. A similar ribonucleolytic activity was also observed with NV VPg. The ribonucleolytic activity of VPg proteins may contribute to the disappearance of most mRNAs previously observed during potyvirus infection and to the transient inhibition of translation documented for host cell mRNAs during picornavirus infection.

Results

Expression and purification of VPg, VPgPro and eIF(iso)4E proteins

TuMV VPgPro was expressed as a fusion protein with the S-tag, glutathione-S-transferase (GST) and histidines fused at the N-terminus (a fusion tag of 33 kDa). SDS-PAGE analysis of the protein extracts obtained from soluble fractions following GST affinity chromatography showed that protein bands corresponding to both the VPg and VPgPro proteins were present. This is consistent with the data of Ménard et al. (1995) that showed that recombinant TuMV VPgPro self-cleaves into two functional domains, the VPg and Pro domains. A prominent 55 kDa band [VPg domain (22 kDa) + N-terminal tag (33 kDa)] and a fainter 82 kDa band [VPgPro domain (49 kDa) + N-terminal tag (33 kDa)] (Fig. 1) corresponding to GST–VPg and GST–VPgPro, respectively, were found in the preparation. Both the GST–VPg and GST–VPgPro fusions were obtained since the GST domain was fused at the N-terminus of the protein. The D77N mutant of VPgPro was similarly expressed and purified (Fig. 1). The identity of the proteins was confirmed by Western blot analysis using an anti-GST monoclonal antibody and a polyclonal anti-VPgPro serum (data not shown). The lower molecular weight products resulted from degradation of the fused GST moiety. NV and TuMV VPg proteins were also expressed in *E. coli* cells as N-terminal GST fusions (a fusion tag of 26 kDa); fusion proteins of 53 kDa (Fig. 1) [NV VPg domain (27 kDa) + N-terminal tag (26 kDa)] and a 48 kDa [VPg domain (22 kDa) + N-terminal tag (26 kDa)] were obtained for NV and TuMV, respectively. *Triticum aestivum* eIF(iso)4E protein was expressed as an N-terminal T7 fusion protein of 28 kDa (Fig. 1).

VPgPro inhibits in vitro translation of capped reporter RNA

A capped luciferase reporter RNA was translated either in wheat germ extract (WGE) (Fig. 2A) or in RRL (Fig. 2B) translation systems in the presence of increasing concentrations of TuMV GST–VPgPro or of GST protein. Luciferase luminescence was used to measure translation efficiency of the m^7G-luciferase RNA. Translation of the reporter mRNA decreased sharply when increasing amounts of GST–VPgPro were added to the translation systems. As a control for the presence of contaminants in our protein purifications, the same amounts of GST protein alone, produced and purified using the same procedure as GST–VPgPro, were added to the in vitro translation systems; no inhibition of translation was observed.

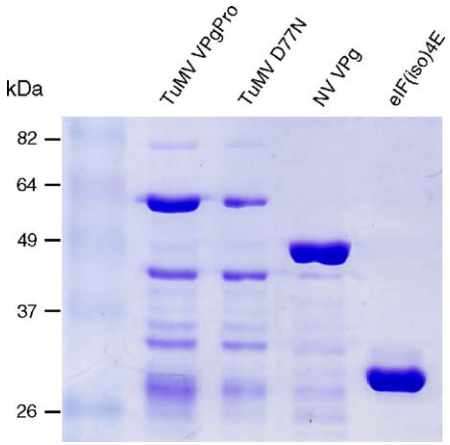

Fig. 1. Expression and purification of GST–VPgPro and D77N of TuMV, GST–VPg of NV and wheat eIF(iso)4E as described under Materials and methods. In both TuMV VPgPro and D77N protein preparations the prominent 55 kDa band corresponds to the self-cleavage product of VPgPro (or D77N) while the 82 kDa fainter band corresponds to the full length VPgPro species. Samples were loaded on a SDS-polyacrylamide gel and were stained with Coomassie blue.

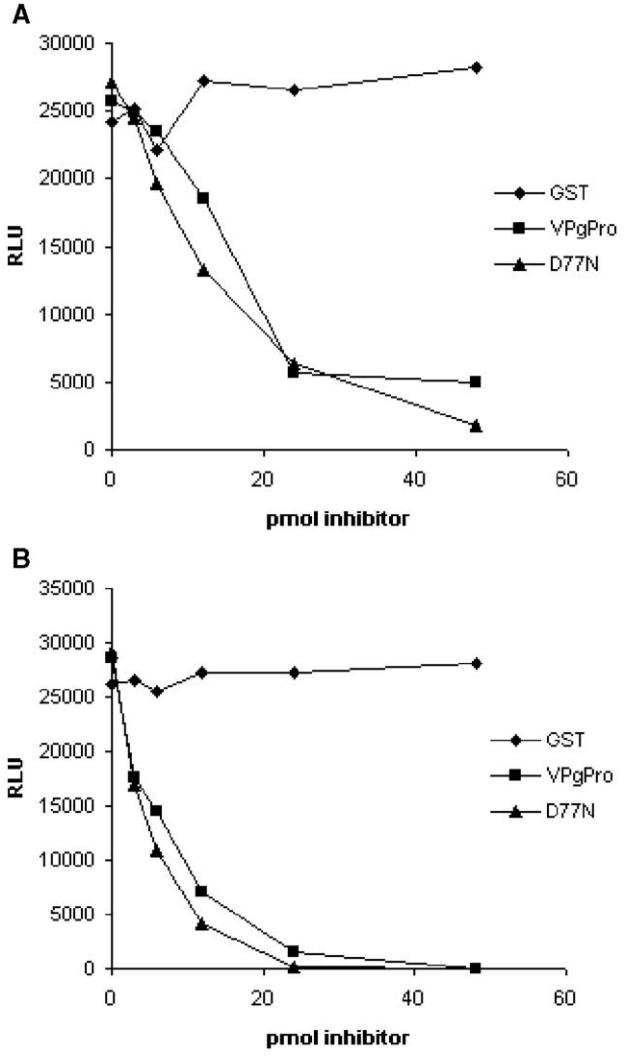

Fig. 2. Translation inhibition of reporter RNA by GST–VPgPro. (A) Relative light units (RLU) of luciferase obtained when adding capped luciferase RNA in WGE translation system in the presence of GST, GST–VPgPro or GST–D77N. (B) Capped luciferase RNA was added to RRL translation system in the presence of GST, GST–VPgPro or GST–D77N and luciferase RLU was measured. These experiments were repeated at least three times.

Role of the eIF(iso)4E–VPgPro interaction in translation inhibition

We tested the D77N mutant of VPgPro on its capacity to interfere with translation in vitro. The D77N mutant contains an asparagine at position 77 of the protein instead of an aspartic acid residue; the resulting protein is unable to interact with eIF(iso)4E and virus infectivity is abolished (Léonard et al., 2000). We reasoned that if the eIF(iso)4E–VPgPro interaction is essential for the inhibition of translation, the addition of D77N into WGE or RRL translation systems would not interfere with translation of the m^7G-luciferase RNA. Increasing amounts of GST–VPgPro, GST or GST–D77N were added to translation reactions (WGE and RRL). GST–VPgPro or GST–D77N both decreased translation of the reporter gene in the same fashion, in both the RRL and WGE systems. Addition of GST had no effect on translation of the reporter RNA (Fig. 2).

Furthermore, to test whether the VPgPro-mediated translation inhibition was linked to the sequestration of eIF(iso)4E by VPgPro, different concentrations of VPgPro were pre-incubated with *T. aestivum* eIF(iso)4E before addition to the translation reaction. Addition of 24 or 48 pmol of VPgPro inhibited capped luciferase RNA translation even in the presence of excess eIF (iso)4E (Fig. 3A). Myoshi et al. (2005) have previously reported that the GST portion of GST–VPg interferes with the binding of TuMV VPg to eIF(iso)4E from *A. thaliana* in pull-down assays. However, in our study, GST-tagged TuMV VPgPro did interact with wheat eIF(iso)4E in ELISA-based binding assays (Fig. 3B). Binding was specific as no signal was detected in absence of primary antibody or when eIF(iso)4E was replaced with T7-tagged β-galactosidase.

TuMV VPgPro and NV VPg degrade reporter RNA

We monitored the fate of reporter RNA during in vitro translation to investigate how VPgs affect protein translation.

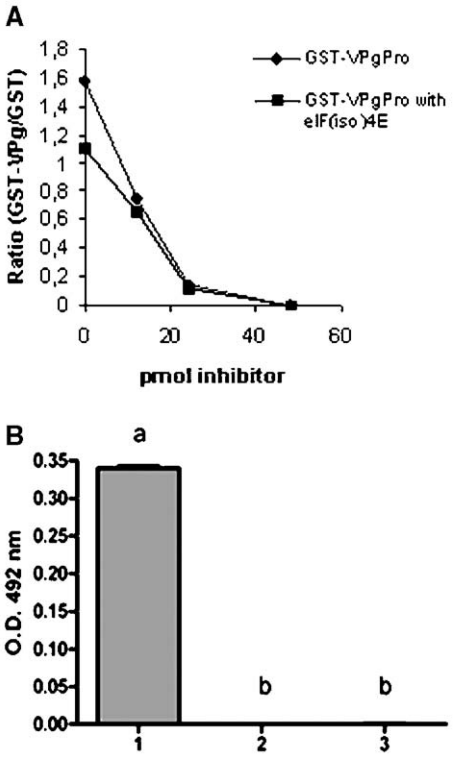

Fig. 3. Inhibition of translation of luciferase reporter RNA by GST–VPgPro in the presence of eIF(iso)4E. (A) Ratio of the luciferase light units (synthesized from capped luciferase RNA) from translation reactions containing GST–VPgPro over that of reactions containing GST in WGE incubated or not with eIF (iso)4E. (B) GST–VPgPro interaction with wheat T7-eIF(iso)4E as demonstrated by ELISA-based binding assay. Wells were coated with 1.5 μg of purified GST–VPgPro and incubated with 1.5 μg of *E. coli* recombinant T7-tagged eIF (iso)4E (bar 1) or T7-tagged β-galactosidase (bar 3). Protein retention was detected using a monoclonal anti-T7-tag antibody. Non-specific binding of the secondary antibody was verified by incubating VPgPro and eIF(iso)4E in absence of anti-T7 tag antibody (bar 2). Error bars represent the standard error of the mean. Groups a and b are statistically different ($P < 0.001$).

After addition of luciferase RNA in the RRL in presence of GST or GST–VPgPro from TuMV, or in presence of NV VPg, RNA was collected at different times and purified. Fig. 4 shows that reporter RNA is degraded when TuMV GST–VPgPro or NV GST–VPg, but not GST, is added to the translation assay. An incubation period as short as 5 min was sufficient to allow TuMV GST–VPgPro to degrade the reporter RNA. Ribosomal RNA was not affected by the addition of either protein to the in vitro RRL.

mRNA turnover can be substantially increased when translation is arrested (Stanssens et al., 1986). We tested whether or not RNA degradation was triggered by the absence of translation. Reporter RNA was incubated without added protein or with 48 pmol of GST or GST–VPgPro from TuMV in WGE in presence of cycloheximide. The addition of 600 μM of cycloheximide to the in vitro translation system completely inhibited translation of the reporter luciferase RNA since no light emission was detected (data not shown). Samples were collected at different times and RNA was electrophoresed, blotted and hybridized with a ^{32}P-labelled RNA probe complementary to the luciferase RNA. Fig. 5 shows that the reporter luciferase RNA was degraded more rapidly in the presence of TuMV GST–VPgPro compared to controls where no protein was added or where GST was added.

TuMV VPgPro and NV VPg degrade total plant RNA

To test the involvement of cellular factors for the VPgPro ribonucleolytic activity, 48 pmol of TuMV GST–VPgPro, NV GST–VPg and GST were incubated with total plant RNA. Fig. 6 shows that TuMV GST–VPgPro and NV GST–VPg degraded total plant RNA within 30 min. In contrast with the RNA degradation observed in the in vitro translation system (see Fig. 4), ribosomal RNA was degraded by GST–VPgPro and GST–VPg. Total plant RNA incubated with GST was not degraded nor was the RNA sample incubated with no added protein.

The VPg domain of TuMV is sufficient for the degradation of total plant RNA

VPgPro auto-catalytically cleaves itself into two functional domains: VPg and Pro (Laliberté et al., 1992). Since NV VPg

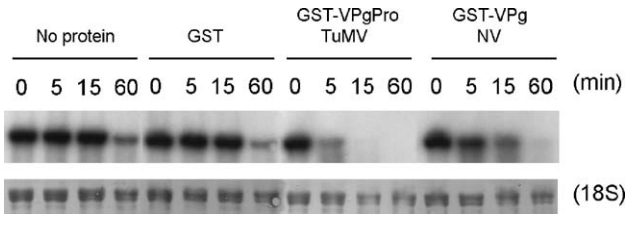

Fig. 4. Reporter RNA degradation in the presence of GST, TuMV GST–VPgPro or NV GST–VPg in a RNA stability assay. The proteins were incubated in a RRL in the presence of capped luciferase RNA and RNA samples were collected at different time points. Total RNA was run on an agarose-formaldehyde gel and transferred to a nylon membrane which was incubated with a ^{32}P-labelled RNA probe complementary to luciferase RNA. The 18S rRNA was used as a loading reference.

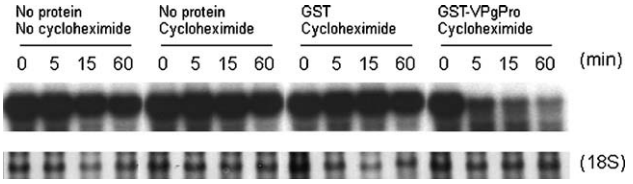

Fig. 5. Reporter RNA degradation in the presence of GST or TuMV GST–VPgPro in absence of translation. 48 pmol of GST or TuMV GST–VPgPro proteins were incubated in WGE in the presence of capped luciferase RNA with 600 μM of cycloheximide to arrest translation of the reporter RNA. Samples were collected at different times and RNA was purified. Total RNA was run on an agarose-formaldehyde gel and transferred to nylon. The membrane was incubated with a ^{32}P-labelled RNA probe complementary to luciferase RNA. 18S rRNA was used as a loading reference.

degraded RNA, we verified if the VPg domain of VPgPro was responsible for the observed ribonucleolytic activity. We constructed and purified the VPg domain of TuMV VPgPro and added the protein to total plant RNA. TuMV GST–VPg was able to degrade total plant RNA as efficiently as GST–VPgPro (Fig. 7). No RNA degradation was observed when total RNA was incubated without protein or with GST.

Effect of EDTA and heat treatment on TuMV VPgPro ribonucleolytic activity

The requirement for divalent cations for VPgPro catalytic activity was tested by incubating total plant RNA with TuMV GST–VPgPro in increasing concentrations of EDTA. The results showed that GST–VPgPro nuclease activity was not completely inactivated by the addition of EDTA concentrations ranging from 1 to 10 mM (Fig. 8A). However, degradation products increased in size with increasing amounts of EDTA. To investigate whether RNA degradation is due to enzymatic

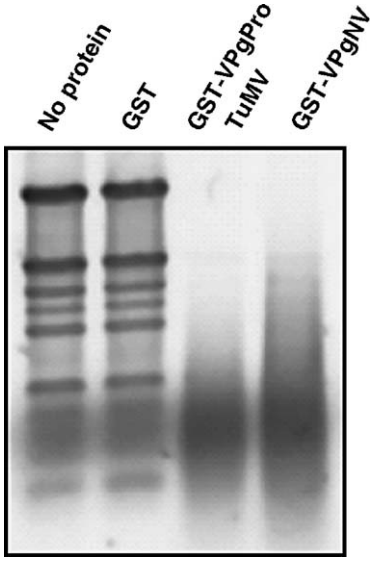

Fig. 6. Plant total RNA degradation by GST–VPgPro of TuMV and GST–VPg of NV. Total RNA was incubated without protein or with 48 pmol of GST, TuMV GST–VPgPro or NV GST–VPg for 30 min. The RNA was purified, run on an agarose gel and stained with ethidium bromide.

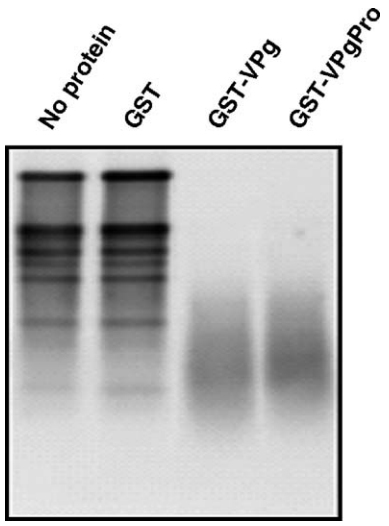

Fig. 7. Plant total RNA degradation by GST–VPg domain of TuMV. Total RNA was incubated without protein or with 48 pmol of GST, TuMV GST–VPg or TuMV GST–VPgPro for 30 min. The RNA was purified, run on an agarose gel and stained with ethidium bromide.

cleavage, heat-denatured GST–VPgPro was incubated with total plant RNA; no RNA degradation was observed (Fig. 8B).

Discussion

One of the roles of the cap structure found at the 5′ end of most eukaryotic mRNAs and some viral RNAs is to interact with the cellular host translation initiation machinery, namely the eIF4F complex (Pestova and Hellen, 2000). eIF4E is part of this complex and recognizes the 5′ cap structure which will eventually lead to the recruitment of the small ribosomal subunit (reviewed by Sachs et al., 1997). The presence of a VPg at the 5′ end of many viral RNAs suggests that a different mechanism is at play for translation initiation of these viral genomes.

Potyvirus VPgs have been shown to interact with eIF4E isoforms (Kang et al., 2005; Myoshi et al., 2005; Wittmann et al., 1997), as is also the case for calicivirus VPg (Goodfellow et al., 2005). The VPg–eIF4E interaction is important for virus infection since mutations in either VPg or eIF4E of potyviruses lead to the reduction of symptoms or the absence of infection (Duprat et al., 2002; Lellis et al., 2002; Léonard et al., 2000). TuMV VPgPro can also interact with PABP in planta (Léonard et al., 2004). Interaction of VPg proteins with translation factors has lead to the suggestion that they may play a critical role in assembly of the viral translation initiation complex (Daughenbaugh et al., 2003; Léonard et al., 2004). The VPg of caliciviruses was shown to act as a ‘cap substitute’ for viral RNA translation and the effect is dependent on the interaction with eIF4E. However, it is paradoxical that the addition of NV VPg to an in vitro translation system was found to inhibit translation of reporter RNAs (Daughenbaugh et al., 2003).

We report here on the effect of TuMV VPgPro on mRNA translation in vitro. We show that recombinant VPgPro inhibits translation of capped reporter RNA, both in plant and animal in vitro translation systems. One hypothesis that would explain the effect of VPg on translation is that sequestration of eIF(iso)4E by VPgPro in the extract could interfere with translation. eIF4E has been reported to be the least abundant translation factor, and perhaps the rate limiting one, in animal cells (Duncan et al., 1987). However, the inhibition of translation was not relieved by addition of supplementary eIF(iso)4E in the translation system. A VPgPro mutant that could no longer interact with eIF(iso)4E (Léonard et al., 2000) was also tested. The mutant could still inhibit translation. Our results do not support the hypothesis that the interaction between VPgPro–eIF(iso)4E is responsible for the inhibition of translation.

Given these results, we investigated whether the presence of VPg or VPgPro in the translation system could have a destabilizing effect on the reporter RNA. The addition of TuMV GST–VPgPro led to the degradation of reporter RNA over time. RNA remained intact when GST protein purified in the same way as VPgPro was added to the translation system. The effect was linked to the presence of VPgPro as the rest of the fusion protein expressed without viral sequences did not lead to RNA degradation, therefore eliminating the possibility

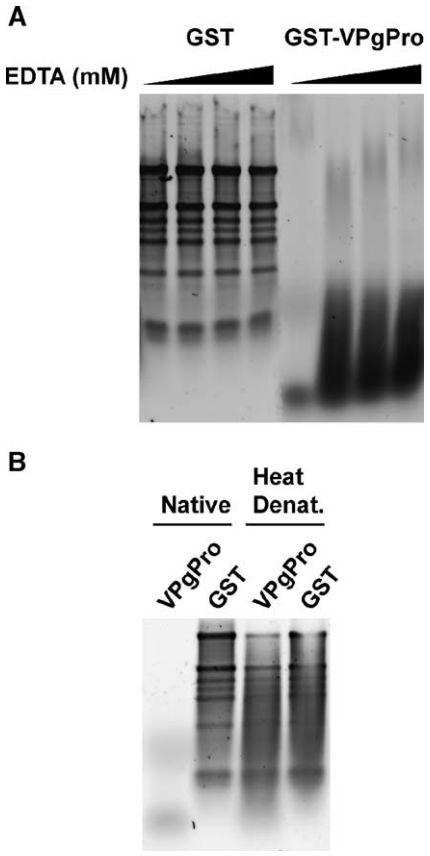

Fig. 8. Effect of EDTA and heat denaturation on the RNase activity of TuMV VPgPro. (A) Total RNA was incubated with 48 pmol of GST or TuMV GST–VPgPro for 30 min at 25 °C with increasing concentrations of EDTA (0, 1, 5, and 10 mM). (B) Total RNA was incubated with 48 pmol either native or heat denatured (95 °C for 15 min) of GST or TuMV GST–VPgPro for 30 min at 25 °C.

that a contaminating RNase from *E. coli* was co-purified along with the fusion tags. RNA degradation was not the result of translation inhibition as it was observed in translation reactions arrested by the addition of cycloheximide; RNA degradation was observed only in samples that contained VPgPro protein.

Daughenbaugh et al. (2003) have also observed an inhibition of translation when NV VPg was added to an in vitro translation system. We speculated that both the NV VPg and TuMV VPgPro proteins inhibit translation through RNA destabilization and/or degradation. In our experiments, the addition of NV to the in vitro translation system lead to the degradation of reporter RNAs as was observed for TuMV. Given that the TuMV VPgPro and NV VPg proteins were purified according to different protocols and share little homology, it is unlikely that both proteins interact with the same contaminating RNAse from *E. coli*. Since the VPg protein was always predominant relative to the full length VPgPro protein in the protein preparations, we examined the capacity of the VPg domain alone to degrade RNA. As seen with NV, we also observed that the VPg domain of TuMV was sufficient for RNA degradation. The proteinase portion of VPgPro was not required for ribonuclease activity.

We studied the effect of purified recombinant proteins incubated with total plant RNA, without the components of a translation reaction. Purified RNA was degraded in the presence of either TuMV GST–VPgPro or NV GST–VPg. This suggests that both proteins have a ribonucleolytic activity in vitro without the need for cellular factors. The ribonuclease activity is therefore associated with the VPg, and is not the result of the activation of a latent RNAse activity from a cellular protein present in the translation system.

Since both NV and TuMV VPg displayed RNAse activity, we examined the two sequences for homology; alignment of the two sequences revealed less than 13% overall identity (Fig. S1). A stretch of 15 amino acids is well conserved between the two proteins (60% identity and 80% similarity). This region overlaps with the nucleotide tri-phosphate binding domain identified and experimentally confirmed for *Potato virus A*, another potyvirus (Puustinen and Mäkinen, 2004). A phosphate-binding site may be correlated with a ribonuclease activity as was observed in other plant ribonucleases with no clear homology to known ribonucleases (Bantignies et al., 2000; Hoffmann-Sommergruber et al., 1997).

The RNAse activity displays the characteristic feature of an enzyme, i.e. sensitivity to heat denaturation. The ribonuclease activity of VPgPro was not completely inactivated by the addition of EDTA. This is not uncommon as EDTA insensitive RNases are widespread and have been previously reported in different organisms (Mishra, 2002; Yen and Green, 1991).

It was previously shown that potyvirus replication is associated with disappearance of cellular mRNAs. It was suggested that host mRNA shutoff could be achieved, in part, through the degradation of host transcripts (Aranda et al., 1996). Interestingly, viral proteins inducing host shutoff through their ability to degrade host mRNAs have been reported for many animal viruses (Hulst and Moormann, 2001; Laidlaw et al., 1998; Smiley et al., 2001). For example, proteins of RNA viruses such as the *Influenza virus*, *Leishmania RNA virus 1–4*, of flaviviruses and of coronaviruses were shown to have ribonuclease activity (Bhardwaj et al., 2004; Li et al., 2005; Hulst and Moormann, 2001; Klumpp et al., 2001; Ro and Patterson, 2000). We hypothesize that TuMV VPg contributes to host mRNA degradation through its ribonucleolytic activity. TuMV could enhance its access to the translation and replication components of the cell by degrading host mRNAs. For example, *Influenza virus* proteins, using a similar strategy, bind to capped mRNA and hnRNA molecules in the nucleus of infected cells and cleave the capped host RNA molecules (Klumpp et al., 2001).

The VPgPro of PVBV was shown to have DNase activity (Anindya and Savithri, 2004) and could explain in part the transcriptional shutdown associated with potyviral infection (Aranda and Maule, 1998). The D77N mutation, which impairs eIF(iso)4E binding, did not result in reduction of translation inhibition or ribonuclease activity. This is interesting in light of the results obtained by Anyanda et al., 2004) with the D81N mutant of PVBV (equivalent to D77N mutant of TuMV) which showed reduced DNase activity. The eIF4E-interacting domain does not seem to be important for VPg ribonuclease activity; this suggests that distinct regions of VPg might be important for its nuclease activities.

It is interesting that rRNA was degraded when VPgPro was added to purified plant RNA (i.e. where proteins had been removed). However, rRNA remained intact but reporter RNA was degraded when VPg was added to RRL translation system. The unspecific RNase activity displayed by VPg raises the question of how TuMV protects its own RNA against degradation. It is possible that cellular factors present in infected plant cells (and absent from the in vitro translation systems) regulate VPg's ribonucleolytic activity. Consistent with this hypothesis is the observation that the RNase activity associated with the *Herpes simplex virus* (HSV) vhs protein exhibits a higher specificity in vivo (Krikorian and Read, 1991; Kwong and Frenkel, 1987; Oroskar and Read, 1989; Zelus et al., 1996) than in an in vitro RRL (Lu et al., 2001). Vhs-mediated host shutoff is characterized by disruption of pre-existing polyribosomes, and accelerated turnover of host mRNA. Vhs displays little sequence specificity in vitro and targets most, if not all, cellular and viral mRNAs, in vivo other cytoplasmic transcripts such as rRNA, tRNA and 7SL RNA are spared during infection. We speculate that rRNA was not degraded in our in vitro translation system because of the protection offered by the ribosome ribo-nucleoprotein complex. The number of host proteins involved in replication/translation of virus RNA is growing and the involvement of a large ribonucleoprotein complex is an emerging theme across positive-strand RNA viruses (reviewed by Thivierge et al., 2005). It is also possible that the ribonucleoprotein complex formed during replication and translation of the viral RNA may protect that RNA from degradation, as we observed with rRNA embedded in ribosomes. The association of viral RNA with different cellular factors, perhaps as a result of different subcellular localizations, may regulate the specificity of the

nucleolytic activity. It is also possible that the conditions used in vitro altered the specificity of the activity. The localization of ribonuclease activity to the nucleus, and not the cytoplasm, would allow an extensive reprogramming of host gene expression while protecting viral RNA. The VPgPro protein of potyviruses is normally found in the nucleus of infected cells (Schaad et al., 1996).

In this report, we have shown in vitro that TuMV VPg exhibits an RNase activity. The involvement of VPg as stimulator of viral translation by acting as a cap substitute and its participation in host mRNA translation shutoff by acting as a nuclease are not mutually exclusive activities. VPg may interact with eIF4E isoforms to facilitate the recruitment of the host translation apparatus to its RNA, while removing host mRNAs to reduce competition. Further work is needed however to assess the role of VPg RNase activity during potyvirus infection *in planta* and how TuMV's RNA, if its the case, avoids degradation.

Materials and methods

Expression and purification of recombinant proteins

Sequences encoding TuMV VPgPro and TuMV D77N were PCR-amplified from the full-length TuMV cDNA clone (p35Tunos) and its mutant form p35Tunos-D77N, respectively (Sanchez et al., 1998; Léonard et al., 2000), in order to construct vectors for the expression of GST-fused VPgPro and D77N proteins. Primers used for amplification were VPgPro–*Nco*I (5′-ATCGTACCATGGCGAAAGGTAAGAGGCAAAG-3′) and VPgPro–*Eco*RI (5′-ATCTTCGAATTCTTATTGTGCTA-GACTGCCGTG-3′). The amplified fragments were digested with *Nco*I/*Eco*RI and cloned into similarly digested pET41(b) (Novagen). Both constructions resulted in the expression of a fusion protein containing a S-Tag, six histidine residues and a GST tag at the N terminus.

For construction of the vector coding for the N-terminus GST–VPg fusion protein, TuMV VPg sequences were PCR-amplified from p35Tunos using primers VPg–*Eco*RI (5′-ATCC-GAATTCCGGAAAGGTAAGAGGCAAAG-3′) and VPg–*Not*I (5′-CTTCGCGGCCGCTTACTCGTGGTCCACTGG-GAC-3′). The amplified fragments were digested with *Eco*RI/*Not*I and cloned into similarly digested pGEX-6P1 (Amersham Biosciences).

BL21(DE3) (for pET41(b)-based constructs) and BL21 (for the pGEX-6P1-derived construct) *E. coli* cells containing recombinant plasmids were cultured at 37 °C to an OD_{600} of 0.6 and protein expression was induced with 1 mM IPTG for 3 h at 30 °C. Bacterial cells were collected by centrifugation and resuspended in buffer A (4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.3). The cells were disrupted by sonication and the lysate was centrifuged at 39,000 × *g* for 20 min. The supernatant was used for affinity purification of either GST–VPgPro, GST–VPg or GST–D77N.

The protein extract was added to GST-Bind resin (Novagen) according to the manufacturer's protocol and incubated at room temperature with agitation for 30 min. Beads were washed three times with buffer A and collected by centrifugation for five min at 500×*g*. The fusion proteins were eluted from the resin in a buffer containing 10 mM reduced glutathione and 50 mM Tris–HCl pH 8.0. Protein concentration was measured using a Bradford assay (Bio-Rad) using bovine serum albumin as standard. GST controls (the pET41(b) or pGEX-6P1 vectors without inserts) were expressed and purified using the same conditions.

The bacterial clones for expression of NV GST–VPg (pGEX–4T1 NV GST–VPg) and wheat eIF(iso)4E (pETtag (iso)4E*Ta*) were kindly provided by M.E. Hardy (Montana State University, MT, USA) and J.-F. Laliberté (INRS-Armand Frappier, QC, Canada) respectively. NV GST–VPg and wheat eIF(iso)4E were expressed and purified as described previously (Daughenbaugh et al., 2003; Léonard et al., 2000).

In vitro translation

The pGEM-luc vector (Promega) containing a luciferase cDNA was linearized with *Xho*I and used as template for synthesis of capped RNA. m^7G-luciferase RNA was transcribed using the mMessage mMachine SP6 system (Ambion). RNA was denatured for three min at 65 °C before use. One μg of reporter RNA was translated in a 50-μl reaction containing 25 μl of wheat germ extract (WGE), 40 U of RNAGuard RNase inhibitor (Amersham Biosciences) and 10 μM of amino acid mixture (Promega). Different concentrations of GST, TuMV GST–VPgPro, TuMV GST–D77N and NV GST–VPg protein were added to the translation mix. The reactions were incubated at 25 °C for 2 h and light emission was measured after the addition of 100 μl of luciferase substrate (Promega). The experiment was conducted at least three times. The in vitro translation assays using RRL were performed similarly but incubated at 30 °C for 90 min. Experiments on the effect of the VPgPro–eIF(iso)4E interaction on translation of reporter RNA in WGE were conducted using the same protocol but the GST and GST–VPgPro proteins were pre-incubated with eIF(iso)4E at 25 °C for 15 min before addition to the translation system. In these assays, concentrations of 24, 48 or 96 pmol of eIF(iso)4E were used with 12, 24 or 48 pmol of GST or of GST–VPgPro.

VPgPro–eIF(iso)4E ELISA binding assays

GST–VPgPro protein (100 μl of protein at 15 ng μl^{-1} in PBS buffer) was adsorbed to wells of a polystyrene plate (Costar) by overnight incubation at 4 °C and wells were blocked with 5% milk PBS solution for 2 h at room temperature. T7-labelled wheat eIF(iso)4E or β-galactosidase proteins were diluted in PBS with 1% milk and 0.1% Tween 20 and incubated for 1.5 h at 4 °C in the previously coated wells. Detection of retained protein was achieved with a mouse monoclonal anti-T7-tag antibody (Novagen) and horseradish peroxidase-coupled goat anti-mouse immunoglobulin (Pierce). Between each incubation, wells were washed five times with PBS supplemented with 0.04% Tween 20. Enzymatic reactions were performed in 100 μl of OPD citrate buffer (50 mM citric acid, 100 mM sodium phosphate dibasic,

pH 5.0, 0.5 mg/ml o-phenylenediamine dihydrochloride (OPD) and 0.1% hydrogen peroxide) and stopped with a solution of 3 M H_2SO_4. Absorbance was measured at 492 nm. Statistical analyses were performed using the GLM procedure of SAS in a randomized complete block design (RCBD). ANOVA was used to detect statistical differences and LSD method used to determine significant differences among means. SEM and statistics were calculated for three biological replicates from a minimum of three technical replicates (replicate of the assay on the same microplate).

RNA stability assays

To assess RNA stability in translation reactions that were arrested (in presence or not of VPgPro protein), in vitro translation of luciferase RNA in WGE was performed in presence of 600 μM of cycloheximide, an inhibitor of ribosome translocation. Five μl of each translation reaction were removed at 0, 5, 15 and 60 min after the addition of luciferase RNA and RNA degradation was monitored using Northern blot hybridizations with a ^{32}P-labelled luciferase RNA probe.

Total plant RNA degradation assays

RNA was extracted from *Brassica perviridis* using the RNeasy Plant Mini Kit (Qiagen). Five μg of RNA (eluted in RNase-DNase free water) were incubated with 48 pmol of GST, TuMV GST–VPgPro, GST–VPg or NV GST–VPg (eluted in 10 mM reduced glutathione and 50 mM Tris–HCl pH 8.0) for 30 min at 25 °C. The volume of the reaction was completed with RNase-DNase free water. RNA degradation experiments following addition of EDTA and heat denaturation (15 min at 95 °C) were carried out similarly. Samples were run on an agarose gel and stained with ethidium bromide.

Agarose gel electrophoresis and Northern blot analysis

RNA samples were purified using the RNeasy MinElute cleanup kit (Qiagen). A wash step with 200 μl of the RW1 buffer (Qiagen) was added after the application of the sample on the column to remove residual protein. RNA samples were eluted in 14 μl of RNase-free water and combined with four μl of RNA sample buffer (20 mM HEPES, 1 mM EDTA, pH 7.8, with 50% formamide and 6% formaldehyde). Following a 10 min incubation at 65 °C, 2 μl of RNA loading buffer (95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 18 mM EDTA pH 8, 0.025% SDS) were added and the RNA samples were separated through a 1.5% agarose gel containing 6% formaldehyde in running buffer (20 mM HEPES, 1 mM EDTA, pH 7.8, 6% formaldehyde). The gel was washed for 1 h in diethyl pyrocarbonate-treated water. RNA was transferred to a nylon membrane (Zeta-Probe, Bio-Rad) in 10× SSC pH 7 (1.5 M sodium chloride, 0.15 M sodium citrate). Following UV cross-linking, the membrane was stained for five min in a solution of 0.02% methylene blue and 0.3 M sodium acetate and washed in water. The membrane was scanned and the coloration was removed in 1 mM EDTA pH 8, 1% SDS. The membrane was incubated for 4 h at 65 °C in 10 ml of hybridization buffer (1 mg/ml BSA, 50% formamide, 5% SDS, 1 mM EDTA, 400 mM $NaPO_4$ pH 7.2) and incubated for 16 h with the ^{32}P-labelled riboprobe. The membrane was washed twice with washing buffer (0.1× SSC pH 7, 0.1% SDS, 1 mM EDTA) and exposed to Kodak Biomax MS film.

Synthesis of ^{32}P-labelled riboprobes

The plasmid pGEM-luc (Promega) was linearized with *Eco*RV. The riboprobe was synthesized for 2 h at 37 °C in T7 polymerase buffer (10 mM of DTT, 20 U of RNAGuard RNase Inhibitor (Amersham Biosciences), 500 μM of CTP, GTP and ATP (Invitrogen), 50 μCi of [α-^{32}P]-UTP (Amersham Biosciences), 500 ng of pGEM-luc/*Eco*R*V*) and 50 units of T7 polymerase (Invitrogen). RNase-free DNase I (10 U; Qiagen) was added and incubated for 15 min at 37 °C. The probe was purified with the QIAquick nucleotide removal kit (Qiagen).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version at, doi:10.1016/j.virol.2006.03.019.

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