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## Short Communication

# Central domain of a potyvirus VPg is involved in the interaction with the host translation initiation factor eIF4E and the viral protein HcPro

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Using recombinant proteins produced in bacteria or in infected plants, interactions between the VPg and HcPro of *Lettuce mosaic potyvirus* (LMV) and between LMV VPg and the lettuce translation initiation factor 4E, the cap-binding protein (eIF4E), were demonstrated *in vitro*. Interaction with eIF4E and HcPro both involved the same VPg central domain. The structure of this domain in the VPg context was predicted to include an amphiphilic  $\alpha$ -helix, with the amino acids related to biological functions in various potyviruses exposed at the hydrophilic side.

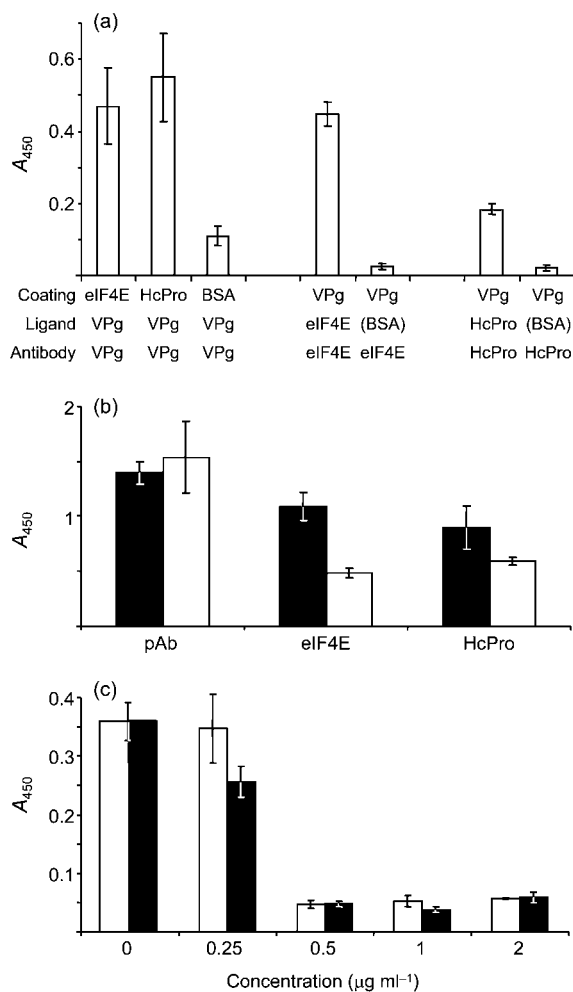
Viral infection relies on the availability of host factors to interact with virus proteins. Limited information is available on the proteins involved in the cycle of plant viruses, but interactions between host and virus proteins have been documented in recent years. In the genus *Potyvirus*, the interaction between VPg (a small virus-encoded protein bound to the 5' end of the virus RNA genome) and the host cap-binding protein, eIF4E (Wittmann *et al.*, 1997), has received more attention since eIF4E defects were associated with an inability of several hosts to support potyvirus infection (Gao *et al.*, 2004; Lellis *et al.*, 2002; Nicaise *et al.*, 2003; Ruffel *et al.*, 2002). This suggested a biological significance for the eIF4E–VPg interaction, which was confirmed recently (Kang *et al.*, 2005). In lettuce, eIF4E is encoded by *mol1*, a recessive resistance gene against *Lettuce mosaic potyvirus* (LMV) (Nicaise *et al.*, 2003). VPg also interacts with other proteins encoded by the genome of the host (Dunoyer *et al.*, 2004; Schaad *et al.*, 1997; Yambao *et al.*, 2003) or of the virus (Guo *et al.*, 2001; Hong *et al.*, 1995), including the multifunctional protein HcPro (Yambao *et al.*, 2003). In several potyviruses, variations in the VPg central domain are associated with resistance-breaking (Borgström & Johansen, 2001; Keller *et al.*, 1998; Masuta *et al.*, 1999; Moury *et al.*, 2004; Nicolas *et al.*, 1997; Rajamäki & Valkonen, 1999; Schaad *et al.*, 1997). In this work, we investigated the interactions of LMV VPg, in particular its central domain, with lettuce eIF4E and LMV HcPro.

Recombinant lettuce (*Lactuca sativa*) eIF4E was expressed in bacteria from pDest17 (Invitrogen). Recombinant LMV VPg and HcPro were expressed in bacteria from pTrcHis (Invitrogen). These three proteins were purified from

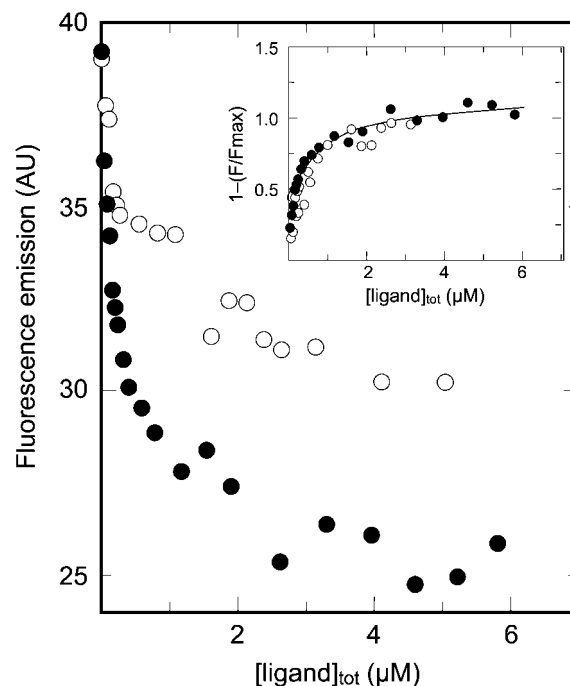
bacterial extracts as poly(His) fusions. In some experiments, His-tagged LMV HcPro purified from infected plants (Plisson *et al.*, 2003) was used, yielding essentially similar results to those shown below (data not shown). Protein purity was controlled by SDS-PAGE (not shown). Rabbit polyclonal antibodies were obtained against VPg and eIF4E. Mouse monoclonal antibodies 1H5 and 21M were raised against VPg and VPg-CD (a synthetic peptide corresponding to the VPg central domain with a non-viral C-terminal extension, VFSDIGLVQDAFGKERLHAAAHAY), respectively. These mAbs were assessed by Western blotting and ELISA using infected lettuce or recombinant VPg (not shown); in addition, the reactivity of 21M for the VPg central domain was confirmed, as increasing concentrations of VPg-CD resulted in loss of ELISA signal (not shown). A monoclonal antibody against HcPro, 3C5, has been described previously (Roudet-Tavert *et al.*, 2002). An ELISA-derived (Clark & Adams, 1977; Léonard *et al.*, 2000) interaction assay was used. The surface was coated with eIF4E or HcPro [4  $\mu\text{g}$  (ml carbonate buffer)<sup>-1</sup> overnight at 4 °C] and saturated with fetal bovine serum (FBS; 30 min at room temperature) before incubation with VPg [8  $\mu\text{g}$  (ml PBS/0.2 % Tween)<sup>-1</sup>, 0.2 % FBS for 1 h at 4 °C]. Interactions were revealed with 1H5 [5  $\mu\text{g}$  (ml PBS/0.2 % Tween)<sup>-1</sup> for 2 h at 37 °C], followed by antibodies conjugated to alkaline phosphatase. In reverse experiments, the wells were coated with VPg (4  $\mu\text{g}$  ml<sup>-1</sup>) and the interaction with eIF4E or HcPro (8  $\mu\text{g}$  ml<sup>-1</sup>) was revealed similarly using anti-eIF4E or 3C5.

A specific interaction was observed between VPg and eIF4E, as well as between VPg and HcPro (Fig. 1a). An N-terminally truncated version of HcPro (Plisson *et al.*, 2003) also interacted with VPg (not shown), indicating that the first 99 N-terminal residues of HcPro are dispensable for the interaction with VPg. The interaction

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**Fig. 1.** Immunochemical characterization of the interaction between VPg and eIF4E or HcPro. (a) VPg interacts with eIF4E and HcPro. The bars represent the  $A_{405}$  values observed in ELISA with the coating and ligand proteins and the antibody indicated below each bar. BSA was used as a control protein. mAbs against LMV VPg (1H5) and HcPro (3C5) and polyclonal antibodies raised against recombinant lettuce eIF4E purified from *Escherichia coli* after overexpression were used. The means  $\pm$  SD of three replicates obtained in a typical experiment is shown. (b) Anti-VPg mAb inhibits binding of VPg to eIF4E and HcPro. Wells were coated with an anti-VPg polyclonal antibody (pAb), eIF4E or HcPro, and the interaction with VPg was assayed, with (open bars) or without (filled bars) a preliminary incubation with the anti-VPg-CD mAb 21M. Interactions were revealed using the anti-VPg mAb 1H5. Means  $\pm$  SD of  $A_{405}$  values (mean of four replicates) of a typical experiment are shown. (c) eIF4E and HcPro compete for interaction with VPg. Wells were coated with HcPro ( $5 \mu\text{g ml}^{-1}$ ), and interaction with VPg ( $1 \mu\text{g ml}^{-1}$ ) was assayed after a preliminary incubation with serial concentrations of eIF4E (open bars) or HcPro (filled bars) at the concentrations indicated ( $\mu\text{g ml}^{-1}$ ). Interactions were revealed using the anti-VPg Mab 1H5. Means  $\pm$  SD of  $A_{405}$  values (mean of three replicates) of a typical experiment are shown.



**Fig. 2.** Spectroscopic characterization of the interaction between eIF4E and VPg. eIF4E tryptophan fluorescence was measured following the addition of VPg ( $\circ$ ) or VPg-CD ( $\bullet$ ) ( $0.05$ – $5 \mu\text{M}$ ) was added to a  $2.5 \mu\text{M}$  eIF4E solution in 1 ml of 20 mM HEPES/KOH (pH 7.6), 25 mM KCl, 1 mM DTT, 10 % glycerol. The mixture was incubated (5 min at  $25 \text{ }^\circ\text{C}$ ) until a steady-state fluorescence at 342 nm was reached upon excitation at 280 nm. AU, arbitrary units. Inset: variation of eIF4E fluorescence as a function of increasing concentrations of VPg ( $\circ$ ) or VPg-CD ( $\bullet$ ).

between eIF4E and VPg was confirmed by a spectrofluorometric approach, whereby binding of a ligand results in quenching of the fluorescence of eIF4E tryptophans. A decrease in eIF4E tryptophan fluorescence was observed in the presence of increasing concentrations of VPg (Fig. 2) but not of BSA used as a control (not shown). The data suggested a stoichiometry of one molecule bound per eIF4E and a dissociation constant in the same range as that of the cap–eIF4E interaction (Khan *et al.*, 2006; Michon *et al.*, 2006).

In *Turnip mosaic virus* (TuMV) (Léonard *et al.*, 2000), although not in *Tobacco etch virus* (TEV) (Schaad *et al.*, 2000), amino acid changes in the VPg central region that impair eIF4E interaction are lethal, suggesting a direct interaction with eIF4E. The VPg central domain is also involved in HcPro binding (Yambao *et al.*, 2003). Therefore, the involvement of the LMV VPg central domain in both HC-Pro and eIF4E interactions was assayed *in vitro* as described above. The anti-VPg-CD mAb 21M did not reveal an interaction between VPg and eIF4E (not shown), suggesting that the VPg central domain was not accessible to 21M once the VPg–eIF4E interaction had taken place

(a)

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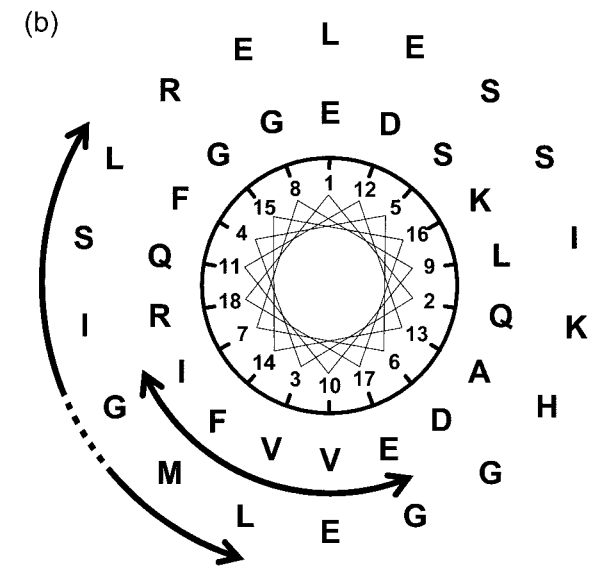
LMV (AJ278854)       GKGKRQR-QKLRYRQARDNKGIEVYGGD---ATMEHYFGAAYTEKGGKSGTKGKMGTK-NRKFVNMYGY
                       .....HH-HHHHHHHHHHH.....bbbbbb.....---HHHHHHHHHHHH.....b.....---.bbbbbb..
PVY (NC_001616)     .....HHHHHHHHHHHH.....bbb.....---HHHHHHHHHHHH.....bbbbbb.....---.bbbbbb..
PVA (NC_004039)     .....H-HHHHHHHHHHHH.....bbb.....---.bbbbbb.....bb.....---.bbbbbbbb..
TuMV (NC_002509)    .....HH-HHHHHHHHHHH.....bbbbbb.....---.bbbbbb.....bbbbbb.....---.bbbbbbbb..
TVMV (NC_001768)    .....-HHHHHHH.HHHHHbbbbbb.....---.HHHHHHHHHH.....bbbbbb.....---.bbbbbbbb..
PsbMV (NC_001671)   .....H---HHHHHHHH.....HHHHHH.....---.HHHHHH.HHHHH.....bbb.bb---bbbbbbbbb..
TEV (NC_001555)     .....--.HHHHHHHHHHH.....HHHHHH.H---HHHHHH.....bbbbbb.....---.bbbbbb..
PPV (NC_001445)     .....H-HHHHHHHHHHHHHHHHbbbbbb.....---.HHHHHH.....bb.....b---bbbbbbbbb..
BaMMV (NC_003483)   .....bb-----.....HHHHHHHHHHHH.....bbb......HHHHHHHHHHHH.....
BaYMV (NC_002990)   .....-----..HHHHHHHH.....-HHHHHHHHHHHHHHHH.....HHHHHHHH---.....bbb.
    
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LMV (AJ278854)       NPEDFSFIRFLDPLTGKTMDEQVFSDIGLVQDAFGKERLKLKLSGGEIESEHMRSG---IRAYLVKNLTAA
                       .....bbbbbb.....H---HHH.....HHHHHHHHHHHHHH.....HHHHHHHH---HHHHHH.....HHH
PVY (NC_001616)     .....bbbbbb.....HH--HHHHHHHHHHHHHHHHHHHHHH.....HHHHHH.....bbbbbb.....bb
PVA (NC_004039)     .....bbbbbb.....--.....HHHHHHHHHHHHHHHHHHHHHHHH.....bbbbbb.....bb
TuMV (NC_002509)    .....bbbbbb.....-.....HHHHHH.....HHHHHH.....bbbbbb.....HHHHHH.bbbbbbbbbb.....b
TVMV (NC_001768)    .....bbbbbbbbb.....bb.--.....HHHHHHHHHHHHHHHHHHHHHH.H---HHH.....bbbbbb.....b
PsbMV (NC_001671)   .....bbbbbb.....bbb.--.....HHHHHHHHHH.....HHHHHHHHHHHHHHHH.....HHHH.....b
TEV (NC_001555)     .....bbbbbb.....---.....HHHHHH.....bbbbbb.....bbbbbb.....bb
PPV (NC_001445)     .....bbbbbb.....--.....HHHHHHHHHHHH.....HHHHHH.....HHHHHHHHHHHH.....
BaMMV (NC_003483)   .....HHHHHHHHHH.....bbbbbb.....HHHHHHHHHHH.....-.....HHHHHH.....bbbbbbbbb.....H
BaYMV (NC_002990)   .....HHHHbb.....bb.....HHHHHHHHHHHH.HHHHHH---HHHHHHHHHHHHHH.....
    
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LMV (AJ278854)       LEIDMTPHNSCOLGIKTNNIAGFVDREYELRQTGEARVVPALIPKDNPITDEDIPVKHE
                       HHH.....bb.....HHHHHHHHHH.HHHHH.....bbbb..
PVY (NC_001616)     bbb.....bbb.....HHHHHHHH.....bbbb.....bbb..
PVA (NC_004039)     bbb.....bbb.....HHHHHH.....HHHHHH.....---.bb.  H
TuMV (NC_002509)    bbb.....bbb.....HHHHHHHH.....bb.-bbb..
TVMV (NC_001768)    bbb.....bbb.....HHHHHHHH.....---.bbb..
PsbMV (NC_001671)   bbb.....bb.....HHHHHHHH.....bb.....HHHHHHH.bbbb
TEV (NC_001555)     bbb.....HHHHHHHHHH.....HHHHHH.....bb.bb.....bbb..
PPV (NC_001445)     HHH.....HHHHHHHH.....HHHHHH.....bbb.....bbb..
BaMMV (NC_003483)   HHHHHH.HHHHH.....-bb.....HHH.....bbb---b..
BaYMV (NC_002990)   bbbbbb.....bb.....bb.....HHH.....bbb.....bbb.....-bbb..
    
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**Fig. 3.** Prediction of the VPg secondary structure. (a) Multiple alignment of the two-dimensional structures of LMV and other potyviruses. The two-dimensional structures of the VPg of ten members of the family *Potyviridae* were predicted using GOR-IV (Garnier *et al.*, 1996). Each amino acid along a sequence alignment (Thompson *et al.*, 1997) was replaced with a letter indicating its predicted involvement in an  $\alpha$ -helix (H) or a  $\beta$ -sheet (b). Hyphens indicate gaps in the alignment. Above the LMV sequence, R indicates the RNA-bound tyrosine (Murphy *et al.*, 1991) and 4 the aspartate involved in interaction with eIF4E in TuMV (Léonard *et al.*, 2000). The variable central domain is underlined and italicized. Amino acids on the hydrophobic side of the predicted amphiphilic helix are highlighted with asterisks. (b) Axial view of the predicted  $\alpha$ -helix formed by the LMV VPg central domain (underlined in a). The inner circle represents the N-terminal half of this 36 aa peptide and the outer circle represents its C-terminal half. The hydrophobic side of each helix is arrowed. These arrows account for the observation that, in the multiple alignment in (a), the residue at position 25 (glycine in LMV) is usually not hydrophobic, whilst that at position 17 (glutamate in LMV) is usually hydrophobic. PVY, *Potato virus Y*; PVA, *Potato virus A*; TVMV, *Tobacco vein mottling virus*; PsbMV, *Pea seed-borne mosaic virus*; PPV, *Plum pox virus*; BaMMV, *Barley mild mosaic virus*; BaYMV, *Barley yellow mosaic virus*.

and hence that this domain of VPg was involved directly in eIF4E interaction.

To test the hypothesis that eIF4E, HcPro and 21M bound in the same area on VPg, VPg was pre-incubated with 21M (1 : 3, w/w, for 2 h at 37 °C) and assayed for eIF4E binding. Such pre-treatment of VPg significantly diminished binding to eIF4E, as well as to HcPro (Fig. 1b). When an anti-VPg polyclonal antibody was used for immunocapture of VPg in a control experiment, no reduction in signal was observed after pre-treatment, indicating that 21M did not interfere with recognition by 1H5, the mAb used to reveal the interaction. Taken together, this confirmed that 21M, when bound to the VPg central domain, interferes with further interaction with eIF4E or HcPro, and thus demonstrated that the central domain must remain available for specific interactions with eIF4E and HcPro to occur.

In order to confirm the localization of the interaction domain on VPg, the wells were coated with the synthetic peptide VPg-CD (5 µg ml<sup>-1</sup>) and incubated with eIF4E. The results were revealed using a mAb (2B12) directed against the N-terminal tag of the recombinant eIF4E. In a typical experiment,  $A_{405}$  values averaged  $0.264 \pm 0.040$  for VPg-CD compared with  $0.028 \pm 0.006$  for BSA, confirming a specific interaction. The interaction between eIF4E and VPg-CD was confirmed by spectrofluorometry, as above (Fig. 2), with the same dissociation constant as for VPg. Similarly, in a binding assay revealed using the anti-HcPro mAb 3C5, VPg-CD interacted with HcPro ( $A_{405} = 0.420 \pm 0.190$  vs  $0.028 \pm 0.025$ ).

VPg-CD overlaps the region involved in the interaction between eIF4E and TuMV VPg (Léonard *et al.*, 2000) and between HcPro and VPg of *Clover yellow vein virus* (Yambao *et al.*, 2003). The present work therefore provides the first evidence that, for a single given potyvirus, HcPro and eIF4E bind to the same central domain of VPg (residues 89–105). This clearly suggests the possibility that they could compete for binding to VPg. In order to assess this possibility, we conducted an ELISA-based competition assay in which the effect of lettuce eIF4E on LMV HcPro–VPg binding was studied. In such experiments, the wells were coated with HcPro (5 µg ml<sup>-1</sup>) followed by VPg (1 µg ml<sup>-1</sup>) pre-incubated with either eIF4E or HcPro (1 h at 4 °C). In both cases, inhibition of HcPro–VPg interaction could be observed (Fig. 1c), confirming that HcPro and eIF4E compete for VPg binding.

A three-dimensional model of VPg from *Potato virus Y* has been predicted based on that of an unrelated protein showing a similar distribution of hydrophobic/hydrophilic residues (Płochocka *et al.*, 1996). VPg appeared as an egg-shaped protein, with the domain involved in the interaction exposed at one pole. Although this model was obtained through an unusual approach, it is consistent with an involvement in protein interactions.

To understand better the possible relationships between VPg function and structure, attempts were made to predict

the folding of LMV VPg. Homology-based modelling was unsuccessful due to the lack of homologous proteins of known structure in databases (not shown). To predict the secondary structure of the interaction domain (Fig. 3), ten VPg sequences were aligned and the secondary structure was predicted for each of them using GOR-IV (Garnier *et al.*, 1996). Other algorithms operated online (<http://www.expasy.org> and <http://npsa-pbil.ibcp.fr>) provided essentially similar results (not shown). A hydrophobic (Kyte & Doolittle, 1982)  $\beta$ -sheet was returned for all sequences except those of bymoviruses, between the RNA-bound Tyr-64 (Murphy *et al.*, 1991) and Asp-78, a residue associated with eIF4E-binding in TuMV (Léonard *et al.*, 2000). For all viruses analysed, the downstream portion (residues 90–120) was predicted to be folded in a long amphiphilic  $\alpha$ -helix (Fig. 3). The amino acid variations associated with different biological properties (Borgström & Johansen, 2001; Masuta *et al.*, 1999; Moury *et al.*, 2004; Nicolas *et al.*, 1997; Rajamäki & Valkonen, 1999; Schaad *et al.*, 1997), where identified unambiguously, mapped to the hydrophilic – presumably exposed – side of this helix, which is consistent with a role in protein interactions.

Despite obvious discrepancies between this two-dimensional prediction and the available three-dimensional model (Płochocka *et al.*, 1996), both are compatible with a surface exposition of the VPg central domain. Further structural and biochemical studies are needed to clarify the VPg modelling and the roles of the VPg central domain in the success of virus infection through interactions with eIF4E, HcPro and possibly other proteins. The biological implications of the duality of the interaction in which the VPg central domain is engaged with the host protein eIF4E and the virus protein HcPro will also require investigation. For instance, it has been established that changes in the VPg central domain that affect long-distance movement of TEV in tobacco are not necessarily associated with a loss of eIF4E interaction (Schaad *et al.*, 2000). It is possible that this type of phenotype could be related to changes in affinity with HcPro, a viral protein involved in long-distance movement (Cronin *et al.*, 1995).

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