Potyvirus genome-linked protein, VPg, directly affects wheat germ in vitro translation

INTERACTIONS WITH TRANSLATION INITIATION FACTORS eIF4F AND eIFiso4F†

Received for publication, April 23, 2007, and in revised form, November 6, 2007. Published, JBC Papers in Press, November 28, 2007, DOI 10.1074/jbc.M703356200

Mateen A. Khan‡, Hiroshi Miyoshi§, Daniel R. Gallie¶, and Dixie J. Goss††

From the ‡Department of Chemistry, Hunter College and the Graduate Center of the City University of New York, New York, New York 10065, §Department of Microbiology, St. Marianna University School of Medicine, Kawasaki 216-8511, Japan, and ¶Department of Biochemistry, University of California, Riverside, California 92521-0129

Potyvirus genome linked protein, VPg, interacts with translation initiation factors eIF4E and eIFiso4E, but its role in protein synthesis has not been elucidated. We show that addition of VPg to wheat germ extract leads to enhancement of uncapped viral mRNA translation and inhibition of capped viral mRNA translation. This provides a significant competitive advantage to the uncapped viral mRNA. To understand the molecular basis of these effects, we have characterized the interaction of VPg with eIF4F, eIFiso4F, and a structured RNA derived from tobacco etch virus (TEV RNA). When VPg formed a complex with eIF4F, the affinity for TEV RNA increased more than 4-fold compared with eIF4F alone (19.4 and 79.0 nM, respectively). The binding affinity of eIF4F to TEV RNA correlates with translation efficiency. VPg enhanced eIFiso4F binding to TEV RNA 1.6-fold (178 nM compared with 108 nM). Kinetic studies of eIF4F and eIFiso4F with VPg show ~2.6-fold faster association for eIFiso4F/VPg as compared with eIF4F/VPg. The dissociation rate was ~2.9-fold slower for eIFiso4F than eIF4F with VPg. These data demonstrate that eIFiso4F can kinetically compete with eIF4F for VPg binding. The quantitative data presented here suggest a model where eIF4F/VPg interaction enhances cap-independent translation by increasing the affinity of eIF4F for TEV RNA. This is the first evidence of direct participation of VPg in translation initiation.

Most eukaryotic mRNAs contain a cap structure m7GpppX2 (where X is any nucleotide) at the 5′-end that is required for efficient initiation of translation. The cap serves as the binding site for initiation factors eIF4F or eIFiso4F, an isozyme form of eIF4F present in higher plants, either of which is involved in the assembly of the initiation complex. Alternative translation mechanisms have been shown to take place under a unique set of circumstances. One such example is that of viral infection. Viruses employ different strategies for preferential translation of their mRNAs often using internal ribosome entry sites (IRES).

Recently attention has focused on the possible role in translation initiation of the viral protein linked to the genome (VPg) of potyvirus (1, 2). It has been suggested that VPg may serve as an analog of the m7G cap of the mRNAs and plays a role in mRNA translation because it interacts with the cap-binding proteins eIF4E and eIFiso4E (3, 4), subunits of eIF4F and eIFiso4F. Potyviruses have a messenger-polarity single-strand RNA genome, a poly(A) tail, and VPg covalently attached to the 5′-terminus (1, 5), which upon infection of a suitable host is cleaved from the genome RNA by an unknown cellular enzyme. The 5′-pUpU-processed RNA serves as a template for viral protein synthesis as well as early rounds of RNA synthesis (6). Potyviruses have been shown to be capable of cap-independent translation (7) making the role of VPg in translation uncertain. Studies support a biological role for the VPg linked to the viral RNA in virions. The covalent tyrosine residue-mediated linkage between the VPg and viral RNA is necessary for infectivity (8). VPg has also been implicated indirectly in cell-to-cell movement of the virus through plasmodesmata and, more directly implicated by mutagenesis, in the long distance translocation of the virus (9–14). Additionally, the potyvirus VPg protein has been shown to be the avirulence factor for recessive resistance genes in various plants (10, 15).

Wheat germ and other plants express two related but distinct forms of the cap-binding protein designated as eIF4F and eIFiso4F (16). Each consists of two subunits, eIF4E and eIF4G and subunit eIFiso4E and eIFiso4G. The amino acid sequence of eIFiso4E, the cap-binding subunit, is ~50% similar to eIF4E from wheat and (~38%) similar to the cap-binding proteins from yeast and mammals and retains all of the reported conserved tryptophan residues (17, 18). Wheat germ eIFiso4F can substitute for mammalian eIF4F with respect to supporting RNA-dependent ATPase activity and in cross-linking of mammalian eIF4A to the cap of oxidized mRNA (19). The two plant proteins (eIF4F and eIFiso4F) have a number of functional similarities in that both support translation in vitro and facilitate ATP-
dependent helicase activity (19–22). eIF4F was found to support translation of RNA containing secondary structure in the noncoding region in a more efficient manner than did eIFiso4F (23). Moreover, binding studies with oligonucleotides suggest that eIF4F exhibits binding preference for secondary structure and that eIFiso4F prefers linear structures (24). Further studies showed that in vitro translation using the tobacco etch virus 5'-leader, which contains an internal ribosome entry site, to direct cap-independent translation specifically depended on eIF4G and not eIFiso4G (25). Direct binding studies showed that binding affinity of eIF4G and eIFiso4G correlated with translational efficiency (26).

Robaglia and Caranta (27) showed the importance of the link between translation initiation factors eIF4E, eIF4G, and plant RNA virus infection. These observations suggest VPg is important in initiation of protein synthesis, perhaps functioning as a cap analogue, as proposed by Herbert et al. (28). VPg not covalently attached to the viral RNA is present at least in some viral stages (29) and may affect translation. Earlier studies (4, 30) have shown that VPg and cap bind competitively to plant eIF4E and eIFiso4F. Whether this contributes to a direct role of VPg in translation or acts as a mechanism for competitive inhibition of host cell initiation factors has not been elucidated. The observation (26) that translation of TEV RNA requires eIF4F and not eIFiso4G raises the possibility that the eIFiso4F-VPg interaction does not have a direct role in translation. To further understand the potential role of VPg in translation initiation, we have investigated the interaction of VPg with eIF4F and the effects of this interaction on TEV binding and translation in vitro. The kinetics of binding eIFiso4F to VPg led us to propose a mechanism where VPg substituted for the cap and enhanced formation of an eIFiso4F complex with the IRES (4). In this report we show that VPg has an even stronger effect on eIF4F, enhancing the interaction with TEV IRES about 4-fold. Furthermore, the addition of VPg to an in vitro translation system increased translation of an uncapped TEV RNA construct by almost 3-fold. Taken together with our earlier data (4), these results suggest direct participation of VPg in translation initiation by increasing the affinity of eIF4F for TEV IRES and, thus, confers a competitive advantage.

**EXPERIMENTAL PROCEDURES**

**Materials**—Wheat germ (partially crushed) was purchased from Bob's Red Mill, Natural Foods, Inc. (Milwaukee, WI) and was stored at −20°C until used. The TuMV VPg full-length cDNA clone and construction of the expression vector for TuMV VPg was described previously (30, 31). diethylaminoethy cellulose was purchased from Whatman International Ltd., Maidstone, England. Sephadex G-25 was purchased from GE Healthcare. m7GTP-Sepharose and glutathione-Sepharose-4B were purchased from Amersham Biosciences. Nickel-nitri locetic acid Superflow was purchased from Qiagen. The HitTrap SP, HiTrap Mono Q Column, and PreScission Protease were purchased from Amersham Biosciences. The pET21a and pET28a expression vectors were purchased from Novagen, an affiliate of Merck. Luciferin was obtained from Promega, Madison, WI.

**Purification of Proteins**—Wheat germ eIF4F was isolated from the 0–40% ammonium sulfate fraction of the 120 mM KCl post-ribosomal supernatant fraction as described previously (32). The eIF4G was obtained from a plasmid expressed in *Escherichia coli* as described elsewhere (33). Some samples of eIF4G were generously provided by Dr. Karen Browning (University of Texas, Austin, TX).

The recombinant proteins, eIF4E, eIFiso4E, and eIFiso4G contained in pET3d vector constructs were expressed in BL21 (DE3) pLysS E. coli as described elsewhere (33) with slight modifications. Protein was eluted from the m7GTP column with buffer B-50 containing 100 mM GTP. After purification, the purity was confirmed by 10% SDS-PAGE. All steps were carried out in a cold box at 4°C.

**Construction of Expression Vector for Wild Type VPg and N-terminal-truncated VPg71**—The TuMV VPg gene was amplified by PCR from the TuMV cDNA clone using primer 3 (5′-AGAATTCATATGGCGCAAGGCCAGGCAAGAAG-3′) and primer 4 (5′-AAAGTCGACTCTGTCGTTTTGTGAT-3′) and primer 5 (5′-AACATATGCTGTCTGTTGGTGTGAT-3′) and 4 (5′-AAAGTCGACTCTGTCGTTTTGTGAT-3′) and primer 4. The resultant product was digested with NdeI and XhoI and ligated into the same sites in pET21a to create an expression vector with a His6 tag. The TEV RNA virus infection. These observations suggest VPg is important in initiation of protein synthesis, perhaps functioning as a cap analogue, as proposed by Herbert et al. (28). VPg not covalently attached to the viral RNA is present at least in some viral stages (29) and may affect translation. Earlier studies (4, 30) have shown that VPg and cap bind competitively to plant eIF4E and eIFiso4F. Whether this contributes to a direct role of VPg in translation or acts as a mechanism for competitive inhibition of host cell initiation factors has not been elucidated. The observation (26) that translation of TEV RNA requires eIF4F and not eIFiso4G raises the possibility that the eIFiso4F-VPg interaction does not have a direct role in translation. To further understand the potential role of VPg in translation initiation, we have investigated the interaction of VPg with eIF4F and the effects of this interaction on TEV binding and translation in vitro. The kinetics of binding eIFiso4F to VPg led us to propose a mechanism where VPg substituted for the cap and enhanced formation of an eIFiso4F complex with the IRES (4). In this report we show that VPg has an even stronger effect on eIF4F, enhancing the interaction with TEV IRES about 4-fold. Furthermore, the addition of VPg to an in vitro translation system increased translation of an uncapped TEV RNA construct by almost 3-fold. Taken together with our earlier data (4), these results suggest direct participation of VPg in translation initiation by increasing the affinity of eIF4F for TEV IRES and, thus, confers a competitive advantage.

**EXPERIMENTAL PROCEDURES**

**Materials**—Wheat germ (partially crushed) was purchased from Bob’s Red Mill, Natural Foods, Inc. (Milwaukee, WI) and was stored at −20°C until used. The TuMV VPg full-length cDNA clone and construction of the expression vector for TuMV VPg was described previously (30, 31). diethylaminoethy cellulose was purchased from Whatman International Ltd., Maidstone, England. Sephadex G-25 was purchased from GE Healthcare. m7GTP-Sepharose and glutathione-Sepharose-4B were purchased from Amersham Biosciences. Nickel-nitri locetic acid Superflow was purchased from Qiagen. The HitTrap SP, HiTrap Mono Q Column, and PreScission Protease were purchased from Amersham Biosciences. The pET21a and pET28a expression vectors were purchased from Novagen, an affiliate of Merck. Luciferin was obtained from Promega, Madison, WI.
Interactions of VPg with eIF4F and eIFiso4F

RNA was synthesized with 10 μg of template in the same reaction mixture as described for uncapped RNA synthesis except that GTP used was 3 mM, and 40 mM m7GpppG was included in the reaction mixture. Under these conditions more than 90% of the mRNA was capped. The concentration of RNA was determined by measuring the optical density at 260 nm. The purity of synthesized RNA was confirmed by 1% agarose gel electrophoresis and measuring the absorbance ratio A260/A280 nm in diethylpyrocarbonate-treated water.

In Vitro Translation Assay—Wheat germ extract was isolated as described previously (36). For extract depleted in cap binding complex, wheat germ extract (200 μl) was mixed with m7GTP-Sepharose-4B resin (300 l) and incubated at 4 °C for 30 min with continuous mixing. The lysate was collected by high speed centrifugation. The resin was washed with 500 μl of buffer B-40 followed by resuspension of the resin in buffer B-40 containing 100 mM GTP. The GTP eluted fractions (eIF4E, eIF4G, eIFiso4E, and eIFiso4G depleted from wheat germ extract) were verified by running 10% SDS-PAGE. Translation was determined in luciferase assay buffer (25 mM Tricine, pH 8.0, 5 mM MgCl2, 0.1 mM EDTA supplemented with 33 mM dithiothreitol, 0.25 mM coenzyme A, and 0.5 mM ATP) for luciferase activity. A 1.0-μg sample of TEV1–143-luc-A50 RNA or TEV51–13-luc-A50 was translated in a 200-μl reaction mixture containing 50 μl of complete or depleted wheat germ extract, 50 units of RNase inhibitor, and a 10 μM complete amino acid mixture (Promega). Luciferase activity for brome mosaic virus RNA provided by Promega was used as a control. Different concentrations of wt VPg or VPg71 variant protein were added to the translation mixture as described under “Results.” Depleted wheat germ lysates were supplemented with initiation factors eIF4F and eIFiso4F, purified from wheat germ extract and recombinant initiation factors. Translational activity of recombinant eIFs was the same or better than factors isolated from wheat germ.3 The optimum concentration of eIF4F/iso4F added to the depleted lysate was 10 nM. Additional protein did not significantly increase translation. The reaction mixtures were incubated for 2 h at 22 °C, and light emission was measured after the addition of 0.5 μl luciferin.

Steady State Fluorescence Measurements—Steady state fluorescence measurements were performed using a Spex Fluorolog 72 spectrofluorimeter equipped with excitation and emission polarizers. The formation of the binary protein–protein and protein–RNA complexes were studied by direct fluorescence titration using a 10-mm path length quartz cuvette. The excitation wavelength for eIF4F and eIFiso4F was 280 nm, and emission was monitored at 332 nm. The excitation and emission slits were 4 and 5 nm, respectively. The excitation slits were chosen to avoid photobleaching, and the absorbance of the sample at the excitation wavelength was less than 0.02 to minimize the inner-filter effect. Emission spectra were corrected for the wavelength-dependent lamp intensity and monochromator sensitivities. Titrations were performed in 20 mM HEPES/KOH, pH 7.6, 100 mM KCl, 1 mM MgCl2, and 1 mM dithiothreitol (titration buffer). The sample temperature was maintained at 25 °C for all experiments unless otherwise stated. For each data point three samples were prepared. The fluorescence intensity of a solution containing 100 nM eIF4F was measured. A second sample with a specific amount of VPg protein was also measured, and the corrected intensities of the two samples were summed together (Fcorr). A third sample containing the same amount of eIF4F and VPg protein was mixed together, and the corrected fluorescence intensity for this complex was obtained (Fcorr). The difference in fluorescence intensity related to the complex was defined as ΔF = Fcorr – Fcorr. Similar measurements were also done for the interaction of eIFiso4F and VPg titrations. The inner filter correction for the TEV RNA experiment was performed as described previously (26) using the equation (37),

\[ F_{corr} = F_{obs} \text{antilog}(A_{ex} + A_{em})/2 \]  
(Eq. 1)

where Fcorr and Fobs are the corrected and observed fluorescence intensities, respectively. Aex and Aem are the absorbance of the excitation and emission wavelength, respectively. The absorbance of the sample was measured using an Ultrospec 1100 Pro UV-visible absorption spectrophotometer. The normalized fluorescence difference (ΔF/ΔFmax) between the protein–protein and protein–RNA complex and the sum of the individual fluorescence spectra were used to determine the equilibrium dissociation constant (Kd). A double reciprocal plot was used for determination of ΔFmax. The details of the data fitting are described elsewhere (38, 39). Fluorescence intensities were corrected when necessary for dilution and for the inner filter effect. Nonlinear least squares fitting of the data were performed using KaleidaGraph software (Version 2.1.3; Abelbeck Software).

Competition reactions for VPg and TEV RNA binding to eIF4F and eIFiso4F were analyzed by fluorescence measurements at 25 °C in titration buffer. The competition reactions were performed at a constant eIF4F or eIFiso4F concentration (0.1 μM) and TEV RNA (0.0, 0.1, and 0.5 μM) and increasing amounts of VPg.

Stopped-flow Measurements—Stopped-flow kinetic measurements were performed on an OLIS RSM 1000 stopped-flow system with a 1-μs dead time. The excitation wavelength for eIF4F and eIFiso4F was 280 nm, and the cut-off filter was 324 nm. A reference photomultiplier was used to monitor fluctuations in the lamp intensity. The temperature of the flow cell and solution reservoirs was maintained using a temperature-controlled circulating water bath. VPg binding induced a decrease in eIF4F and eIFiso4F fluorescence. After rapid mixing of 0.5 μM (0.25 μM after mixing) eIF4F or eIFiso4F with 2 μM (1 μM after mixing) VPg, the time course of the fluorescence intensity change was recorded by computer data acquisition. In each experiment 1000 pairs of data were recorded, and sets of data from three experiments were averaged. Each averaged set of stopped-flow data were then fitted to nonlinear analytical equations using Global analysis software provided by OLIS. The data were fitted to the single- and double-exponential functions. Fitted curves correspond to the following single-exponential equation (40),

\[ F(t) = F_{max} \exp(-k_{obs}t) + F_{in} \]  
(Eq. 2)

3 K. Browning, personal communication.
where $F_t$ is the fluorescence observed at any time, $t$, the fluorescence when the reaction achieves equilibrium, $F_{\infty}$ is the final value of fluorescence, and $k_{obs1}$ is the observed first-order rate constant. For double-exponential fits, the kinetic data were fit to the equation,

$$F_t = \Delta F_1 \exp(-k_{obs1}t) + \Delta F_2 \exp(-k_{obs2}t) + F_{\infty}$$

(Eq. 3)

where $\Delta F_1$ and $\Delta F_2$ are the amplitudes for the first and second components of two exponentials with rate constants $k_{obs1}$ and $k_{obs2}$, respectively. The residuals were measured by the differences between the calculated fit and the experimental data. The fitted rate constants were used to construct an Arrhenius plot according to the equation

$$\ln k = \frac{-E_a}{RT} + \ln A$$

(Eq. 4)

where $k$ is the rate constant, $E_a$ is the activation energy, and $A$ is the Arrhenius pre-exponential term. The activation energy was calculated from the slope of the fitted linear plot of $\ln k$ versus $1/T$ (kelvin).

Dissociation Rate Constants—To measure the dissociation rate constants, a complex of eIF4F or eIFiso4F with VPg was rapidly diluted 15-fold in a spectrophotometer cuvette, and the resulting increase in fluorescence was measured. Because of the high binding affinity of the protein-protein complex, a large dilution, which could not be accomplished by stopped flow, was necessary. The concentrations of the reactants before mixing were 2 $\mu$M VPg and 0.5 $\mu$M eIF4F or eIFiso4F, respectively. The dissociation rates were determined from the fits of a single-exponential equation (Equation 2) to the data using the nonlinear least-square fitting program KaleidaGraph (Version 2.1.3, Abelbeck software). Equilibrium data indicate that at the end of the reaction for VPg-eIF4F and VPg-eIFiso4F, only 2% and 4%, respectively, of the complex remains. Essentially complete dissociation occurs under these conditions.

RESULTS

Interactions of eIFiso4E with Wild Type VPg and Truncated VPg71—To determine the specificity of VPg, VPg71, a truncated variant of wt VPg (N-terminal 1–70 amino acids) was used. Pulldown assays, as described for wt VPg (30), were used (supplemental Fig. S1). Pulldown data showed that VPg71 did not compete with eIF4F4 for cap analog, and fluorescence titration data (See below) showed essentially no interaction of VPg71 with either eIF4E or eIFiso4E. These results are consistent with earlier work (41) that showed a 1–61 deletion mutant did not compete with either eIF4E or eIFiso4E for cap binding. VPg71 was, therefore, used as a control for translation and binding assays.

Effects of VPg on in Vitro Translation of RNA with Wheat Germ Extract—The 5’-leader of TEV RNA contains an IRES that promotes internal initiation and cap-independent translation (42, 43). In addition to the 5’-IRES, TEV contains a virus-encoded protein (VPg) covalently attached to the 5’ terminus that is cleaved after infection (44) but is still present in the cell (45). To examine whether VPg affects in vitro translation in wheat germ extract, a capped and uncapped RNA construct containing the TEV-untranslated region (143-nt including an IRES) with a luciferase reporter as described elsewhere (7) was used (see “Experimental Procedures”). Fig. 1 shows that the addition of wt VPg increased translation about 2.6-fold for the capped RNA and inhibited translation about 1.8-fold for capped RNA in complete wheat germ extract. In the absence of VPg, capped RNA was translated approximately three times more efficiently than uncapped RNA. However, the addition of VPg gives TEV RNA an ~1.6-fold higher translation compared with capped RNA. To determine whether a functional IRES was necessary for the VPg translational enhancement, TEV S1–3–luc–A50 was assayed. TEV S1–3 does not contain a functional IRES (see “Experimental Procedures”). In the absence of VPg, capped TEV S1–3 RNA translated ~2.5-fold more efficiently than uncapped TEV S1–3 (supplemental Fig. 2). Unlike TEV 1–143 RNA, VPg did not enhance translation of uncapped TEV S1–3 RNA. VPg did, however, inhibit translation of capped TEV S1–3 to a similar extent as the inhibition of capped TEV 1–143 RNA. These data suggest a functional IRES is necessary for VPg enhancement of translation. To determine whether or not this was a specific effect of VPg, presumably related to interaction with eIF4F or eIFiso4F, VPg71 was used. VPg71 does not interact with eIFiso4E/eIF4E (see Fig. 4B). As shown in Fig. 1, this variant had virtually no effect on translation of either capped or uncapped RNA, suggesting that the central domain of VPg, which is involved in the interaction with eIF4E/eIFiso4E, is also involved in translation.

To examine the effects of eIF4F/eIFiso4F in translation, it was essential to generate eIF4F/eIFiso4F-depleted wheat germ extract. The level of initiation factors eIF4F (consisting of eIF4E and eIF4G subunits) and eIFiso4F (consisting of eIFiso4E and eIFiso4G subunits) in wheat germ extract was reduced through their binding to mGTP-Sepharose. The reduction of eIF4E and eIF4G and of eIFiso4E and eIFiso4G (Fig. 2, lane 2) was con-

![Figure 1. Translation of luciferase reporter TEV RNA constructs in wheat germ extracts.](image-url)
Interaction of Vpg with eIF4F and eIFiso4F

![Figure 2: Depletion of eIF4F and eIFiso4F from wheat germ extract with m'GTP-Sepharose binding.](image)

Wheat germ extract was incubated with m'GTP-Sepharose, and depletion of eIF4F (consisting of two subunits, eIF4E and eIF4G) and eIFiso4F (consisting of eIFiso4E and eIFiso4G) was confirmed by running 10% SDS-polyacrylamide gel electrophoresis. Lane 1, fractions eluted from m'GTP-Sepharose with 100 mM GTP; lane 2, wheat germ lysate fraction after incubation with m'GTP-Sepharose-4B resin; lane 3, complete wheat germ lysate before incubation with m'GTP resin.

Firmed by running 10% SDS-PAGE. Initiation factors eIF4E and eIF4G as well as eIFiso4E and eIFiso4G were observed (Fig. 2, lane 1) in the fraction eluted from the affinity column with GTP, indicating that eIF4F and eIFiso4F were depleted from wheat germ extract. For both capped and uncapped TEV RNA, depleted wheat germ lysate (supplemental Fig. 3B) showed about a 5-fold decrease in translation as compared with unfractionated wheat germ lysate (supplemental Fig. 3A). Capped TEV RNA showed about a 3-fold higher translation than did uncapped TEV RNA for both unfractonated and eIF4F/eIFiso4F-depleted wheat germ lysate. The addition of Vpg to uncapped TEV RNA increases translation about 2.6- and 1.3-fold for unfractonated and depleted wheat germ lysate, respectively. Vpg inhibited translation of capped TEV RNA by 1.8- and 1.3-fold for unfractonated and depleted wheat germ lysate, respectivly. Vpg71 had no effect on translation of uncapped or capped TEV RNA in unfractonated and depleted wheat germ extract. Cotton et al. (46) have previously reported that the VpgPro inhibits the translation of capped RNA in wheat germ extract and rabbit reticulocyte lysate. Our results are consistent with the inhibition of capped RNA and in addition show that Vpg enhances the translation of uncapped TEV RNA in wheat germ extract.

Fig. 3A shows that the addition of eIF4F to depleted lysate increases translation for uncapped TEV RNA by 5.4-fold, whereas a 1.6-fold increase was observed when eIFiso4F was added. Additional eIF4F/iso4F above 10 nM did not significantly increase translation (data not shown). A 13.5-fold increase in translation of uncapped TEV was observed in the presence of eIF4F and Vpg together. However, only a 2.4-fold increase was observed for eIFiso4F with Vpg. This small stimulation may be partially due to residual eIF4F in the depleted lysate. The data suggest that Vpg considerably enhances the translation of uncapped TEV RNA in the presence of eIF4F as compared with eIFiso4F. The data also suggested that eIF4F and eIFiso4F differ in the extent of supporting translation as was previously shown by Gallie and Browning (23). Fig. 3B shows the addition of Vpg inhibits translation of capped TEV RNA. The addition of 50 nM eIF4F to depleted wheat germ lysate was able to overcome the Vpg inhibition of capped RNA translation. The addition of 50 nM eIFiso4F showed restoration of capped TEV RNA translation as well. These data show that an excess amount of eIF4F and eIFiso4F can overcome the inhibition of translation of capped TEV RNA by Vpg. This is consistent with Vpg acting as a cap analog to inhibit eIFiso4F and eIF4F cap binding. We would, therefore, expect higher levels of eIF4F/iso4F to be necessary to overcome Vpg inhibition compared with the levels required for optimum translation since some eIF4F/iso4F would be bound to Vpg.
Interaction of VPg with eIF4F and eIFiso4F

Steady State Fluorescence Measurements of the Binding of eIF4F and eIFiso4F with VPg—We have previously shown (4) that eIFiso4F interacts with VPg and that TEV1–143 RNA stabilizes this reaction. Furthermore, eIFiso4F interaction with VPg was kinetically favored over the interaction of eIFiso4F with the m7G cap. However, TEV RNA preferentially interacts with eIF4F over eIFiso4F, and this interaction correlates with translation efficiency (26). It was, therefore, of interest to determine the extent to which VPg could interact with eIF4F and how this interaction affected the affinity of the protein complex for TEV RNA. To examine the binding affinity for the potyviral VPg, wheat germ translation initiation factors eIF4F and eIFiso4F (100 nM each) were titrated with varying concentrations of VPg in the absence and presence of TEV1–143 RNA (100 nM). Fig. 4A shows a representative binding plot \( \Delta F/\Delta F_{\text{max}} \) versus VPg concentration for determination of the equilibrium dissociation constant. The inset of Fig. 4A shows the double reciprocal plots of 1/\( \Delta F \) versus 1/[VPg], extrapolated to the ordinate from which the value of \( \Delta F_{\text{max}} \) was determined. The dissociation constant of eIF4F as compared with eIFiso4F with VPg shows ~2.7-fold higher binding affinity for eIFiso4F with VPg (Table 1). The binding stoichiometry of eIF4F and eIFiso4F with VPg remained one to one as shown in our previous results (4). We further examined the interaction of VPg/VPg71 with eIF4E and eIF4G (subunits of eIF4F) because VPg interacts with eIF4F and eIFiso4F. Fig. 4B shows the binding isotherm of eIF4E and eIF4G with VPg/VPg71. eIF4E shows 7.5-fold higher binding affinity as compared with eIF4G with VPg. VPg showed about 2.7-fold higher binding affinity for eIFiso4E (4) than eIF4E (Table 1). VPg has previously been shown not to bind to eIFiso4G. However, no fluorescence increase was observed for VPg71 with eIF4E and eIFiso4E as expected.

Fig. 5A shows the effect of uncapped TEV1–143 RNA on the binding of eIF4F with VPg. The addition of 100 nM TEV1–143 RNA enhanced the binding of eIF4F + VPg significantly. The binding affinity of eIF4F + VPg increased about 4.1-fold in the presence of TEV1–143 RNA, and the binding affinity of eIF4G-TEV + VPg was 42-fold lower than eIF4F-TEV + VPg (Table 1). The binding constants for eIF4F and TEV RNA were the same for wild type and recombinant eIF4F (data not shown.). The higher binding affinity of eIF4F compared with eIF4G is consistent with the previous data showing VPg interacts with the eIF4E subunit of eIF4F. Similarly, the binding affinity of eIFiso4F with VPg in the presence of TEV1–143 RNA is shown in Fig. 5B. In the presence of 100 nM TEV the binding affinity of eIFiso4F + VPg increased about 1.6-fold (Table 1), whereas no significant increase was observed for eIFiso4G. These data suggest cooperative binding between VPg and TEV for sites on eIF4F or eIFiso4F. Control experiments were run for eIF4F/eIFiso4F with truncated VPg71 (see Fig. 5, A and B), and no change in TEV binding was observed.

To further characterize the binding interaction, the interaction between either eIF4F or eIFiso4F and VPg was measured in the presence of different TEV concentrations. Fluorescence data with different concentrations of TEV RNA were represented as Lineweaver-Burk plots (inset of Fig. 5, A and B). Lineweaver-Burk plots that yield parallel lines, as shown, are indicative of noncompetitive or uncompetitive binding between VPg and TEV RNA.

Stopped-flow Kinetics for the Binding of eIF4F and eIFiso4F with VPg—Fig. 6A shows the stopped-flow fluorescence change for the binding of eIF4F and eIFiso4F with VPg. Fluorescence intensity versus time data were fitted by nonlinear regression analysis (40) as a single- and double-exponential as described under “Experimental Procedures.” The stopped-flow kinetic data were fit with a single-exponential function at 25 °C, and the binding of eIFiso4F showed about 2.6-fold higher rate constant than eIF4F for binding to VPg (Table S-1) (Fig. 6A). Treatment of the data using a double-exponential function did not...
Interaction of VPG with eIF4F and eIFiso4F

TABLE 1
Equilibrium dissociation constants (Kd) for the interactions of eIF4F and eIFiso4F with VPG and TEV1–143 RNA

| Complex                        | Kd  | Kd* |
|--------------------------------|-----|-----|
| eIF4F + VPG                    | 314 | ND  |
| eIF4G + VPG                    | 2350| ND  |
| eIF4F + VPG                    | 219 | K1  |
| eIF4F + TEV1–143               | 79.0| K2  |
| eIF4F + VPg + TEV1–143         | 55.0| K3  |
| eIFiso4F + VPG                 | 19.4| K4  |
| eIFiso4F + TEV1–143           | 81.3| K5  |
| eIFiso4F–TEV1–143 + VPg        | 178.0| K6  |
| eIFiso4F–VPG + TEV1–143        | 49.3| K7  |
| eIFiso4F–VPG                  | 108 | K8  |

* See Kd as designated in Fig. 9.
* Values were obtained from S. Ray et al. (26).
* Calculated from the thermodynamic cycle.
* Values were obtained from M.A. Khan et al. (4).

The data were obtained using equilibrium dialysis procedures (4) and 1D NMR. The dissociation constants were obtained at 25 °C. Titration of TEV1–143 with VPG showed no interaction. ND, values were not determined by the thermodynamic cycle.

Under pseudo-first order conditions, where VPg was in excess, the observed rate was predicted to be a linear function of the VPg concentration. However, as observed previously (4), the binding rates show little dependence on VPg concentration over a concentration range of 1–5 μM, which constitutes a 4–20-fold excess (Fig. 6). Stopped-flow experiments were conducted using a high concentration of VPg and limiting concentrations of eIF4F and eIFiso4F to ensure that the bimolecular combination of VPg with eIF4F was pseudo-first order. As we proposed previously (4), the binding mechanisms can be explained by a one- and two-step process. The one-step process is

$$k_1$$

$$eIF4F + VPg \rightleftharpoons eIF4F-VPg$$

**REACTION 1**

where $$k_1$$ and $$k_1^{-1}$$ are forward and reverse rate constants, respectively. Under the pseudo-first order condition, the observed rate is predicted to be a linear function of substrate concentration, i.e. $$k_{obs} = k_1 [C] + k_1^{-1}$$. The two-step process involves a fast association of eIF4F/eIFiso4F and VPg followed by a slow change in conformation from the first association complex (eIF-VPG)* to the stable complex (eIF-VPG) as shown.

$$k_1$$

$$eIF4F + VPg \rightleftharpoons (eIF4F-VPG)* \rightleftharpoons eIF4F-VPg$$

**REACTION 2**

The binding rates are related to the concentration of substrate as described previously (40), $$1/k_{obs} = 1/k_2 + k_1/k_2[C]$$, where $$k_{obs}$$ is the observed first-order rate constant, $$k_2$$ is the forward rate constant for the second step, $$k_1$$ is the association equilibrium constant for the first step, and [C] is the concentration of VPg. Fig. 6B is a plot of $$1/k_{obs}$$ versus $$1/[C]$$ for eIF4F and eIFiso4F with VPg, which shows the predicted linear relationship. From the intercept of $$1/k_2$$, $$k_2$$ was found to be 36.4 ± 1.9 and 86.8 ± 2.7 s−1 for eIF4F and eIFiso4F, respectively.

The value of the rate constant ($$k_2$$) for VPg binding to eIF4F and eIFiso4F was calculated from the data sets collected at different temperatures (supplemental Table S1) and used to construct Arrhenius plots (Fig. 7). The activation energies were calculated from the fitted slopes of ln $$k$$ versus 1/T according to Equation 4. The activation energies for VPg binding to eIF4F and eIFiso4F

![FIGURE 5. Binding plots for the interaction of initiation factors with VPg in the absence and presence of TEV RNA. Panel A, the change in fluorescence data are indicated as eIF4F + VPg ( ), eIF4F–TEV + VPg ( ), eIF4G–TEV + VPg ( ), and eIF4F–TEV + VPg71 ( ). Panel B, eIFiso4F + VPg ( ), eIFiso4F–TEV + VPg ( ), eIFiso4G–TEV + VPg ( ), and eIFiso4–TEV + VPg71 ( ). Initiation factor and RNA concentrations were 0.1 μM. The curves were fit to obtain dissociation constants ($$K_d$$) as described under “Experimental Procedures.” The inset shows the Lineweaver-Burk plot for competition of TEV RNA and VPg with eIF4F ( ), eIF4F–TEV ( ), 0.1 μM TEV ( ), and 0.5 μM TEV ( ). Panel B, eIFiso4F + VPg ( ), eIFiso4F–TEV + VPg ( ), eIFiso4G–TEV + VPg ( ), and eIFiso4–TEV + VPg71 ( ). Initiation factor and RNA concentrations were 0.1 μM. The curves were fit to obtain dissociation constants ($$K_d$$) as described under “Experimental Procedures.” The inset shows the Lineweaver-Burk plot for competition of TEV RNA and VPg with eIFiso4F ( ), 0.0 μM TEV ( ), 0.1 μM TEV ( ), 0.5 μM TEV ( ). The experimental conditions were the same as described for Fig. 4.](image-url)
The activation energy is related to the enthalpy of reaction, suggesting hydrogen bonds or ionic interactions rather than hydrophobic forces, which are usually entropically favorable, are involved.

Fig. 8 shows the dissociation reactions as a result of dilution as described under “Experimental Procedures.” The dissociation rate constants were obtained from the fitted curves. Equilibrium calculations showed that less than 4% of the proteins remained as a complex after dilution so that the reverse reaction could be neglected. Data fitting using a relaxation expression did not improve the quality of fitting. elf4F shows a 3-fold higher dissociation rate from VPg as compared with elfiso4F-VPg (supplemental Table S1). These results show that elfiso4F binds more rapidly and dissociates more slowly with VPg than elf4F, suggesting that VPg competes with cap more effectively for elfiso4F than elf4F.

DISCUSSION

In this study we have shown that VPg stimulates the in vitro translation of uncapped IRES-containing RNA and inhibits capped RNA translation in wheat germ extract. These effects appear to depend on VPg-elf4F or VPg-elfiso4E interactions. Roudet-Tavert et al. (47) showed that the central domain of potyvirus VPg was involved in the interaction with translation initiation factor elf4E. Moreover, Leonard et al. (41) showed that alteration of Asp-77 of VPg abolished the interaction with elfiso4F and the infectivity of the virus. Similarly, truncation of the N-terminal 61 amino acid residues of VPg renders the protein unable to interact with elfiso4E (41). We have shown that VPg71, a variant with 70 amino acids truncated, was unable to bind with either elf4E or elfiso4E. VPg71 did not show any effect on translation of either capped or uncapped RNA. Furthermore, an uncapped RNA with a non-functional IRES was not translationally stimulated. Experiments with elf4F/elfiso4F depleted lysates showed that translation of both the capped and uncapped TEV \(^{1–143}\) RNA depended more on elf4F compared with elfiso4F, consistent with earlier work (23) showing that mRNA with significant secondary structure was more dependent on elf4F and that uncapped TEV \(^{1–143}\) RNA translation depended on elf4G and not elfiso4G (25, 26). The addition of either elf4F or elfiso4F to the VPg inhibited translation of capped TEV \(^{1–143}\) RNA was able to restore translation to levels measured in the absence of VPg. These data suggest VPg competes for the cap-binding site for both proteins. Binding studies (4) have previously shown that VPg and cap bind competitively to elfiso4E. To further understand these interac-

were \(41 \pm 3\) and \(80 \pm 3\) kJ mol\(^{-1}\) respectively. The overall lower activation energy for VPg binding to elf4F suggests a number of more favorable hydrogen bonds are formed in the transition state.

FIGURE 6. Stopped-flow fluorescence changes for the interaction of elf4F and elfiso4F with VPg. A, the curve represents a single exponential fit to the fluorescence data points. Residuals for the fits are shown in the lower panels. A solution of 0.5 \(\mu\)M (0.25 \(\mu\)M after mixing) elf4F or elfiso4F was mixed with 2 \(\mu\)M (1 \(\mu\)M after mixing) VPg. The experimental conditions are described under “Experimental Procedures.” B, plots of \(1/k_{obs}\) versus \(1/[C]\) for the interaction of elf4F and elfiso4F with VPg. Shown are elf4F (○) and elfiso4F (□) (0.25 \(\mu\)M each) with varying concentrations of VPg (1, 2, and 5 \(\mu\)M). The rate constant \(k_2\) was obtained as the reciprocal of the y intercept. See “Experimental Procedures” for details.

FIGURE 7. Arrhenius plots for determining the activation energy of the interaction of elf4F and elfiso4F with VPg. The rate constant values for elf4F (○) and elfiso4F (□) with VPg at different temperatures \(5, 15, \) and \(25^\circ C\) were used to construct an Arrhenius plot according to Equation 4. The activation energy was calculated from the slope of the fitted linear plot of \(\ln k\) versus \(1/T\) (kelvin).

The activation energy is related to the enthalpy of reaction, suggesting hydrogen bonds or ionic interactions rather than hydrophobic forces, which are usually entropically favor-
Interaction of VPg with eIF4F and eIFiso4F

FIGURE 8. Kinetics of the VPg dissociation from eIF4F-VPg and eIFiso4F-VPg complexes. Off rates for eIF4F-VPg (○) and eIFiso4F-VPg (△) complexes were monitored by rapidly diluting 100 μl of the complex with 1500 μl of titration buffer. The concentrations of reactants before mixing were 2 μM VPg and 0.5 μM eIF4F or eIFiso4F. The temperature was 22°C.

FIGURE 9. Schematic diagram of the eIF4F/eIFiso4F, VPg, and TEV interactions. The model shows the two pathways K1K2 and K3K4 for the formation of eIF4F-VPg-TEV complex. The same interactions can be written for eIFiso4F.

is sufficient to direct cap-independent translation in vitro (7). In a virally infected cell, both host cell mRNA, largely capped, and IRES-containing uncapped viral mRNA are present. VPg is present covalently attached to viral RNA but is rapidly cleaved and is presumably present in the cell. Furthermore, because the potyvirus gene products arise from cleavage of a single polyprotein, they are presumed to be present in equimolar amounts (29). Formation of viral particles requires a large number of copies of a single capsid protein and only one VPg, suggesting the presence of excess VPg at least during the encapsidation stage.

Viral RNA, which is generally translated less efficiently than capped RNA, must compete for available cell components for translation. In the presence of both capped and uncapped mRNA, VPg will compete for cap binding with both eIF4F and eIFiso4F. Many host cell mRNA are more dependent on eIFiso4F for translation because they do not have extensive secondary structure (23), and eIFiso4F is thought to be more abundant (48). Formation of an eIFiso4F-VPg complex would lead to a non-productive complex that reduces host cell translation. In contrast, an eIF4F-VPg complex would also lead to inhibition of capped mRNA translation, but in this case the complex will bind more tightly to IRES-containing mRNA and lead to production of viral proteins. We have previously shown (26) that eIF4F binding to TEV correlated well with translational efficiency. These data demonstrate a dual role for VPg: competition with eIFiso4F and eIF4F for cap, and direct enhancement of TEV-mediated cap-independent translation through interaction with eIF4F. These complementary functions provide a significant competitive advantage for viral RNA.

REFERENCES

1. Goodfellow, I., Chaudhry, Y., Gioldasi, L., Gerondopoulos, A., Natoni, A., Labrie, L., Laliberte, J. F., and Roberts, L. (2005) EMBO Rep. 6, 968–972
2. Daughenbaugh, K. F., Fraser, C. S., Hershey, J. W., and Hardy, M. E. (2003) EMBO J. 22, 2852–2859
3. Wittmann, S., Chatel, H., Fortin, M. G., and Laliberte, J. F. (1997) Virology 234, 84–92
4. Khan, M. A., Miyoshi, H., Ray, S., Natsukai, T., Suehiro, N., and Goss, D. J. (2006) J. Biol. Chem. 281, 28002–28010
5. Oruetxebarria, L., Guo, D., Merits, A., Makinen, K., Saarma, M., and Valkonen, J. P. (2001) Virus Res. 73, 103–112
6. Harris, K. S., Xiang, W., Alexander, L., Lane, W. S., Paul, A. V., and Wimmer, E. (1994) J. Biol. Chem. 269, 27004–27014
7. Niepel, M., and Gallie, D. (1999) J. Virol. 73, 9080–9088
8. Murphy, J. F., Klein, P. G., Hunt, A. G., and Shaw, J. G. (1996) Virology 220, 535–538
9. Dunoyer, P., Thomas, C., Harrison, S., Revers, F., and Maule, A. (2004) J. Virol. 78, 2301–2309
10. Nicolas, O., Dunnington, S. W., Gotow, L. F., Pirone, T. P., and Hellmann, G. M. (1997) Virology 237, 452–459
11. Rajamaki, M. L., and Valkonen, J. P. (1999) Mol. Plant-Microbe Interact. 12, 1074–1081
12. Rajamaki, M. L., and Valkonen, J. P. (2002) Mol. Plant-Microbe Interact. 15, 138–149
13. Schaad, M. C., and Carrington, J. C. (1996) J. Virol. 70, 2556–2561
14. Schaad, M. C., Lellis, A. D., and Carrington, J. C. (1997) J. Virol. 71, 8624–8631
15. Keller, K. E., Johansen, I. E., Martin, R. R., and Hampton, R. O. (1998) Mol. Plant-Microbe Interact. 11, 124–130
16. Browning, K. S., Webster, C., Roberts, J. K., and Ravel, J. M. (1992) J. Biol. Chem. 267, 10096–10100
17. Allen, M. L., Metz, A. M., Timmer, R. T., Rhoads, R. E., and Browning, K. S. (1992) J. Biol. Chem. 267, 23232–23236
Interaction of VPg with eIF4F and eIFiso4F

Metz, A. M., Timmer, R. T., and Browning, K. S. (1992) *Nucleic Acids Res.* 20, 4096

Abramson, R. D., Browning, K. S., Dever, T. E., Lawson, T. G., Thach, R. E., Ravel, J. M., and Merrick, W. C. (1988) *J. Biol. Chem.* 263, 5462–5467

Lax, S. R., Browning, K. S., Maia, D. M., and Ravel, J. M. (1986) *J. Biol. Chem.* 261, 15632–15636

Browning, K. S., Lax, S. R., and Ravel, J. M. (1987) *J. Biol. Chem.* 262, 11228–11232

Lax, S., Fritz, W., Browning, K., and Ravel, J. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 330–333

Gallie, D. R., and Browning, K. S. (2001) *J. Biol. Chem.* 276, 36951–36960

Carberry, S. E., and Goss, D. J. (1991) *Biochemistry* 30, 4542–4545

Gallie, D. R. (2001) *J. Virol.* 75, 12141–12152

Ray, S., Yumak, H., Domashevskiy, A., Khan, M. A., Gallie, D. R., and Goss, D. J. (2006) *Biochemistry* 43, 9092–9097

Robaglia, C., and Caranta, C. (2006) *Trends Plant Sci.* 11, 40–45

Herbert, T. P., Brierley, L., and Brown, T. D. (1997) *J. Gen. Virol.* 78, 1033–1040

Riechmann, J. L., Lain, S., and Garcia, J. A. (1992) *J. Gen. Virol.* 73, 1–16

Miyoshi, H., Suehiro, N., Tomoo, K., Muto, S., Takahashi, T., Tsukamoto, T., Ohmori, T., and Natsuki, T. (2006) *Biochimie (Paris)* 88, 329–340

Suehiro, N., Natsuki, T., Watanabe, T., and Okuda, S. (2004) *J. Gen. Virol.* 85, 2087–2098

Lax, S. R., Lauer, S. J., Browning, K. S., and Ravel, J. M. (1986) *Methods Enzymol.* 118, 109–128

van Heerden, A., and Browning, K. S. (1994) *J. Biol. Chem.* 269, 17454–17457

Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254

Zeenko, V., and Gallie, D. R. (2005) *J. Biol. Chem.* 280, 26813–26824

Anderson, C. W., Straus, J. W., and Dudock, B. S. (1983) *Methods Enzymol.* 101, 635–644

Lakowicz, J. R. (1999) *Principles of Fluorescence Spectroscopy*, 2nd Ed., p. 54, Plenum Publishing Corp., New York

Firpo, M. A., Connelly, M. B., Goss, D. J., and Dahlberg, A. E. (1996) *J. Biol. Chem.* 271, 4693–4698

Khan, M. A., and Goss, D. J. (2004) *Biochemistry* 43, 9092–9097

Olsen, K., Christensen, U., Sierks, M. R., and Svensson, B. (1993) *Biochemistry* 32, 9686–9693

Leonard, S., Plante, D., Wittmann, S., Daigneault, N., Fortin, M. G., and Laliberte, J. F. (2000) *J. Virol.* 74, 7730–7737

Carrington, J. C., and Freed, D. D. (1990) *J. Virol.* 64, 1590–1597

Gallie, D., Tanguay, R., and Leathers, V. (1995) *Gene (Amst.)* 165, 233–238

Dougherty, W., and Carrington, J. (1988) *Annu. Rev. Phytopathol.* 26, 123–143

Carrington, J. C., Haldeman, R., Dolja, V. V., and Restrepo-Hartwig, M. A. (1993) *J. Virol.* 67, 6995–7000

Cotton, S., Dufresne, P. J., Thivierge, K., Ide, C., and Fortin, M. G. (2006) *Virology* 351, 92–100

Roudert-Tavert, G., Michon, T., Walter, J., Delaunay, T., Redondo, E., and Le Gall, O. (2007) *J. Gen. Virol.* 88, 1029–1033

Browning, K. S., Humphreys, J., Hobbs, W., Smith, G. B., and Ravel, J. M. (1990) *J. Biol. Chem.* 265, 17967–17973