Interaction in vitro between the proteinase of Tomato ringspot virus (genus Nepovirus) and the eukaryotic translation initiation factor iso4E from Arabidopsis thaliana

Simon Léonard, 1 Joan Chisholm, 2 Jean-François Laliberté 1 and Hélène Sanfaçon2

1 Centre de Microbiologie et Biotechnologie, INRS-Institut Armand-Frappier, 531 Boulevard des Prairies, Ville de Laval, Québec, Canada H7V 1B7
2 Pacific Agri-Food Research Centre, 4200 Highway 97, Summerland, BC, Canada V0H 1Z0

Eukaryotic initiation factor eIF(iso)4E binds to the cap structure of mRNAs leading to assembly of the translation complex. This factor also interacts with the potyvirus VPg and this interaction has been correlated with virus infectivity. In this study, we show an interaction between eIF(iso)4E and the proteinase (Pro) of a nepovirus (Tomato ringspot virus; ToRSV) in vitro. The ToRSV VPg did not interact with eIF(iso)4E although its presence on the VPg-Pro precursor increased the binding affinity of Pro for the initiation factor. A major determinant of the interaction was mapped to the first 93 residues of Pro. Formation of the complex was inhibited by addition of m7GTP (a cap analogue), suggesting that Pro-containing molecules compete with cellular mRNAs for eIF(iso)4E binding. The possible implications of this interaction for translation and/or replication of the virus genome are discussed.

A key step in the replication cycle of viruses is translation of the viral genome. Optimal translation is achieved by recruiting, and in some cases selectively altering, host translation factors (Thompson & Sarnow, 2000; Gale et al., 2000). This in turn often results in the inhibition of host mRNA translation. Most viral RNAs differ in structure from cellular mRNAs. This provides opportunities for viruses to redirect the host translation machinery in favour of viral protein synthesis. For example, the 5′ end of the genomic RNA from picorna-like viruses (including animal and insect picornaviruses, and plant poty-, como- and nepoviruses) does not have a cap structure (m7GpppN, where N is any nucleotide) as found in cellular mRNAs. Instead, the RNA is covalently linked to a virus-encoded protein termed VPg. Translation proceeds in a cap-independent manner through the use of an internal ribosome-entry site (IRES) (Martínez-Salas et al., 2001; Gallie, 2001).

Viral proteins are likely to participate in the regulation of viral genome translation (Thompson & Sarnow, 2000; Gale et al., 2000). A case in point is the VPg of Turnip mosaic virus (TuMV; genus Potyvirus), which interacts with the eukaryotic initiation factor eIF(iso)4E of Arabidopsis thaliana (Wittmann et al., 1997; Léonard et al., 2000). eIF(iso)4E is a plant isomer of eIF4E (Rodriguez et al., 1998) which binds the cap structure of cellular mRNAs and plays an important role in the regulation of translation initiation (Sonenberg & Gingras, 1998). The cap analogue m7GTP, but not GTP, inhibits VPg–eIF(iso)4E complex formation, suggesting that VPg and cellular mRNAs compete for eIF(iso)4E binding. Plants inoculated with TuMV infectious cDNA containing a mutation in the eIF(iso)4E binding domain of VPg remain symptomless and do not show accumulation of virus coat protein, indicating that there is a correlation between VPg–eIF4E binding in vitro and virus viability in planta (Léonard et al., 2000). The NIa protein (also called VPg-Pro) of Tobacco etch virus also interacts with eIF4E from tobacco and tomato, and the interaction was shown to enhance genome amplification (Schaad et al., 2000). Although the precise biological function of the VPg (or VPg-Pro)–eIF4E interaction remains to be elucidated, it may either play a role in recruiting host factors for the translation and/or replication of the viral RNA or be involved in host translational shut-down, possibly through disruption of the interaction between cellular mRNAs and cap-binding translation initiation factors.

Nepoviruses are closely related to potyviruses in terms of their genomic structure and genome expression strategy but differ from potyviruses in at least two significant aspects. First, the nepovirus genome is bipartite, with RNA1 encoding most of the proteins involved in virus replication (including VPg and Pro). Second, nepovirus VPgs are much smaller than potyvirus VPgs, which range from 22 to 24 kDa (Riechmann et al., 1992). For example, the VPg of Tomato ringspot virus (ToRSV) is composed of 27 residues (Wang et al., 1999). There is no amino acid sequence homology between potyvirus and nepovirus VPgs (Mayo & Fritsch, 1994). It was therefore of interest to determine if the nepovirus VPg, or larger precursor forms,
that the presence of VPg increases the affinity of the viral protein for eIF(iso)4E. In contrast, the interacting domain of the TuMV VPg-Pro resides within VPg, and the TuMV VPg-Pro and VPg have the same binding affinity for eIF(iso)4E (Wittmann et al., 1997).

The domain(s) of the ToRSV VPg-Pro involved in the interaction with eIF(iso)4E were mapped by Far-Western experiments. Mutants (Fig. 2a) were generated by amplifying cDNA fragments from plasmid pET21d-VPg-Pro (Chisholm et al., 2001) using specific oligonucleotides. The PCR products were inserted into plasmids pET21d (Novagen) or pTrxFus (Invitrogen). Exceptions to this are plasmid pET15bVPg-Pro-delBamHI, which was constructed by deleting a small BamHI fragment from plasmid pET15bVPg-Pro-N-Pol (Wang et al., 1999), and plasmid pET21dVPg-Pro-delClal, which was constructed by inserting a Ncol–Clal fragment from plasmid pET21d-VPg-Pro into plasmid pET21d. Protease mutants were expressed in E. coli as described by the supplier (Novagen). E. coli proteins were separated by SDS–PAGE and electrobotted to PVDF membranes (Bio-Rad). The membranes were probed with approximately 60 µg of the purified eIF(iso)4E essentially as described (Kao et al., 1992). Binding of eIF(iso)4E was revealed by immunodetection using the anti-T7 tag monoclonal antibody and a goat anti-mouse secondary antibody linked to alkaline phosphatase (Sigma). The different proteinase forms were seen as a predominant band in E. coli extracts, as shown by Coomassie blue staining (Fig. 2b). The identity of these proteins was confirmed by immunodetection with polyclonal antibodies raised against the recombinant VPg-Pro (data not shown). Interaction of VPg-Pro with eIF(iso)4E was observed (Fig. 2b, c, lane 1) and no additional interactions were detected with E. coli proteins from the extract, indicating that the interaction was specific. An interaction was also detected with Pro (lane 7) but not with a protein containing the VPg domain fused to thioredoxin (lane 12).

Point mutations in the putative catalytic triad (H128D) and in the putative substrate-binding pocket (H145L) that abolished the proteolytic activity of the proteinase (Hans & Sanfaçon, 1995) did not affect the binding affinity for the factor (Fig. 2c, lanes 2 and 3), indicating that the determinants for the interaction were distinct from those for proteolytic activity. All proteins containing the first 93 amino acids of the Pro domain were shown to interact with eIF(iso)4E (lanes 1–7 and 11). Truncated proteinases containing a deletion of the N-terminal 93 residues could interact with the factor (lanes 8 and 10), but a truncated protein harbouring the last 81 amino acids of Pro did not (lane 9). Taken together these results indicate that a major determinant of the interaction resides within the first 93 amino acids of the Pro domain, although an additional domain within the C-terminal two-thirds of Pro may contribute to the binding.

To test whether ToRSV proteins and the cap structure of cellular mRNAs compete for eIF(iso)4E binding, the influence of the cap analogue m7GTP on the formation of the VPg-

interacts with eIF(iso)4E. One possible precursor of VPg is VPg-Pro. VPg-Pro (the functional equivalent of the potyvirus Nla) was found to accumulate during in vitro translation of larger precursors as a result of inefficient processing of the VPg-Pro cleavage site (Wang et al., 1999; Wang & Sanfaçon, 2000), although accumulation of VPg-Pro in infected plants has not yet been demonstrated.

The interaction between VPg-Pro as well as Pro of ToRSV and eIF(iso)4E of A. thaliana was tested using an ELISA-based binding assay (Wittmann et al., 1997; Léonard et al., 2000). ToRSV has the ability to replicate in A. thaliana (R. I. Hamilton, personal communication). eIF(iso)4E was produced in E. coli and purified by m7GTP–Sepharose chromatography (Wittmann et al., 1997). The factor was fused to the N-terminal peptide of the T7 gene-10 protein (T7 tag), which allows its recognition by an anti-T7 tag monoclonal antibody (Novagen). ToRSV proteins were produced in E. coli, purified (Chisholm et al., 2001) and adsorbed to wells of ELISA plates (1-0 µg per well) by overnight incubation at 4 °C. Purified eIF(iso)4E (2 µg) diluted in 1% BLOTTO in PBS containing 0.2% Tween was incubated for 1 h at 4 °C in the coated wells. Detection of bound initiation factor was achieved in an ELISA with the anti-T7 tag antibody and peroxidase-labelled goat anti-mouse immunoglobulin G (KPL). VPg-Pro from TuMV was purified as described previously (Wittmann et al., 1997) and used as positive control. As shown previously (Wittmann et al., 1997), the VPg-Pro of TuMV interacted with eIF(iso)4E (Fig. 1). VPg-Pro of ToRSV also interacted with eIF(iso)4E. The interaction was specific for the viral protein since the factor was not retained when wells were coated with an E. coli lysate not containing any VPg-Pro (Wittmann et al., 1997). An interaction was also detected between the mature Pro and eIF(iso)4E, although retention of the factor was less than for VPg-Pro (0.55 OD units for Pro vs 1.2 OD units for VPg-Pro). This suggests that the interacting domain resides within Pro, but
Interaction between eIF(iso)4E and ToRSV Pro

Fig. 2. Definition of the domains in ToRSV VPg-Pro involved in the interaction with eIF(iso)4E using a Far-Western assay.

(a) Schematic representation of the ToRSV protease mutants. The white boxes represent regions of the Pro domain, the hatched boxes represent the VPg domain and the black box represents the thioredoxin protein (from vector pTrxFus; Invitrogen) fused in-frame with the VPg coding region. A histidine tail (His) at the C termini of the proteins is shown when present. The amino acids of the ToRSV RNA1-encoded polyprotein (P1) present in each recombinant protein are indicated (numbering from the first amino acid of the polyprotein according to Rott et al. (1995)).

(b and c) Analysis of the interaction of the ToRSV protease mutants with eIF(iso)4E using the Far-Western assay. After induction of the expression of viral proteins, E. coli cells were resuspended in 50 mM NaH₂PO₄, pH 8.0, 0.3 M NaCl, 0.1% Triton X-100. After sonication the extracts were separated by SDS–PAGE and either stained by Coomassie blue staining (b) or electroblotted to PVDF membranes (Bio-Rad). Interaction with eIF(iso)4E was tested using the Far-Western method as described in the text (c). The migration of molecular mass standards is indicated on the left of the gels. The following proteins were tested in each lane. Lane 1, VPg-Pro; lane 2, VPg-Pro^{H1283D}; lane 3, VPg-Pro^{H1451L}; lane 4, VPg-Pro-del BamHI; lane 5, VPg-Pro-del Clal; lane 6, VPg-Pro-del 4; lane 7, Pro; lane 8, Pro-del 2; lane 9, Pro-del 3; lane 10, Pro-del 5; lane 11, Pro-del 6; lane 12, Thio-VPg.

Pro–eIF(iso)4E and Pro–eIF(iso)4E complexes was tested. ELISA plate wells were coated with 1–10 µg of viral proteins and incubated with 2–20 µg of eIF(iso)4E and either 10 or 20 mM m⁷GTP. The cap analogue inhibited the formation of the complexes by approximately 30% at a concentration of 20 µM (Fig. 3). No inhibition was observed with 20 µM of GTP, indicating that the inhibition was cap-related. As previously shown, 20 µM of m⁷GTP inhibited the interaction of eIF(iso)4E with the VPg-Pro of TuMV by 60% (Léonard et al., 2000).

One possible role for the interaction between the nepovirus Pro and eIF(iso)4E is that it could promote assembly of the translation complex on the viral RNA. In the circular translation model, efficient translation of cellular mRNAs requires interactions between initiation factors bound to the 5’ cap structure and the poly(A)-binding protein (PABP) bound to the 3’ poly(A) tail (Sachs, 2000). Similarly, translation of several viruses has been shown to be mediated by protein bridges between the 5’ and 3’ termini of the RNA which involve viral
proteins and cellular translation initiation factors. For instance, the rotavirus NSP3 protein promotes genome circularization and efficient translation of the viral RNA by simultaneously binding to the 3′ untranslated region of the non-polyadenylated viral RNA and to eIF4G, which in turn binds to eIF4E, eIF4G and eIF3 (Ali et al., 2001; Borman et al., 2001; Lopez de Quinto et al., 2001). Given that eIF4G interacts with PABP and picornavirus RNA is polyadenylated, circularization was expected. Indeed, eIF4G–PABP interaction was required for poly(A) tail-mediated stimulation of IRES translation (Michel et al., 2001).

The formation of protein bridges leading to genome circularization may also be an important feature of the translation of plant virus genomes. Recently, the coat protein of *Alfalfa mosaic virus* was shown to stimulate translation of viral RNAs, presumably by acting as a functional analogue of PABP (Neeleman et al., 2001). The nepovirus Pro could similarly participate in genome circularization by acting as a bridging element between host initiation factors and the viral RNAs. Pro is a member of the 3C-like proteinase family and is likely to have RNA-binding properties (Blair et al., 1998). It could thus interact with the 5′ end of the viral genome, as shown for the picornavirus 3C (Gamarnik & Andino, 2000; Kusov et al., 1997; Kusov & Gaus-Muller, 1997; Harris et al., 1994; Walker et al., 1995) and the potyvirus Nla proteinases (Daros & Carrington, 1997). Alternatively, Pro as a precursor protein with VPg may be covalently linked to viral RNAs. In addition to possibly improving translation of the viral RNAs, genome circularization could provide several advantages for virus replication, including the coordination of translation and RNA synthesis, the localization of the viral polymerase at the appropriate start site and a control mechanism for the integrity of the viral genome (Herold & Andino, 2001). Finally, the interaction of the ToRSV Pro with eIF(iso)4E may also have other biological functions, one of which may be a direct or indirect role in a possible shut-down of host translation.

The presence of the VPg domain on a precursor of the ToRSV Pro regulates the different activity of the proteinase as it enhances its ability to interact with eIF(iso)4E (this study) and decreases its ability to release the movement protein and coat protein from RNA2-derived substrates (Chisholm et al., 2001). The results presented here provide additional support for our previous suggestion that the inefficient cleavage at the VPg-Pro site may play an important role in the biology of ToRSV (Chisholm et al., 2001).

We wish to thank Dr Aiming Wang (in the Sanfacon laboratory) for the construction of plasmid pThio-VPg. We are also grateful to Drs Marc Fortin and D’Ann Rochon for critical reading of the manuscript. This work was supported in part by NSERC research grants awarded to J.F.L. and H.S.

**References**


