Eukaryotic translation initiation factor 4G (eIF4G) coordinates interactions with eIF4A, eIF4B, and eIF4E in binding and translation of the barley yellow dwarf virus 3’ cap-independent translation element (BTE) *

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Barley yellow dwarf virus RNA, lacking a 5’ cap and a 3’ poly(A) tail, contains a cap-independent translation element (BTE) in the 3’-untranslated region that interacts with host translation initiation factor eIF4G. To determine how eIF4G recruits the mRNA, three eIF4G deletion mutants were constructed: (i) eIF4G601–1196, containing amino acids 601–1196, including the putative BTE-binding region, and binding domains for eIF4E, eIF4A, and eIF4B; (ii) eIF4G601–1488, which contains an additional C-terminal eIF4A-binding domain; and (iii) eIF4G742–1196, which lacks the eIF4E-binding site. eIF4G601–1196 binds BTE tightly and supports efficient translation. The helicase complex, consisting of eIF4A, eIF4B, and ATP, stimulated BTE binding with eIF4G601–1196 but not eIF4G601–1488, suggesting that the eIF4A binding domains may serve a regulatory role, with the C-terminal binding site having negative effects. eIF4E binding to eIF4G601–1196 induced a conformational change, significantly increasing the binding affinity to BTE. A comparison of the binding of eIF4G deletion mutants with BTEs containing mutations showed a general correlation between binding affinity and ability to facilitate translation. In summary, these results reveal a main; and (iii) eIF4G742–1196, which lacks the eIF4E-binding site; (ii) eIF4G601–1488, but not eIF4G601–1488, suggesting that the eIF4A binding domains may serve a regulatory role, with the C-terminal binding site having negative effects. eIF4E binding to eIF4G601–1196 induced a conformational change, significantly increasing the binding affinity to BTE. A comparison of the binding of eIF4G deletion mutants with BTEs containing mutations showed a general correlation between binding affinity and ability to facilitate translation. In summary, these results reveal a new role for the helicase complex in 3’ cap-independent translation element-mediated translation and show that the functional core domain of eIF4G plus an adjacent probable RNA-binding domain mediate translation initiation.

Cap recognition is the rate-limiting step for canonical eukaryotic translation (1), in which the mRNA 5’terminal 7-methylguanosine cap structure (m7GpppN) of cellular mRNAs is recognized and bound by cap-binding protein eukaryotic translation initiation factor 4E (eIF4E) 2(2, 3). eIF4E is one subunit of eIF4F. In plants, eIF4F exists as a heterodimer composed of eIF4E and eukaryotic translation initiation factor 4G (eIF4G) (4). eIF4G serves as a scaffolding protein to recruit other translation initiation factors such as eIF4A, eIF4B, and poly(A)-binding protein (PABP) (5, 6). The poly(A) tail on cellular mRNA is recognized by PABP (7, 8), which simultaneously binds eIF4G. In this way, the eIF4F/eIF4G directed initiation factor complex circularizes the cellular mRNA linking its 5’ and 3’ ends (9, 10). This “closed loop” conformation of cellular mRNA is assumed as the efficient translation initiation model for canonical cap-dependent translation (11, 12).

Many viral RNAs lack both the 5’ cap structure and the poly(A) tail, utilizing different mechanisms to recruit ribosomes efficiently (13). To compensate for the absence of a 5’ cap, many plant viruses contain a cap-independent translation enhancer element, usually located in the 3’-UTR of the viral mRNA (14, 15). One of the well characterized 3’ cap-independent translation elements is the barley yellow dwarf virus (BYDV)-like cap-independent translation element (BTE) (16). A BTE is present in the 3’-UTRs of all members of the Luteovirus, Dianthovirus, Alphanecrovirus, and Betanecrovirus genera and in some members of genus Umbraviruses (18).

The 5677-nucleotide (nt), positive sense RNA genome of BYDV has no 5’ cap or poly(A) tail. Its 3’ BTE (nt 4890–4918) is required for efficient translation and thus for viral replication (16). The BYDV BTE forms a cruciform secondary structure with three major stem loops (SL-I, SL-II, and SL-III) and flanked by stem IV, which connects to the rest of the viral RNA (see Fig. 1A). Each structured stem loop is necessary for cap-independent translation (16, 19), but the necroviruses lack SL-II, and the dianthoviruses contain two extra stem loops (20, 21). All the BTEs have a 17-nt conserved sequence GGAUC-CUGGAAAACAGG that includes SL-I (underlined bases are paired) (22). The loop of SL-III base pairs to a loop in the 5’-UTR to form a long distance “kissing” stem loop interaction (see Fig. 1A) (23). eIFs and 40S ribosomal subunits recruited by the 3’ BTE may

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be delivered to the 5′-UTR by this long distance interaction (24, 25). The 3′ BTE specifically interacts with eIF4G or eIF4F in wheat germ extract (WGE), and BTE-mediated translation is primarily eIF4F-dependent (21, 26, 27). eIF4G binds to the BTE at SL-I and the proximal end of SL-III (see Fig. 1A) (21).

Wheat expresses two eIF4G isoforms: eIF4G and eIFiso4G, which are different in sequence and size (28, 29). BYDV prefers to utilize eIF4G for the viral mRNA translation (26). Wheat eIF4G is a 160-kDa protein with 1488 amino acid residues (28, 29). eIF4G contains one binding site for eIF4E (residues 710–721) and two binding regions for eIF4A, eIF4B, and PAPB (see Fig. 1B). The first eIF4B or PAPB binding site is near the N terminus followed by the eIF4E binding site in the central region and two HEAT domains located in the C-terminal region, which overlap with two eIF4A binding sites (29). Compared with human eIF4G, wheat eIF4G does not have the third HEAT domain (30, 31). The region between amino acid residues 766 and 836 of eIF4G is required for binding to BTE and facilitating BTE-mediated translation (21).

Other translation initiation factors also participate in the interaction between eIF4G, 40S ribosomal subunits, and BTE. Although eIF4E, the cap-binding protein, does not interact directly with the BTE, it stimulates eIF4G-dependent BTE-mediated translation (26). Unlike human eIF4A, which is a subunit of eIF4F, plant eIF4A does not co-purify with the eIF4G-eIF4E complex and thus is not considered part of eIF4F (32). eIF4A with eIF4B and ATP acts as a helicase utilizing energy from ATP hydrolysis to unwind double-stranded RNAs (4, 33, 34). Addition of the helicase complex (eIF4F-eIF4A-eIF4B-ATP) significantly increases the binding affinity between the BTE and the 40S ribosomal subunit (25).

The functions of different eIF4G domains in the interaction with BTE are still unknown, as is the influence of other translation initiation factors on this binding. To answer these questions, we investigate the translational activities and binding affinities of the following eIF4G deletion mutants, with numbering indicating positions of amino acids indicated in the protein: eIF4G601–1196, eIF4G742–1196, and eIF4G601–1488. Because of the instability of full-length eIF4G, previous studies have used the C-terminal two-thirds of eIF4G (eIF4G587–1488 and eIF4G601–1488), which facilitate BTE-mediated translation as efficiently as full-length eIF4G (21, 26, 29). Here we find that eIF4G601–1196 (containing eIF4G amino acid residues 601–1196) acts as a core domain both in translation and in binding to BTE. eIF4A, eIF4B, and eIF4E, to different extents, stimulated the eIF4G-dependent BTE-mediated translation. The helicase complex eIF4A-eIF4B-ATP and eIF4E also contribute to the tight binding between eIF4G and BTE, mainly through protein-protein interactions, whereas PABP had little effect on binding.

Results

The central domain of eIF4G is sufficient for BTE-mediated translation

Previous studies showed that the C-terminal eIF4G fragment (eIF4G766–1488) functioned as well as full-length eIF4G in translation assays and could rescue BTE-mediated translation in eIF4F-depleted wheat germ extract (21, 26). In this study, the translation stimulation efficiencies of three eIF4G deletion mutants were determined. eIF4G601–1196 is the eIF4G fragment containing amino acid residues 601–1196, including the eIF4E binding site (aa 710–721), one eIF4A binding site, one eIF4B binding site, and a region required for BTE binding (aa 766–863). eIF4G601–1488 is a longer fragment with one additional C-terminal eIF4A binding domain (aa 1300–1488); eIF4G742–1196 is a shorter deletion mutant lacking the eIF4E binding sequence, as well as the second eIF4A site (Fig. 1B).

BlucB is a translation reporter construct in which the firefly luciferase reporter gene is flanked by the BYDV 5′- and 3′-UTRs (16, 21, 26). The 3′-UTR contains the BTE. eIF4F-depleted wheat germ extract was used as the in vitro translation system (28). Western blot assays showed that both eIF4G and eIFiso4G were reduced to undetectable levels in the eIF4F-depleted wheat germ extract; eIF4E and eIFiso4E were reduced to a slightly smaller extent than eIF4G and eIFiso4G (Fig. 2A). eIF4A and eIF4B levels were also partially reduced in eIF4F-depleted wheat germ extracts because they bind eIF4F (Fig. 2A). As expected, BlucB showed significantly reduced expression in
Figure 2. Function of eIF4G mutants in BTE-mediated translation. 

A, Western blot analysis of eIF4G, elFiso4G, eIF4E, eIFiso4E, eIF4A, and eIF4B in wheat germ extract before (left) and after (right) depletion on m7G-Sepharose. Equal amounts of total protein (8 μg) were loaded in each lane.

B, eIF4G deletion mutants rescue BlucB translation in eIF4F-depleted wheat germ extract. The left lane represents translation of 20 nM BlucB RNA in 25 μl of non-depleted wheat germ extract. 60 nM each of eIF4F, eIF4G601–1196, eIF4G601–1488, or eIF4G742–1196 were tested separately in 25 μl of depleted wheat germ extract for translation of 20 nM BlucB mRNA. The reactions were incubated at 25 °C for 90 min. The relative luciferase intensity units (RLU) were obtained to represent translation activity. The luciferase activity obtained after translation in the presence of 60 nM eIF4F of 20 nM BlucB during the 90-min reaction was set as 100% and used to normalize other activities.

C, levels of translation rescue obtained from eIF4F and eIF4G mutants (eIF4G601–1196, eIF4G601–1488, and eIF4G742–1196) after translation of 20 nM BlucB in eIF4F-depleted wheat germ extract for the indicated times. The luciferase activity generated by translation of 20 nM BlucB mRNA after 40 min in the presence of 60 nM added eIF4F was defined as 100%.

D, effect of eIF4A and eIF4B on BTE-mediated translation. 1.8 μM eIF4A and 600 nM eIF4B were preincubated with 60 nM eIF4F or eIF4G mutants to form the protein complex and then added to eIF4F-depleted WGE. The in vitro translation experiments were performed as described under “Experimental procedures.” The data were normalized to translation in the presence of eIF4F after 40 min. E, effect of eIF4E on BTE-mediated translation. 60 nM eIF4F, eIF4G mutant, or eIF4G mutant with eIF4E complex were added into 25 μl of eIF4F-depleted wheat germ extract for translation of 20 nM BlucB mRNA in 40 min. The in vitro translation experiments were performed as described under “Experimental procedures.” The fluorescence intensity readings were obtained at different times as indicated.
**BTE interaction with eIF4G**

The addition of recombinant eIF4F partially rescued translation of uncapped BlucB RNA in the eIF4F-depleted wheat germ extract (26). The luciferase activity obtained after translation of 20 nM BlucB RNA for 90 min in the presence of 60 nM eIF4F added to the eIF4F-depleted wheat germ extract was defined as 100%. Additional recombinant eIF4F did not increase the translation rescue. After 90 min of translation, 60 nM recombinant eIF4F rescued BlucB translation to ~27% of the level in non-depleted WGE. Because eIF4A and eIF4B are also partially depleted when eIF4F is reduced, varying amounts of additional eIF4A and eIF4B were added to the lysate. Optimum translation occurred with the addition of eIF4A (1.8 μM) and eIF4B (600 nM) with eIF4F, which restored BlucB translation to 79% of the non-depleted WGE levels. 60 nM eIF4G601–1196 restored BlucB translation to 66 ± 5% of the level obtained with eIF4F (Fig. 2B). eIF4G601–1488, which includes one additional eIF4A binding site in the C-terminal region, had similar translation stimulating ability, restoring BlucB translation to 72 ± 4% of the eIF4F level, whereas the shorter fragment, eIF4G742–1196, restored translation to 63 ± 10% (Fig. 2B). After a 40-min reaction, the BlucB translation rescued by eIF4G mutants was close to saturation (supplemental Fig. S1).

**Table 1**

<table>
<thead>
<tr>
<th>BTE-eIF4G mutants</th>
<th>+ 4A 4B ATP</th>
<th>+ 4A 4B ADPPNP</th>
<th>+ 4A 4B ATP + PABP</th>
<th>+ 4A 4B ADPPNP + PABP</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTE-eIF4G601–1196</td>
<td>40 ± 4</td>
<td>13 ± 3</td>
<td>29 ± 1</td>
<td>11 ± 4</td>
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<tr>
<td>BTE-eIF4G601–1196</td>
<td>6.8 ± 1.5</td>
<td>4.6 ± 0.5</td>
<td>9.0 ± 1.6</td>
<td>4.4 ± 1.8</td>
</tr>
<tr>
<td>BTE-eIF4G601–1196</td>
<td>91 ± 12</td>
<td>59 ± 5</td>
<td>76 ± 2</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>BTE-eIF4G742–1196</td>
<td>101 ± 2</td>
<td>64 ± 3</td>
<td>81 ± 4</td>
<td>61 ± 2</td>
</tr>
<tr>
<td>BTE-eIF4G601–1488</td>
<td>68 ± 5</td>
<td>58 ± 1</td>
<td>NA</td>
<td>54 ± 2</td>
</tr>
<tr>
<td>BTE-eIF4G601–1488</td>
<td>28 ± 6</td>
<td>20 ± 1</td>
<td>NA</td>
<td>19 ± 3</td>
</tr>
</tbody>
</table>

When eIF4A and eIF4B were added along with eIF4F or eIF4G mutants into the eIF4F-depleted WGE, BlucB translation was at least double the level obtained in the absence of eIF4A and eIF4B after 40 min (Fig. 2D). Additional eIF4A and eIF4B had stronger effects on translation in the presence of eIF4G601–1196 and eIF4G742–1196 than in the presence of eIF4G601–1488. Supplementation of additional eIF4A and eIF4B increased both eIF4G601–1196- and eIF4G742–1196-mediated translation of BlucB ~2.8 ± 0.05- and 3.1 ± 0.06-fold, respectively, at 40 min of *in vitro* translation, whereas for eIF4G601–1488 the increase was 2.6 ± 0.01-fold (Fig. 2D). At 60 min of translation, the difference of eIF4A and eIF4B effects was larger. With additional eIF4A and eIF4B, both eIF4G601-1196- and eIF4G742–1196-mediated BTE translation increased to ~3.5 ± 0.1- and 3.6 ± 0.08-fold, respectively, whereas for eIF4G601–1488 the increase was 2.7 ± 0.05-fold (supplemental Fig. S1).

**eIF4A and eIF4B stimulate eIF4G mutants in rescuing BTE-mediated translation and increase binding affinities of eIF4G mutants to the BTE.**

To investigate the effects of translation initiation factors eIF4A, eIF4B, and eIF4E on the interaction between eIF4G mutants and the BTE, the translation rescue abilities and the dissociation equilibrium constants were measured. Wheat eIF4A (45 kDa) exhibits ATPase hydrolysis activity and RNA helicase activity when combined with eIF4B and ATP. The helicase complex (eIF4A-eIF4B-ATP) in the presence of eIF4G can unwind double-stranded RNA (34).

**eIF4G601–1196 has stronger binding affinity than full-length eIF4G to the BTE**

Previously we correlated eIF4F binding affinity with translational efficiency (27). To determine whether the mutants had affinity differences, fluorescein-labeled BTE was titrated with each of the eIF4G mutants (protein fluorescence quenching titrations revealed that fluorophore-labeled BTE and non-labeled BTE have the same binding affinities to eIF4Gs). The anisotropy changes of labeled BTE during titration were used to determine the equilibrium dissociation constant of the RNA-protein binding. The equilibrium dissociation constant ($K_d$) for binding to BTE by eIF4G601–1196 (40 ± 4 nM) (Table 1 and Fig. 3) was less (tighter binding) than the $K_d$ of BTE binding by full-length eIF4G (177 ± 10 nM, determined by filter binding assay) (21) or by eIF4G601–1488 ($K_d = 68 ± 5$ nM), eIF4G742–1196, which lacks the eIF4E binding region, had a weaker binding affinity to the BTE ($K_d = 91 ± 12$ nM) compared with other two eIF4G deletion mutants (Table 1 and Fig. 3). These results demonstrate that the N- and C-terminal regions of eIF4G are not required for binding to BTE. Unlike the small differences in translation efficiency mediated by the three eIF4G mutants, the binding affinities of the three eIF4G mutants to the BTE are robust but quite different.
eIF4A binding domain but lacks the eIF4E binding region (Table 1). Like the translation data, eIF4A and eIF4B had less influence on the eIF4G mutant with two eIF4A binding domains than the mutants with one eIF4A binding domain. eIF4G601–1488, when incubated with helicase complex, did not show a similar increase in binding affinity, suggesting that the second eIF4A binding site on eIF4G counteracted the helicase complex effect on the interaction between the first eIF4A binding domain of eIF4G and BTE (Table 1), whereas the addition of PAPB in the presence of eIF4A, eIF4B, and ATP showed no significant effect on BTE binding (Table 1).

Neither eIF4A nor eIF4B bound to the BTE in the absence of other proteins (Fig. 3). The helicase complex could affect RNA unwinding and/or protein-protein interactions. To establish whether the increase in binding was due to helicase activity or protein interaction, a non-hydrolyzable ATP analog was used in place of ATP. The equilibrium dissociation constant between eIF4G601–1196 and BTE decreased (29 \nM compared with 40 \nM) (Table 1), indicating that in addition to possible RNA unwinding, binding is affected by protein-protein interactions between eIF4A, eIF4B, and eIF4G.

**eIF4E changes the conformation of eIF4G deletion mutants and increases their binding affinity to the BTE**

eIF4E, the cap-binding protein, slightly increased eIF4G-dependent BlucB translation. Treder et al. (26) found that eIF4E increased ability of eIF4G to restore BlucB translation in depleted WGE by 20–30%. Here eIF4E increased the ability of two eIF4G deletion mutants, eIF4G610–1196 and eIF4G601–1488, to rescue BlucB translation by 22 \% (Fig. 2E). As expected, eIF4E did not enhance BlucB translation in the presence of eIF4G742–1196, which lacks the eIF4E binding site (Fig. 2E). Therefore, the eIF4E effect is specifically dependent on the eIF4E binding region on eIF4G.

In contrast to the relatively small influence on BTE-mediated translation, eIF4E had more obvious effects on the binding between eIF4G mutants and the BTE. eIF4E increased the binding between the BTE and eIF4G601–1196 almost 6-fold (Table 2). The dissociation constant of the protein complex eIF4G601–1196eIF4E binding to the BTE was 6.8 ± 1.5 nM, smaller than the K_D (29 ± 3 nM) of eIF4F binding to the BTE (27). A similar but smaller effect was observed when eIF4E was added to eIF4G601–1488 (K_D changed from 68 ± 5 to 28 ± 6 nM; Table 2). As expected, eIF4E did not enhance binding of eIF4G742–1196 to the BTE (Table 2).

The three eIF4G deletion mutants were further tested for binding to BTE mutants (Fig. 1A). These BTE mutants vary in translation efficiency and binding affinity to eIF4F (16, 27). Mutant BTEBF contains a GAUC insertion in the 17-nt conserved sequence. BTEBF abolishes translation but had similar binding affinity to eIF4F (26). Mutant SL-II-m1 has a mutation
that disrupts the secondary structure of stem loop II. Mutant SL-III-swap has a larger stem loop III derived from the BYDV-PAS virus (formerly known as PAV-129) (16) and reduces translation efficiency (16, 27). Mutant SL-II-m1 and SL-III-swap showed weaker binding than the BTE by eIF4F (Table 2). Mutant SL-III-3 has a mutation (GUC to CAG) within the loop of SL-III (Fig. 1A) that disrupts base pairing of SL-III to the 5′-UTR and thus reduces translation to background levels (24). However, when it was inserted in the 5′-UTR of a reporter gene, in which the long distance base pairing is not necessary, the BTE with SL-III-3 mutation increased translation efficiency (16, 27). Consistent with this, the SL-III-3 mutant had a tighter binding affinity than wild type BTE to eIF4G601–1196 and eIF4G601–1488 (Table 2). Adding eIF4E increased the binding of eIF4G601–1196 or eIF4G601–1488 to all BTE mutants with the exception of the SL-III-3 interaction with eIF4G601–1196. Again, as a control eIF4G601–1196 without the eIF4E binding site showed little or no increased affinity upon addition eIF4E for any of the BTE mutants (Table 2), further suggesting that eIF4E effects on eIF4G deletion mutants resulted from interactions between the two proteins and not interaction of eIF4E with the RNA. However, even in the absence of eIF4E, at least a portion of the region between aa 601 and 742 in eIF4G contributes to binding to the BTE: binding of eIF4G742–1196 to wild type and mutant BTEs in the absence of eIF4E was much weaker than was binding of eIF4G601–1196 in the absence of eIF4E (Table 2).

To examine the possible eIF4E-induced conformational changes in eIF4G mutants, CD measurements were used to examine the secondary structure of eIF4G601–1196 and eIF4E when they formed a protein complex. For CD spectra, 

\[ \Delta A = A_R - A_L = (\epsilon_R - \epsilon_L) c l = \Delta \epsilon c l \]  

(Eq. 1)

where \( A \) is absorbance; \( \epsilon \) is the molar extinction coefficient; the subscripts R and L refer to right and left circularly polarized light, respectively; \( c \) is the molar concentration; and \( l \) is the path-length. For a mixture, the observed absorbance, 

\[ \Delta A = \Delta \epsilon_1 c_1 l + \Delta \epsilon_2 c_2 l \]  

(Eq. 2)

where subscripts 1 and 2 refer to the two components. At a given wavelength, the sum of the \( \Delta A \) for eIF4E and eIF4G601–1196 should be equal to the \( \Delta A \) for the mixture if no interaction has occurred. As observed in Fig. 4, the eIF4G601–1196/eIF4E protein complex showed a reduced signal between 200 and 240 nm compared with the sum of spectra of these two individual proteins (Fig. 4A). The CD spectra showed that after eIF4G601–1196 interacted with eIF4E, the \( \alpha \)-helix content decreased 37 ± 1% (the \( \alpha \)-helix content calculated from the mean residue ellipticity of each protein spectrum at 222 nm). We also examined the effect the eIF4A and eIF4B on eIF4G601–1196 and eIF4G742–1196 conformation. When eIF4G601–1196 was incubated with eIF4A and eIF4B, there were only slight spectra changes (Fig. 4B).

**The binding of eIF4G601–1196 with BTE is both enthalpically and entropically favorable**

The equilibrium dissociation constants were measured at different temperatures (Table 3), and thermodynamic analysis was performed. Enthalpy and entropy were obtained from Van’t Hoff plots (Fig. 5). Free energy, \( \Delta G \), was calculated at 25 °C. The binding between BTE and eIF4G601–1196 was both entropically and enthalpically favorable (Table 4 and Fig. 5). Enthalpy contributed a large part of the \( \Delta G \) (81.9 ± 1.6%). Compared with eIF4G601–1196 alone, adding eIF4E increased the enthalpy contribution to 88.4 ± 1.8% and slightly reduced the entropic contribution. eIF4G601–1196 has a higher entropic contribution to \( \Delta G \) than the eIF4F interaction with BTE (53% enthalpic contribution) (27). In addition, eIF4G601–1196 binding with BTE is also more enthalpically favorable than eIF4 binding to m7GTP (no enthalpic contribution) and to pseudoknot 1 of 5′-UTR on TEV (40.7% enthalpic contribution) (35, 36). The higher enthalpic value suggests increased hydrogen bonds between RNA and the proteins (27, 37). eIF4G601–1196 and eIF4G742–1196 both had significantly lower enthalpic contributions to binding. Our results showed that in contrast, entropy contributed a larger proportion to the free energy \( \Delta G \) (70.5 ± 3.6%) in the binding of BTE to eIF4G601–1196 when eIF4A, eIF4B, and ATP were present (Table 4). Taken together these data indicate a model where eIF4E induces a conformational change resulting in increased enthalpy, suggesting H-bonds, and the second eIF4A binding site is inhibitory, modulating the BTE interaction.

**Discussion**

Human eIF4G central domain (amino acids 642–1091) acts as an active "ribosome recruitment core" and is implicated, with eIF4A, as a critical binding partner to drive mRNA translation in living cells (38). It was also reported that the central domain of human eIF4G preserved the activating effect on the translation of uncapped mRNAs (39). Human eIF4G96–935 has been shown to exert a dominant negative effect on the translation of capped mRNA but stimulated the translation of
uncapped mRNA in vitro (39). The C-terminal third of human eIF4G is dispensable and serves as a regulatory domain in translation (40). Wheat eIF4G fragments p100 (amino acids 587–1488) and p86 (amino acids 766–1488) were able to rescue BTE-mediated translation in 4F-depleted wheat germ extract, whereas p70 (amino acids 863–1488) lost translation ability and failed to bind to BTE (21). Our data show that even without the C-terminal amino acids, the middle region of wheat eIF4G was sufficient for binding and rescue of BTE-mediated cap-independent translation in eIF4F depleted WGE. Therefore, like human eIF4G, the N- and C-terminal amino acids of wheat eIF4G were not required for its core function. However, sequence immediately upstream of the core region is required, as mentioned previously. N-terminal deletion up to aa 863, which does not include the core domain (aa 883–1196; Fig. 1B), eliminates the ability of eIF4G to stimulate BTE-mediated translation (26). The key required region between aa 766 – 863 contains an RNA binding motif and is required for BTE binding (21), so it is likely

BTE interaction with eIF4G

Table 3
Equilibrium constants ($K_D$) for the interaction of $^4$BTE with eIF4G mutants, eIF4E, eIF4A, eIF4B, and ATP at different temperatures

<table>
<thead>
<tr>
<th>Complex</th>
<th>$K_D$ (nM) at 5°C</th>
<th>$K_D$ (nM) at 10°C</th>
<th>$K_D$ (nM) at 15°C</th>
<th>$K_D$ (nM) at 20°C</th>
<th>$K_D$ (nM) at 25°C</th>
<th>$K_D$ (nM) at 30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTE-eIF4G601–1196</td>
<td>7.5 ± 1.2</td>
<td>15 ± 2</td>
<td>23 ± 1</td>
<td>24 ± 2</td>
<td>40.0 ± 4</td>
<td>8.9 ± 1.8</td>
</tr>
<tr>
<td>BTE-eIF4G601–1196/eIF4E</td>
<td>5.2 ± 0.8</td>
<td>9.0 ± 1.6</td>
<td>10 ± 2</td>
<td>7 ± 2</td>
<td>5 ± 2</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>BTE-eIF4G601–1196/eIF4A/eIF4B/eIF4G-ATP</td>
<td>22 ± 3</td>
<td>26 ± 2</td>
<td>29 ± 1</td>
<td>32 ± 2</td>
<td>36 ± 3</td>
<td>40 ± 4</td>
</tr>
<tr>
<td>BTE-eIF4G601–1196/eIF4E/eIF4A/eIF4G-eIF4B/eIF4G-ATP</td>
<td>40 ± 5</td>
<td>45 ± 2</td>
<td>50 ± 3</td>
<td>55 ± 4</td>
<td>60 ± 5</td>
<td>65 ± 6</td>
</tr>
</tbody>
</table>

Figure 5. Van’t Hoff plots for the interaction of BTE with eIF4G601–1196 and eIF4E/eIF4G601–1196 complex. Entropy ($\Delta S$) and enthalpy ($\Delta H$) of the binding of BTE to eIF4G601–1196 or the binding of BTE to eIF4 protein complex were calculated from the intercept and slope, respectively, of the Van’t Hoff plot. Equilibrium values were averaged from at least three independent individual experiments.

The opposite roles of the eIF4A binding sites in eIF4G in stimulating translation may be due to differential ability to stimulate or inhibit helicase activity, as observed with human eIF4G-eIF4A interactions. The central eIF4A binding domain on human eIF4G stimulates ATP-hydrolytic activity, but the C-terminal eIF4A binding domain does not stimulate ATP-hydrolytic efficiency (41). Furthermore, human eIF4G middle domain eIF4G557–1137, which includes the eIF4E binding site and only one eIF4A binding site, has a faster eIF4A-directed helicase RNA unwinding rate than full-length eIF4G or than the longer eIF4G557–1600, which has two eIF4A binding domains (42). The second eIF4A binding domain on mammalian eIF4G possesses an anti-cooperative function for RNA binding (40). A similar modulatory role of the second eIF4A binding site appears to occur in wheat eIF4G. Our translation and binding affinity data support that wheat eIF4A and eIF4B have stronger influence on the eIF4G mutants with one eIF4A binding domain than the mutant with two eIF4A binding domains.

The role of eIF4E in inducing eIF4G conformational changes (Table 1) is interesting. eIF4E plays a role in rhinovirus IRES-mediated translation through inducing an eIF4G conformational change, which increases eIF4G sensitivity to rhinovirus protease cleavage (43). However, there are no reports of BYDV viral protease cleavage of eIF4G or that BYDV encodes a protease at all. Our results suggest that wheat eIF4E also induces a conformational change in eIF4G that increases binding affinity to the BTE. The eIF4E effect of increasing the binding between eIF4G and BTE depended on the eIF4E binding region of eIF4G, not the interaction between eIF4E and RNAs. eIF4E may act as a molecular chaperone for its partner eIF4G, inducing a change in secondary structure of eIF4G601–1196 with less helix content and possibly exposing more eIF4G regions to RNA. In addition, the thermodynamic data showed an increased enthalpic contribution (88.4 ± 1.8%) to free energy of the binding between eIF4G601–1196/eIF4E and BTE, suggesting more hydrogen bond interactions in the complex (27, 37).

We had previously observed that eIF4F binding correlated well with translational activity of the BTE mutants (27). We now report that among the truncated mutants there is a rough correlation in binding with initial translation activity. Taken together, we propose a model in which eIF4E promotes a conformational change in the eIF4G subunit, increasing binding
Thermodynamic analysis of eIF4E, eIF4A, and eIF4B effects on the interaction of \(^{34}\)BTE with eIF4G mutants

<table>
<thead>
<tr>
<th>Complex</th>
<th>(\Delta H)</th>
<th>(\Delta S)</th>
<th>(\Delta G)</th>
<th>T∆S/∆G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(kJ mol^{-1})</td>
<td>(\text{fJ mol}^{-1} K^{-1})</td>
<td>(\text{kJ mol}^{-1})</td>
<td></td>
</tr>
<tr>
<td>BTE-eIF4G601–1196</td>
<td>-34.6 ± 1.2</td>
<td>-12.7 ± 1.0</td>
<td>25.7 ± 1.8</td>
<td>101.9 ± 3.4</td>
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<tr>
<td>BTE-eIF4G601–1196:4E</td>
<td>-41.2 ± 0.9</td>
<td>-14.8 ± 1.9</td>
<td>18.2 ± 2.8</td>
<td>104.3 ± 2.9</td>
</tr>
<tr>
<td>BTE-eIF4G601–1488</td>
<td>-17.2 ± 3.4</td>
<td>-17.8 ± 1.0</td>
<td>78.1 ± 2.9</td>
<td>78.4 ± 2.7</td>
</tr>
<tr>
<td>BTE-eIF4G601–1488:4E</td>
<td>-25.1 ± 0.6</td>
<td>-25.6 ± 1.3</td>
<td>59.0 ± 1.4</td>
<td>59.1 ± 2.2</td>
</tr>
<tr>
<td>BTE-eIF4G742–1196</td>
<td>-20.3 ± 2.1</td>
<td>-19.0 ± 1.1</td>
<td>65.8 ± 3.3</td>
<td>74.3 ± 3.5</td>
</tr>
<tr>
<td>BTE-eIF4G742–1196:4E</td>
<td>-20.3 ± 0.7</td>
<td>-21.3 ± 2.6</td>
<td>65.0 ± 2.6</td>
<td>66.4 ± 3.3</td>
</tr>
</tbody>
</table>

**Experimental procedures**

**Plasmid construction**

eIF4G601–1196 recombinant protein expression construct was a generous gift from Dr. D. R. Gallie (University of California, Riverside, CA). The construct was made as described (29). Plasmid pGEX-2TK was used for expression of eIF4G601–1488 and eIF4G742–1196. The DNA fragments were generated by PCR from eIF4G full-length cDNA template (a generous gift from Dr. Karen Browning, University of Texas, Austin, TX). Forward primer includes a BamHI site in the 5’-end by using Vector labs 5’-end tag labeling protocol, followed by a stop codon and eIF4G ORF (reverse primer for eIF4G601–1488: TTAAAGGATCCCAAGAAGAACGGAAGG; eIF4G ORF sequence underlined); forward primer for eIF4G742–1196: GAAGGATCTCTCAGAAGATTTGCGAG; reverse primer contains another BamHI site at the 5’ end, followed by a stop codon and eIF4G ORF (reverse primer for eIF4G601–1488: GCTGGATCCATTAAAGTCAACTGGAAG; reverse primer for eIF4G742–1196: CCTGATCTCTCAGAAGAACGATTTC). The PCR products were digested with BamHI and cloned into plasmid pGEX-2TK, which also had been digested with the same enzyme. Clones with the correct eIF4G ORF orientation were selected for expressing GST-eIF4G fusion proteins. All constructs were confirmed by sequencing (Geneviz).

**Protein expression and purification**

All the GST-eIF4G deletion mutant constructs were harbored in pGEX-2TK plasmids and expressed in BL21 (DE3) Escherichia coli cells (NEB). The cells were grown at 37 °C in LB medium overnight and then transferred to fresh LB medium with a 1:100 ratio for culturing another 3 h until \(A_{600} = 0.7\). The cultured cells were induced with 0.5 mM isopropyl \(\beta\)-D-thiogalactopyranoside at 30 °C for 2.5 h. Bacterial cells were harvested and lysed by sonication (sonication buffer: 50 mM Tris-HCl, 500 mM NaCl, 10% glycerol) with protease inhibitor tablets (Roche). The cell debris was removed by centrifugation at 4 °C. The supernatants that contained recombinant eIF4G deletion mutants were loaded onto a GST-Trap affinity exchange column (GE Healthcare). Biotin-conjugated thrombin (Merck Millipore) was used for cleavage of the GST tag. Thrombin was removed by streptavidin-agarose. Cleaved GST tag was removed by binding with GST affinity Sepharose resin. The purity of the protein was examined by 8% SDS gel electrophoresis and stained with Coomassie blue. The yield of proteins was determined by Bradford assay (Thermo Scientific).

Recombinant eIF4F and eIF4E were expressed in BL21(DE3) E. coli cells. mGTP-Sepharose columns were used to purify proteins as described before (44). Recombinant His-eIF4A and GST-eIF4B were expressed in BL21 (DE3) pLysE E. coli cells. The purification followed the previous description (35, 45).

**RNA synthesis and 5’-fluorescein labeling**

The RNA oligomers corresponding to the BTE (bases 4815–4917) and BTE mutants: BTEBF, SL-II-m1, SL-III-3, and SL-III-SWAP (Fig. 1A) were transcribed by T7 RNA polymerase from double-stranded DNA templates (purchased from Integrated DNA Technologies Inc.). The procedure followed Megashortscript T7 kit standard protocol. All the in vitro transcription products were purified by phenol-chloroform extraction and ethanol precipitation. Nucaway spin columns from Ambion were used to remove free nucleotides. RNA concentrations were determined by Nano-drop UV-visible spectrometer. The purity of RNA was confirmed by 8% polyacrylamide gel electrophoresis. BTE and BTE mutants were labeled with fluorescein at the 5’ end by using Vector labs 5’ end tag labeling kit and then purified according to the manufacturer’s recommended protocol.

Plasmids pBlucB, pBluc-SLLI-m1, and pBluc-SIII-SWAP were linearized by SmaI and used as templates. To generate mRNA in vitro transcription under the T7 promoter, Megscript T7 kit was used according to the manufacturer’s protocol (Ambion).

**Fluorescence anisotropy measurements**

Fluorescence anisotropy measurements were performed with a Horiba Jobin Yvon Fluorolog-3 FluorEssence™ spectrophotometer equipped with excitation and emission polarizers and an i-format detection configuration. Direct fluorescence anisotropy titration was employed to study protein-RNA interactions. Titration temperature was 25 °C for all experiments except when otherwise indicated (temperature dependent...
study). 50 nm of 5′-fluorescein-labeled BTE or BTE mutant were incubated with increasing concentrations of protein or protein complex in 200 μl of titration buffer (20 mM HEPES buffer, pH 7.4, 100 mM KCl, 1 mM DTT). The helicase complex (eIF4A, eIF4B, and ATP) and eIF complex were preincubated before adding into the titration mix as described (25, 46). The anisotropy of each sample was measured by excitation at 494 nm (4-nm slit), and the emission was measured at 520 nm (5-nm slit). The anisotropy data were fitted to Equation 3, which assumes one binding site, to determine the dissociation equilibrium constant.

\[ r_{\text{obs}} = r_{\text{min}} + \frac{(r_{\text{max}} - r_{\text{min}})}{2 \times [^{[i]BTE/\text{mutants}]}} \left\{ b - \left( \frac{b}{2} - 4 \times [^{[i]BTE/\text{mutants}] \times [\text{eIFs}]} \right) \right\} \quad \text{(Eq. 3)} \]

The \( r_{\text{obs}} \) is the observed anisotropy value for any point in the titration curve; \( r_{\text{min}} \) is the minimal anisotropy value in the absence of protein or protein complex; and \( r_{\text{max}} \) is final saturated anisotropy value. \( b = K_d + [^{[i]BTE/\text{mutants}] + [\text{eIFs}].} \([^{[i]BTE/\text{mutants}] and [\text{eIFs}] \) are the concentration of BTE or BTE mutants and initiation factor concentration, respectively. \( K_d \) is the equilibrium dissociation constant, assuming one protein binding site with RNA. Titration data were nonlinear least squares fitted using KaleidaGraph (Abelbeck Software). Equilibrium values were determined from at least three independent experiments.

**Thermodynamic analyses of eIF4G601–1196, eIF4E interaction with BTE**

Temperature dependence of the association equilibrium constant was used to determine the thermodynamic parameters for eIF4G601–1196 or eIF4G601–1196-eIFs complex to BTE. Enthalpy (\( \Delta H \)), entropy (\( \Delta S \)), and free energy (\( \Delta G \)) were calculated by Van’t Hoff plots of \( -\ln K_{\text{eq}} \) versus 1/\( T \), according to the following Equations 4 and 5,

\[ -RT \ln K_{\text{eq}} = \Delta H - T \Delta S \quad \text{(Eq. 4)} \]
\[ \Delta G = -RT \ln K_{\text{eq}} \quad \text{(Eq. 5)} \]

where \( R \) is the universal gas constant, and \( T \) is the absolute temperature. \( K_{\text{eq}} \) the association equilibrium constant, was determined at different temperatures. \( \Delta H \) and \( \Delta S \) were obtained from the slope and intercept, respectively, of the plot of \( \ln K_{\text{eq}} \) versus 1/\( T \). The titration reactions were thermostatted at 5, 15, 20, 25, and 30 °C. Titrations were performed as described above. A thermocouple inside the cuvette was used to monitor temperature. The temperature dependence experiments for binding between eIF4G601–1196-eIF4E complex and BTE were done in the range 20–30 °C because the tight binding constants for lower temperature make measurements impractical. \( \Delta G \) was calculated at 25 °C.

**CD measurement**

An AVIV 200 CD spectrometer equipped with Peltier thermal controller with 1-mm optical pathlength and 1-nm bandwidth was used for recording CD signals. The spectra were acquired from 200 to 260 nm at 25 °C. 10 μM eIF4E, eIF4G, eIF4G601–1196-eIF4E, and eIF4G601–1196-eIF4A-eIF4B complex were measured in 10 mM phosphate buffer (pH 7.0). Each spectrum was averaged over five scans. eIF4G601–1196 and eIF4E were preincubated to form the protein complex before scanning. Protein peptide \( \alpha \)-helix content is calculated from mean residue ellipticity at 222 nm, based on Equation 6.

\[ \alpha\text{-helix} = \frac{-MRE_{222} - 2340}{30300 \times 100} \quad \text{(Eq. 6)} \]

**In vitro translation**

Wheat germ extract was purchased from Promega. The depleted extract was prepared with m′GTP-Sepharose beads as described (28). The mRNA in vitro translation reaction used the Promega standard protocol. For supplementation in depleted wheat germ extract, proteins were added to a final volume of 50 μl. eIF4F, eIF4Gs, and eIF4E were supplied at a final concentration of 60 nm or as indicated. Translation reactions were incubated at 25 °C for different time duration as indicated. Luciferase assays were performed after translation reactions. 3 μl of the translation mix was added to 50 μl of luciferase assay reagent (Promega) and measured immediately by a Glomax-96 microplate fluorimeter.

**Western blot assay**

To determine initiation factor depletion of wheat germ extract, Western blot assays were performed. Proteins from wheat germ extract and 4F-depleted wheat germ lysates were resolved by 8% SDS-polyacrylamide gel electrophoresis. The proteins were transferred to a 0.45-μm nitrocellulose membrane by immersion electrobolting. 5% milk in TBST buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20) was used to block the nitrocellulose membrane. The primary antibodies (generous gifts from Dr. Karen Browning, University of Texas, Austin, TX) were diluted 1:2000 in TBST buffer with 1% BSA for overnight incubation at 4 °C (28). The blots were washed by TBST three times and then incubated with 800CW infrared dye conjugated goat anti-rabbit antibodies (LI-COR) 1:5000 dilution for overnight incubation at 4 °C (28). The blots were washed three times with TBST buffer, washed with PBS once, and then washed with distilled deionized water once. An Odyssey infrared imager (LI-COR) was used to detect the signals.

**Author contributions**—P. Z. conducted most of the experiments, analyzed the results, and wrote most of the paper. Q. L. conducted experiments on purification of eIF4F, eIF4B, and eIF4A proteins. W. A. M. and D. J. G. conceived the idea for the project, provided advice for experiment design, and revised the paper.

**Acknowledgments**—We thank Dr. Daniel R. Gallie and Dr. Karen S. Browning for providing the clones for eIF4F, eIF4G, eIF4A, and eIF4B.

**References**


36. BTE interaction with eIF4G

BTE interaction with eIF4G