Peptides derived from plant virus VPg protein inhibit eIF4E oncogene

Running title: Viral protein-derived peptides inhibit eIF4E oncogene

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Abstract, Introduction, Results, Discussion, Fig. Legends - 3034 words

List of abbreviations

AcMNPV - Autographa californica multicapsid nucleopolyhedrovirus; CBB - Coomassie Brilliant Blue; CIYVV - Clover yellow vein virus; eIF4E - eukaryotic initiation translation factor 4E; 4E-BP, eIF4E binding protein; FCS - fetal calf serum; LMV – Lettuce Mosaic Virus; MOI – multiplicity of infection; MW – molecular weight, ON – overnight; TMB - 3,3,5,5-tetramethylbenzidine, *T. ni* - *Trichoplusia ni* cells; PBST – PBS buffer containing 0.05% Tween-20; pi – post infection; PI – propidium iodide; TEV – tobacco etch virus; TVMV - tobacco vein mottling virus; PVY – potato virus Y; VPg – genome-linked viral protein.

24 **Abstract**

25 Viruses of the *Potyviridae* family have VPg protein covalently attached to the 5'
 26 end of their linear RNA genome. The protein interacts with the host translation
 27 initiation factor eIF4E that occurs in plant cells in two isoforms, one being the
 28 preferable target of a given VPg, the remaining one still acting in host protein
 29 synthesis. In animal cells only one form of eIF4E is directly involved in protein
 30 synthesis. The human eIF4E is known to be an oncogene; elevated expression of
 31 eIF4E leads to oncogenic transformation, cancers in animal models and poor
 32 prognosis in human cancers, while reduction of the eIF4E level can reverse the
 33 transformed phenotype. We show that VPg protein delivery to cells containing only
 34 one eIF4E isoform involved in protein synthesis resulted in immobilization of eIF4E
 35 in the cytoplasm. The region of VPg involved in the interaction with eIF4E has been
 36 partially identified. Peptides derived from this region interacted better with eIF4E than
 37 complete VPg protein. Here we characterized one of VPg peptides, VPg5 and we
 38 show that VPg5 delivered to colon carcinoma HCT116 cells is able to inhibit cell
 39 growth, which is accompanied by reduction in eIF4E level.
 40

Introduction

Potyvirus VPg is a multifunctional protein implicated in translation, long-distance movement, replication and suppression of host antiviral silencing response (1, 2). The host partner of VPg is the eIF4E, an mRNA 5' cap-binding protein. The eIF4E and VPg are the major determinants of resistance *versus* susceptibility to potyvirus infection; plants are virus-resistant when mutations in eIF4E or in VPg preclude their interaction (3 - 6). However, higher plants produce two isoforms of eIF4E and potyviral VPgs display greater affinity to one or the other eIF4E isoform (7, 8). Thus, although host protein synthesis is weakened upon infection, the virus cell cycle can be completed since the second eIF4E remains active in plant metabolism necessary for virus development.

In human cells only one cap-binding protein, eIF4E-1, directly promotes protein synthesis (9), and a significant fraction of it (sometimes up to 70%) is found in the nucleus (10). In the cytoplasm, eIF4E is involved in the initiation of protein synthesis while in the nucleus it is implicated in transport of certain mRNAs to the cytoplasm. The nuclear eIF4E has an affinity for a class of mRNAs that specify the short-living proteins implicated in cell cycle progression and malignant transformation (11). An unchecked overexpression of eIF4E can cause cell growth and malignant transformation in tissue cultures and is a feature of experimental cancers, whereas reduction of the eIF4E level can reverse the transformed phenotype (12, 13). Elevated expression of eIF4E in human cancers correlates with poor prognosis (11, 14). These effects can partly be explained by a higher level of short-lived proteins involved in malignant transformation, stemming from the increased eIF4E-dependent transport of the appropriate mRNAs to the cytoplasm. The proof of hypothesis was furnished by the human trial in which the inhibition of eIF4E activity in acute myeloid

leukemia patients brought about quite positive results, whereby cancer remission was associated with eIF4E delocalization from the nucleus to the cytoplasm (15). It appears that eIF4E is an oncogene that acts as a node governing proliferation and survival signaling (16 - 18).

We have shown that Potato Virus Y (PVY) VPg interacts *in vitro* with various eIF4Es, including animal eIF4E (19). It was thus interesting to study what will happen in the presence of VPg in cells with only one form of the eIF4E factor active in protein synthesis. We observed that in the presence of VPg the nuclear pool of eIF4E was dramatically depleted since VPg immobilized initiation factor in the cytoplasm. Recently, we partially characterized the site of PVY VPg responsible for interaction with eIF4E, both of plant and human origin, and constructed peptides derived from this region, some of which interacted better with eIF4E than the complete VPg protein. We wished to show that one of VPg-derived peptides is able inhibit the pro-oncogenic role of eIF4E in neoplastic cells. We have chosen peptide VPg5 for further work and also prepared VPg5 mutants interacting weakly with human eIF4E. Delivery of VPg5 to neoplastic cells in culture resulted in cell death accompanied by eIF4E reduction.

84 Results

85 **Localization of eIF4E and VPg in insect cells.** Upon infection with the
86 recombinant baculovirus expressing PVY VPg, the VPg protein was observed only
87 in the cytoplasm (Fig. 1A, VPg column). The endogenous initiation factor eIF4E
88 localized at the beginning of infection to both, the nucleus and the cytoplasm, but at
89 60 h pi. was observed only at the cell periphery (Fig. 1A, eIF4E column). Indeed, at
90 the beginning of infection, a significant fraction of the eIF4E was in the nucleus but
91 diminished in the course of infection; at 60 h pi. eIF4E was observed in the cytoplasm
92 only, showing significant overlap with VPg. To find out if the observed changes in the
93 eIF4E localization were due to the presence of VPg, we analyzed the localization of
94 eIF4E upon infection with empty bacmid and with baculovirus expressing either a
95 non-relevant protein (the domain II of HCV helicase) or VPg (Fig. 1B). At 60 h pi, only
96 cells expressing VPg displayed eIF4E disappearance from the nucleus (Fig. 1B,
97 lowest panel), demonstrating the specificity of VPg effect. To obtain more quantitative
98 data, after infection with the recombinant baculovirus expressing VPg the insect cell
99 nuclei were separated from the cytoplasm, and eIF4E level in both compartments
100 was assessed by Western blot (Fig. 1C) followed by densitometry (Table 1). At 12h
101 a comparable level of eIF4E was present in the cytoplasm and in the nucleus. The
102 nuclear pool of the eIF4E diminished somewhat in the course of infection with bacmid
103 and with the helicase-expressing virus, from 51-53% down to 37-40% (Table 1).
104 However, upon expression of his-tagged or non-tagged VPg forms, the nuclear pool
105 of the eIF4E was much more severely depleted; at 24 and 36h pi it accounted for
106 only 13-18% of total cellular factor. Interestingly, VPg expression was significantly
107 lower than that of HCV helicase, which underlies the strength of VPg effect (Fig. 1C,
108 lower panel on the right).

These experiments demonstrated that (1) about half of the total cellular eIF4E synthesized in the cytoplasm is transported into the nucleus, confirming results seen for other eukaryotic cells (10); (2) VPg expressed alone in insect cells, despite carrying the putative nuclear localization signal, is localized in the cytoplasm only; (3) in the presence of VPg, eIF4E is retained in the cytoplasm, which results in depletion of the nuclear pool of eIF4E.

Interaction of VPg peptides with eIF4E. Previously, upon selection of proteolytically cleaved VPg protein on a column-immobilized wheat eIF(*iso*)4E, we tentatively identified an eIF4E-interacting site on VPg (19). These results suggested that the N-terminal part of the VPg up to tyrosine 40 is dispensable for the interaction, and that interaction site involves downstream region, starting with arginine 41. We have constructed five peptides derived from the putative interaction site and expressed them in frame with the N-terminally attached GST protein, expressing similarly also a complete VPg protein. These proteins after purification have been used in the ELISA sandwich assay, in which peptide attached to eIF4E was recognized with the anti-GST antibody (Fig. 2). The interaction with wheat eIF(*iso*)4E and with human eIF4E were analysed. The best interacting peptides were VPg5 and VPg4 for the plant factor. The human factor interacted best with the same peptides and in addition with the peptide VPg1. This shortest interacting peptide (VPg1, 19 aa) was contained in both VPg5 (26 aa) and VPg4 (42 aa). Also, the VPg2 contained peptide VPg1 but was significantly longer and much more hydrophobic in character. It attached less well to eIF4E and, similarly as peptide VPg3, had a propensity for forming oligomers at high concentration (results not shown). We have chosen to work with peptide VPg5. In parallel we created two VPg5 mutants. Based on comparison of corresponding sequences from other potyviral VPgs (Fig. 3A), we changed either

tyrosine 64 to alanine (pVPg5A1) or tyrosine 64 and glycine 54 to alanines (pVPg5A2). These peptides in fusion with GST were expressed and purified (Fig. 3C). The VPg5 mutants were analysed for their interaction with human eIF4E (Fig. 3D). Both VPg5 mutants interact rather weakly with human eIF4E, only slightly better than with GST. Interestingly, rather weak interaction could be observed also for a complete VPg protein (but which was on the same level as that shown in Fig. 2).

Effect of VPg peptides on neoplastic cells in culture. This effect was investigated on HCT116 colon carcinoma cells in culture, transduced with Tat fusions of VPg5 and VPg5A2. Treatment of neoplastic cells with TAT-VPg5 resulted in marked cell death, while TAT-VPgA2 despite low binding to eIF4E appears to act only somewhat less well than Tat-VPg5 (Fig. 4). Cell death induced by Tat-VPg5 was accompanied by reduction in eIF4E level (Fig.4, BC).

Discussion

Nuclear eIF4E participates in a variety of important RNA processing events including nucleocytoplasmic transport of specific, growth regulatory mRNAs. Indeed, the availability of eIF4E is believed to be critical for nuclear export and translational activation of capped mRNAs with extensive secondary structures in their 5' untranslated regions (UTRs) (20), many of which code for the labile regulatory proteins essential for cell growth or viability. Various molecular mechanisms of cell growth converge on eIF4E, and it appears that eIF4E is a critical node in RNA regulation influencing nearly every stage of cell cycle progression (16). Thus, eIF4E, besides its direct cytoplasmic role in protein synthesis, seems to have another, nuclear function, whereby by promoting the export of messenger RNA of several proteins involved in cell cycle it plays a key role in regulation of cell growth and survival.

Elevated expression of eIF4E leads to oncogenic transformation in tissue culture, cancers in animal models and poor prognosis in some human cancers. Reduction of eIF4E level can reverse the transformed phenotype, which suggests that eIF4E could be exploited as a therapeutic target. A validation of eIF4E as a therapeutic target has been provided by *in vivo* experiments, in which suppression of eIF4E expression in mice by eIF4E-specific antisense oligonucleotides (ASO) reduced tumor growth in mice (21). Despite reducing the eIF4E levels in mouse liver by 80%, administration of eIF4E-specific ASO did not affect the body weight or the liver enzymes. This suggested that the malignant tumors might be more susceptible to eIF4E inhibition than the normal tissues. Most importantly, a human trial on acute myeloid leukemia patients in whom eIF4E was inactivated with ribavirin, a cap structure mimic (and thus an eIF4E inhibitor) resulted in tumor growth inhibition and cancer remission that

were correlated with eIF4E reduction and translocation of nuclear eIF4E to the cytoplasm in blasts (15, 22). Recently, a phase I trial of ribavirin and low-dose cytarabine for the treatment of acute myeloid leukemia with elevated eIF4E has been terminated, showing that the combination was well tolerated, with some patients having a full molecular response with both reduction of eIF4E levels and eIF4E re-localization to cytoplasm (23).

The eIF4E binding partners such as 4E-BPs, Gemin5, Xenopus Maskin and mammalian neuroguidin, have all been shown to exert translational control (24). These factors usually block the interaction between eIF4E and the scaffold-like eIF4G factor, involved in the formation of the large translation initiation complex eIF4F (25). They all contain the dorsal eIF4E-binding motif YxxxxLØ. Another type of eIF4E effectors, the PML protein and the arenaviral protein Z use their respective RING domains to directly interact with the dorsal surface of eIF4E, thus reducing the affinity of eIF4E for its ligand, 5' cap of mRNA (26, 27). These two proteins shuttle between nucleus and cytoplasm (28), and thus can interact with both nuclear and cytoplasmic eIF4E pools. In addition, a transcription factor, homeodomain protein PRH/HEX that binds nuclear eIF4E inhibits eIF4E function only when its NLS is intact, i.e. it must be nuclear to inhibit eIF4E (29), whereas VPg protein delivered alone shows an exclusively cytoplasmic localization. More importantly, VPg does not contain the eIF4E-binding motif YxxxxLØ or the RING domain, which suggests that VPg interaction with eIF4E occurs at different eIF4E site. Some plants became resistant to potyviruses due to the eIF4E mutations abolishing interaction with VPg. Such mutations have been identified in two eIF4E regions; one partially overlapping the interior of the cap-binding pocket (which is in the concave part of eIF4E) and another on the surface of eIF4E facing 90° from the cap-binding site (30, 7). Both

these regions are distant from the dorsal eIF4E site attaching YxxxxLØ peptides.

Another member of eIF4E family of translational initiation factors that interact with the 5'cap structure of mRNA is 4E-HP. However, 4E-HP does not share the role of eIF4E in mRNA transport from nucleus to cytoplasm; it is found exclusively in the cytoplasm whereby it may act as a translational repressor of mRNAs (31). The third member of the eIF4E family, eIF4E3, that share ~25% identity and ~50% similarity with the two other eIF4E family members, and is nuclear and cytoplasmic, also binds the m7G cap, but acts as a tumor suppressor, competing with the growth-promoting functions of eIF4E (18). All these observations together show that VPg protein is novel type of eIF4E inhibitor.

Different type of eIF4E inhibitor in a form of a cap analog was used by us during *in vivo* studies in the animal model of hepatocellular carcinoma. HCC is one of the leading causes of cancer-related death worldwide, one of the most lethal, chemoresistant and prevalent cancers in the human population with limited treatment options (32). It has been shown that HCC development depended on eIF4E overexpression (33). We have demonstrated that eIF4E inhibition with cap analog in the orthotopic rat model of HCC resulted in tumor growth inhibition, accompanied by decrease in the level of two pro-oncogenes, eIF4E and c-myc (34). Of nWojtl et al, Mrch 2018ote, c-myc is amplified in up to 50% of HCC cases (35).

This time we used a peptide-type inhibitor of eIF4E to find out whether inhibition of the eIF4E activity is an interesting therapeutic approach to cancer treatment that conceivably could be extended to other neoplasms driven by eIF4E overexpression. A partially identified site on PVY VPg served here as a source of five peptides, out of which some interacted better with eIF4E than complete VPg protein. We have chosen a peptide VPg5 of 26 amino acid residues that strongly interacted with eIF4E (Figs.

2 and 3). In parallel we designed a mutant VPg5A2, which showed rather low-level interaction with eIF4E. These peptides were delivered to neoplastic HCT116 cells in a form of the N-terminal fusions with transducing Tat peptide (36). As use of Tat peptide might introduce several artefacts, we followed the conditions developed for minimizing the nonspecific Tat peptide activity (37). Viability of neoplastic cells was significantly diminished by addition of Tat-VPg5 peptides, in a dose dependent manner, quite strongly with the original VPg5 peptide, somewhat less with VPg5A2 peptide. Of note, designing the control - eIF4E-nonreacting - peptide is rather difficult, as there is no structural data identifying the amino acid residues of PVY VPg interacting with eIF4E.

It is possible that NLS of VPg (probably starting from ⁴¹Arg) is not functioning when the complete protein (188 amino acid residues) is delivered upon expression in insect cells, as complete VPg brought alone was localized exclusively to cytoplasm (Fig. 1). However, it is quite plausible to think that NLS in the VPg5 peptide could be functional and VPg5 would go directly to the nucleus, interacting there with the pro-oncogenic nuclear eIF4E. Nevertheless, the eIF4E localization seen after treatment with VPg5 (Fig. 4 B,C) shows strong reduction of nuclear eIF4E but nearly complete depletion of factor in the cytoplasm. Further experiments including confocal microscopy would more precisely describe the intracellular effect and localization of VPg5.

In conclusion, results obtained with VPg5 peptide show its activity as a novel, efficient inhibitor of eIF4E, leading to death of neoplastic cells, which is accompanied by reduction in eIF4E level. This kind of specific oncogene eIF4E inhibitors should be analysed in eIF4E-driven cancers; it was thought that about 30% of human malignancies show overexpression of eIF4E (38), however, more detailed data indicate this percent as close to 63% (39).

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Materials and methods

Materials. *T. ni* insect cells (Invitrogen) were grown as described by Grzela et al (19). Anti-eIF4E mAb that recognized human and animal eIF4E was from Santa Cruz Biotechnology (USA). Anti-eIF4E recognizing human, mouse and insect eIF4E, was a gift of Nahum Sonenberg (McGill University, Canada). Anti-VPg rabbit polyclonal antibody was prepared in ESD (France), using as antigen PVY hisVPg expressed in baculovirus (19) and was affinity-purified with VPg immobilized on CNBr-activated Sepharose 4B (Amersham Bioscience). Goat anti-GST-HRP antibody was from Rockland Immunochemicals Inc. Molecular weight markers for SDS-PAGE were from Thermo Scientific. Rabbit polyvalent anti-Dd serum was prepared in the laboratory using as antigen a mixture of well-formed and ultrasound-treated Dd.

Localization of eIF4E and VPg in insect cells. *T. ni* cells (2×10^6 cells/ml) were infected at MOI 10 with recombinant baculoviruses or empty bacmid. Cells were collected at indicated times p.i., washed with PBS, fixed in cold 2% PFA and suspended in 300 μ l PBS. Portions of 100 μ l of fixed cells on round coverslips inserted into wells of a 24-wells plate and rehydrated were permeabilized with cold 0.1% Triton X-100/PBS, rinsed twice with PBS and blocked with 5% serum/PBS for 30 min at RT. Permeabilized cells were incubated for 1 h with anti-his or anti-eIF4E Ab diluted 100-fold, washed and incubated for 1 h with anti-rabbit FITC-conjugated Ab or anti-mouse Texas Red-conjugated Ab (Jackson ImmunoResearch), diluted 1:100. Nuclei were stained with PI in PBS (1 μ g/ml) and PBS-rinsed coverslips were mounted with 50% glycerol. Images were collected with Bio-Rad MRC-600 laser scanning confocal apparatus coupled to a Nikon Optiphot microscope.

Insect cell fractionation. High Five cells (2×10^6 cells/ml) were infected with the baculoviruses expressing VPg, or hisVPg, or domain II of HCV helicase or empty

bacmid. The infected cells were collected at the indicated times p.i. by centrifugations at 2000 rpm for 10 min and nuclei were separated from the soluble fraction as described (Xu et al, 1995). Cytoplasmic and nuclear fractions were run on 15% SDS-PAGE followed by Western blot with anti-eIF4E antibody and the ECL system. Densitometry of eIF4E bands was performed with GelDoc 2000 (BioRad, Quantity One software).

Protein expression and purification. VPg expression in *E. coli* and purification was done according Grzela et al (19). GST protein was expressed in *E. coli* BL21(DE3) Rosetta from pGEX-4Ti and purified on the GST-Sepharose. For the synthesis of the fusion protein GST-VPg5, the VPg5 gene encoding a part of VPg protein between residues 41 – 66, was amplified by PCR using plasmid pGEXVPg (containing the gene encoding the VPg protein of PVY strain 0, acc. no Z29526; Grzela et al., 2006) as template, with the forward primer 5'CGCGGATCCGAAAACCTGTATTTTCAGGGCAGGAAAAAGGGAAAAGGTAAA GG3'

and the reverse primer 5'CCGCCGCTCGAGCTATTAGAACCCGTACATGTTAATGAA3'. The resulting PCR product was then digested by BamHI/XhoI and inserted into pGEX4T-1 vector between BamHI and XhoI sites for expression in *E. coli* as GST-fusion protein.

Two VPg5 mutants, VPg5A1 and VPg5A2 have been prepared in form of a fusion with the GST protein (at the N-terminus of the peptide). To obtain GST-VPg5A2, mutagenesis was done using Q5® Site-Directed Mutagenesis Kit (NEB) and a GST-VPg5 in pGEX4T-1 as a template. In VPg5A1 the Gly54 has been changed to Ala, in VPg5A2 both Gly54 and Tyr64 has been changed to Ala. The following primers (forward and reverse, F and R) have been used for mutagenesis of

the pVPg5 sequence. The correct sequence of mutants was confirmed by DNA sequencing.

VPg5A1

F1A AGCAGGAGGTTTCATTAACATGGCCGGGTTCTAATAGCTCGAG

R1A TGA CTTGCCCATACCAACTGTGGTACCTTTACC

VPg5A2

F2A

AAAGGTACCACAGTTGGTATGGCCAAGTCAAGCAGGAGGTTTCATTAACAT

GGCCGGGTTCTAATAG

R2A

ACCTTTTCCCTTTTTCCTGCCCTGAAAATACAGGTTTTTCGGATCCACGCG

GAACCAG

GST peptides were expressed in *E. coli* Rosetta2(DE3)pLyS as follows. Bacteria were grown in the presence of 100 µg/ml of carbenicillin and 20 µg/ml chloramphenicol, initially at 37°C. Protein production was induced with 400 µM IPTG at OD₆₀₀ equal 0.6, then the temperature was switched to 30°C and 4h later the bacteria were harvested. Cell pellet from 250 ml culture was suspended in 10 ml of 50 mM Hepes buffer, pH 7.4, containing 300 mM NaCl, 5 mM DTT, 0.5 mM EDTA and supplemented with the protease inhibitor cocktail (Complete, Roche) and lysozyme (LabEmpire) at 1mg/ml. The bacteria were lysed by sonication on ice. The lysates were then centrifuged at 14 000 rpm for 30 min at 4°C and the supernatants were filtered through 0.2 µm filter before applying onto 0.5 ml Glutathione-Sepharose 4B column (GE Healthcare). After 4 washes with 10 ml of lysis buffer GST-fusion proteins were eluted with five 1ml portions of 10 mM reduced glutathione in lysis buffer.

Human eIF4E was cloned in plasmid pETM30 (EMBL) with 6xhis tag attached at the N-terminus. The protein was expressed in *E. coli* BL21(DE3) and purified by affinity chromatography on a nickel column (HisTrap HP, GE-Healthcare). Plant eIF4E was prepared in fusion with GST tag attached at the N-terminus in plasmid pGEX4T-1 and expressed in *E. coli* BL21(DE3). The protein was purified on a Glutathione-Sepharose 4B column.

Interaction of GST-VPg peptides with human eIF4E (ELISA). The 96-well plates (Nunc) were coated with human eIF4E in 0.1 M carbonate buffer, pH 9.6 (100 ng/well), and incubated overnight at 4°C. Excess eIF4E was removed and the wells were blocked with 5% milk in PBST for 1 hour at 37°C. After milk removal the plates were washed four times (5 min each) with PBST. Two-fold dilutions in cascade of fusion proteins GSTVPg, GSTVPg5, GSTVPg5A1, GSTVPg5A2 and GST alone (2000 – 0.97 nM) were added to the wells and allowed to incubate for 90 min at RT. After removal of proteins excess the wells were washed two times (5 min each) with PBST. Next, the plates were incubated for 1 hour at 37°C with anti-GST-HRP antibody diluted 1:10 000. Excess antibody was removed, the plates were washed three times with PBST and the color reaction was revealed with TMB (Thermo Scientific) and terminated 20 min later with 2 M H₂SO₄. The absorbance at 450 nm was measured with the BIO-TEK Synergy HT fluorimeter.

TAT-VPg peptides. For delivery to human cells in culture, peptides VPg5 and VPg5A2 have been synthesized by Pepscan Presto BV (The Netherlands) as N-terminal TAT fusion peptides, with over 90% of purity.

Tat-VPg5 – NH₂-RKKRRQRRR- RKKGKGKGTT VGMGKSSRRF INMYGF-CONH₂

Tat-VPg5A2 – NH₂-RKKRRQRRR-RKKGKGKGTT VGMAKSSRRF INMAGF-
CONH₂

Effect of Tat-VPg peptides on viability of neoplastic cells in culture.

HCT116 cells were maintained in RPMI 40 medium containing 10% fetal bovine serum (FBS), and antibiotics (100 mg/mL streptomycin, 100 U/mL penicillin) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. They were seeded into 24-well dishes at 1x10⁵ and left overnight. The medium was removed, cells rinsed with PBA and Tat-VPg peptides dissolved in PBS were added for indicated times. The viability was estimated by trypan blue inclusion. The results are expressed as an average of three measurements.

Level of eIF4E in nuclear and cytoplasmic fraction of HCT116 cells. Before and after treatment with Tat-VPg peptides, HCT116 cells were scraped and fractionated according to REAP method (40). Proteins in cytosol and in the nuclear fraction were fractionated on SDS-PAGE (Mini-protean TGX precast gel from BioRad), transferred to the PVDF membrane using the Trans-Blot Turbo transfer apparatus (BioRad). The eIF4E was revealed with rabbit polyclonal Ab (kind gift of Nahum Sonenberg) at 1:1000 using ECL system (BioRad) and ChemiDoc XRS+ imager (BioRad) for visualisation. Densitometric analysis was done on eIF4E bands using ImageLab software version 4.1 (BioRad).

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Figure legends

Fig. 1. Effect of VPg on distribution of endogenous eIF4E in insect cells.

Expressing insect cells were analyzed by confocal microscopy (x 60). (A) Localization of eIF4E (in green) in the presence of VPg (in red). (B) Cytoplasmic retention of native eIF4E (in green) is specific for VPg. (C) Distribution of endogenous insect eIF4E upon VPg expression (MOI 10). Expressing cells nuclei (n) were separated from the cytoplasm (c) and the eIF4E content was analyzed. Recombinant proteins expression is shown in the lower far right panel.

Fig. 2. Interaction of VPg peptides with eIF4E. (A) Amino acid sequence of VPg

and VPg peptides derived from the VPg region involved in interaction with eIF4E. The putative eIF4E interaction site is marked with bold letter. (B) Interaction of VPg peptides with wheat eIF(iso)4E and human eIF4E. Complete VPg protein is marked in bold.

Fig. 3. VPg5 peptides and their interaction with human eIF4E. (A) Comparison

of primary sequence of some potyviral VPgs. (B) Primary sequence of VPg5 peptides. (C) GST-fusion proteins used in ELISA test, run on 15% tricine polyacrylamide gel (41). Samples of 1-2 µg were stained with CBB. Lane 1 – VPg protein, lane 2 – VPg5; lane 3 - VPg5A1; lane 4 - VPg5A2; lane 5 – his-eIF4E protein; lane 6 - GST protein. (D) Interaction of pVPg peptides with human eIF4E (ELISA sandwich technique).

Fig. 4. Effect of Tat-VPg peptides on HCT116 cell viability. Tat-VPg5 peptides

were applied to HCT116 cells as described in Materials and Methods. (A). Cell viability was measured by trypan blue exclusion. B. Portions of 100 000 cells untreated or treated with Tat-VPg5 underwent fractionated with REAP method (40),

522 run on SDS-PAGE gel, and Western blot was performed with anti-eIF4E antibody.

523 Densitometry was performed as described in Materials and Methodes.

524

Table 1. Distribution of eIF4E in insect cells

Hours pi	baculovirus	in cytoplasm (%)	in nuclei (%)
Non-infected	- none	48.49	51.51
12 h	bacmid	46.5	53.5
	HCV helicase	51.5	48.5
	VPg	55.5	44.5
	hisVPg	53.4	46.6
24 h	bacmid	57.8	42.2
	HCV helicase	63.0	37.0
	VPg	84.7	15.3
	hisVPg	81.3	18.7
36 h	bacmid	59.2	40.8
	HCV helicase	62.5	37.5
	VPg	87.1	12.9
	hisVPg	83.3	16.7
48 h	bacmid	56.0	44.0
	HCV helicase	57.9	42.1
	VPg	73.9	26.1
	hisVPg	74.1	25.9

eIF4E level assessed by Western blot (Fig. 1C) was followed by densitometry.

Fig. 1, Wojtal et al

A ¹GKNKSKRIQA LKFRHARDKR AGFEIDNND
TIEFFGSAY **RKKGKGKGTTVGMGKSSRRF**
INMYGFDPTEYSFIQFVDPL TGAQIEENVY
ADIRDIQERF SEVRKKMVEN DDIEMQALGS
NTTIHAYFRK DWSDKALKID LMPHNPLKVC
DKTNGIAKFP ERELELRQTG PAVEVDVKDI
PAQEVEHE¹⁸⁸

PVY VPg
primary sequence

VPg peptides

VPg1, 19 aa 41-RKKGKGKGTTVGMGKSSRR-59
VPg2, 54 aa 41-RKKGKGKGTTVGMGKSSRRFINMYGFDPT EYSFIQFVDPLTGAQIEENVYADIR-94
VPg3, 34aa 60-FINMYGFDPT EYSFIQFVDPLTGAQIEENVYADIR-94
VPg4, 42aa 41-RKKGKGKGTTVGMGKSSRRFINMYGFDPT EYSFIQFVDPLTG-83
VPg5, 26aa 41-RKKGKGKGTTVGMGKSSRRFINMYGF-66

B

Fig. 2, Wojtal et al

A

PVY	⁴¹ RKKGKGKGTT VGMGKSSRRF INMYGF
TEV	NNKGKRKGTT RGMGAKSRRF INMYGF
CIYVV	SKKGKTKGTV VGLGAKTRRF TNMYGF
TVMV	TKKGKSKGKT HGAGTKAHKF VNMYGV
LMV	TEKGKSKGKT KGMGTKNRRF VNMYGY
TuMV	TKKGKSKGRT RGIGHKNRKF INMYGF
PVA	TKKGKTKGKT HGMGKKNHRF VNMYGF

B

VPg5	RKKGKGKGTT VGMGKSSRRF INMYGF
VPg5A1	RKKGKGKGTT VGMGKSSRRF INM A GF
VPg5A2	RKKGKGKGTT VGM A KSSRRF INM A GF

Fig. 3, Wojtal et al

Fig. 4, Wojtal et al.