Cellular cap-binding protein, eIF4E, promotes picornavirus genome restructuring and translation

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Picornaviruses use internal ribosome entry sites (IRESs) to translate their genomes into protein. A typical feature of these IRESs is their ability to bind directly to the eukaryotic initiation factor (eIF) 4G component of the eIF4F cap-binding complex. Remarkably, the hepatitis A virus (HAV) IRES requires eIF4E for its translation, but no mechanism has been proposed to explain this. Here we demonstrate that eIF4E regulates HAV IRES-mediated translation by two distinct mechanisms. First, eIF4E binding to eIF4G generates a high-affinity binding conformation of the eIF4F complex for the IRES. Second, eIF4E binding to eIF4G strongly stimulates the rate of duplex unwinding by eIF4A on the IRES. Our data also reveal that eIF4E promotes eIF4G binding and increases the rate of restructuring of the picornavirus (PV) IRES. This provides a mechanism to explain why PV IRES-mediated translation is stimulated by eIF4E availability in nuclease-treated cell-free extracts. Using a PV replicon and purified virion RNA, we also show that eIF4E promotes the rate of eIF4G cleavage by the 2A protease. Finally, we show that cleavage of eIF4G by the poliovirus 2A protease generates a high-affinity IRES binding truncation of eIF4G that stimulates eIF4A duplex unwinding independently of eIF4E. Therefore, our data reveal how picornaviruses IRESs use eIF4E-dependent and -independent mechanisms to promote their translation.

Significance

Picornaviruses translate their RNA genomes by a cap-independent mechanism that uses an internal ribosome entry site (IRES) to hijack the host translation machinery. The hepatitis A virus (HAV) and poliovirus (PV) IRESs contain binding sites for the eIF4G component of the cap-binding complex, eIF4F. The HAV IRES also requires eIF4E for efficient translation, but the reason for this is unknown. We now show that eIF4E greatly stimulates the cleavage of eIF4G when 2Apro is expressed and processed from a PV replicon. Thus, our data provide mechanistic insight into how eIF4E can play a positive role in IRES-mediated translation, and reveal an unexpected commonality in the mechanism...
used by HAV and PV IRESs to recruit eIF4F and restructure their RNA domains.

**Results**

**eIF4E Stimulates IRES-Mediated Translation in a Nuclease Treated Cell-Free Extract.** To precisely establish the extent to which eIF4E availability can regulate cap-dependent and cap-independent IRES-mediated translation, we quantitatively manipulated eIF4E availability in a nuclease-treated rabbit reticulocyte lysate (RRL) cell-free extract system. We used three different reporters to monitor the following translation initiation mechanisms: (i) cap-dependent; (ii) HAV IRES-mediated; and (iii) PV IRES-mediated (Fig. 1A). The translation conditions were adjusted to ensure high-fidelity initiation codon selection (18). The RRL was also supplemented with HeLa cytoplasmic extract for all PV IRES translation experiments to ensure high-fidelity PV IRES-mediated translation (19), which was verified using a PV IRES mutant that lacks domain V (dV; SI Appendix, Fig. S1). Using this translation system, we first preincubated the lysate with increasing concentrations of purified recombinant 4E-BP1 to sequester the available eIF4E. Our data clearly reveal a dose-dependent inhibition of cap-dependent and both IRES-mediated translation mechanisms (Fig. 1 B–D). As expected, cap-dependent translation was severely inhibited by ≈80% at high 4E-BP1 concentrations (Fig. 1B). Unexpectedly, 4E-BP1 also inhibited both the HAV and PV IRES-mediated translation mechanisms by ≈40–50% (Fig. 1 C and D). Importantly, the inhibitory effect of 4E-BP1 on translation was specific and a direct effect of eIF4E sequestration, as incubating equimolar amounts of 4E-BP1 and eIF4E in the lysate did not inhibit translation compared with the control lysate (Fig. 1 B–D). In addition, translation directed by the unrelated HCV IRES, which does not require eIF4F, was insensitive to both recombinant 4E-BP1 and eIF4E addition (SI Appendix, Figs. S2A and S3E). We also found that the lysate was limiting for eIF4E, observing a 30–50% increase in cap-dependent, PV, and HAV IRES-mediated translation on addition of recombinant eIF4E (Fig. 1 B–D and SI Appendix, Fig. S3 A–C). Furthermore, we also found that the EMCV IRES and PV IRES behave similarly (Fig. 1D and SI Appendix, Fig. S2B). These data indicate that both cap-dependent and IRES-mediated translation mechanisms are regulated by eIF4E availability.

Cap-dependent and HAV IRES-mediated translation mechanisms are inhibited by cap analogs (15, 17). As expected, the addition of m7GTP, but not of GDP, inhibited cap-dependent translation by 80% and HAV IRES-mediated translation by 60% in our lysate system (Fig. 1B and C). In contrast, PV and EMCV IRES-mediated translation was not inhibited by either m7GTP or GDP (Fig. 1D and SI Appendix, Fig. S2B), supporting the idea that eIF4E stimulates PV and EMCV IRES-mediated translation independent of its cap-binding function. Consistently, the addition of eIF4E with a W56L mutation to disrupt its cap-binding ability inhibits HAV IRES-mediated translation by ≈50%, but stimulates PV and EMCV IRES-mediated translation to a similar extent as wild-type eIF4E (SI Appendix, Fig. S4).

To determine whether intact eIF4G is required for stimulation of translation by eIF4E, we preincubated lysates with recombinant foot-and-mouth disease virus (FMDV) Lb protease, which cleaves eIF4G 7 aa upstream of the PV 2A<sup>PO</sup> site (20). Consistent with previously published results (21), cleavage of eIF4G in the lysate by FMDV Lb protease stimulated translation by ≈2.5-fold (SI Appendix, Fig. S5). After cleavage of eIF4G, the addition of 4E-BP1 or eIF4E had no effect on PV translation (SI Appendix, Fig. S5B). Thus, our data suggest that IRES mechanisms that share a requirement for eIF4G/4A for translation also require eIF4E for efficient translation. This commonality is apparent even though PV and EMCV IRESs are classically considered eIF4E independent.

**eIF4E Stimulates Translation of a PV Replicon and Viral-Mediated Cleavage of eIF4G.** A limitation of the in vitro translation system is that reporter genes containing an IRES do not always faithfully recapitulate translational regulation facilitated by other portions of the mature mRNA. Other features of the genomic PV RNA, including the 3′ UTR and poly(A) tail, can influence translation (22–24). Therefore, we used a more physiologically relevant RNA, a PV replicon in which the P1 region of the PV genome is replaced with a firefly luciferase reporter. This PV-Luc replicon construct contains the viral genes necessary for replication, as well as the PV 3′ UTR and the poly(A) tail (25) (Fig. 1F). 4E-BP1 inhibited translation of the PV-Luc replicon by ≈25% after 30 min, and both eIF4E WT and eIF4E W56L stimulated PV-Luc
replicon translation by 60% (Fig. 1E). These results show that eIF4E stimulates translation of a replication-competent PV RNA.

Previous studies found eIF4E greatly stimulates cleavage of eIF4G in vitro using purified viral proteases (26, 27). Here we found that eIF4E greatly stimulated the rate of eIF4G cleavage from 2Apro expressed and processed from the PV-Luc replicon (Fig. 1F), to verify that 2Apro expressed from the PV-Luc replicon is fully functional, we repeated the experiment using RNA extracted from PV virions, and observed the same result (SI Appendix, Fig. S64). Importantly, the eIF4E-mediated stimulation of eIF4G cleavage also occurred in HeLa lysate alone, albeit at a faster rate (SI Appendix, Fig. S6 B and C). These data demonstrate that functional protease expressed and processed from genomic PV RNA is dependent on eIF4E to efficiently cleave eIF4G.

eIF4E Stimulates eIF4A-Dependent Duplex Unwinding on the HAV and PV IRESs. Binding of human eIF4E to eIF4G promotes RNA restructuring by stimulating eIF4A helicase activity on a short RNA duplex with an unstructured loading strand (28). We generated an IRES-dependent restructuring assay by modifying our fluorescent helicase assay (29) to include either the HAV or PV IRES on the 5' side of the fluorescent-labeled molecular beacon binding sites (Fig. 2A). This assay enables accurate real-time kinetic measurements of strand separation in the region located immediately after the authentic initiation codon of HAV (AUG153) and PV (AUG571). For our study, we used a previously characterized eIF4G truncation that contains the eIF4E-binding site and the C terminus of human eIF4G (28), elF4G557–1599 (Fig. 2B). In the absence of eIF4E, the rate of duplex unwinding by eIF4A in the presence of eIF4B and eIF4G557–1599 was only moderately efficient on both HAV and PV IRESs (Fig. 2 C and E and SI Appendix, Table S1). Strikingly, the addition of eIF4E appreciably stimulated both the initial rate of duplex unwinding and the maximum amplitude of unwinding reactions on both IRESs (Fig. 2 C–F and SI Appendix, Table S1). The unwinding is eIF4A-dependent, given that the addition of 5 μM hippuristanol resulted in an 87% inhibition in the initial rate of duplex unwinding for reactions containing eIF4A, eIF4B, and eIF4G557–1599, and eIF4E (SI Appendix, Fig. S7 A–D). We also found that eIF4A alone or in combination with eIF4B and eIF4E is a poor helicase on HAV and PV IRESs. Moreover, in the absence of eIF4A, a combination of eIF4E, eIF4B, and eIF4G682–1599 did not exhibit appreciable unwinding activity (SI Appendix, Fig. S7 A–D).

To determine whether the cap-binding function of eIF4E is required for the observed stimulation of eIF4A duplex unwinding activity, we repeated our unwinding assay in the presence of either mGTP or eIF4E W56L. The initial rate of HAV IRES duplex unwinding was similar when eIF4E was added in the absence or presence of mGTP or when eIF4E W56L was used (Fig. 2D and SI Appendix, Fig. S8 and Table S1). We also found that the rate of PV IRES-mediated duplex unwinding was stimulated to the same degree when eIF4E was added in the presence of mGTP or when eIF4E W56L was used (Fig. 2F and SI Appendix, Fig. S8B and Table S1). This strongly suggests that the unwinding activity of eIF4E on the HAV and PV IRESs in the presence of eIF4B is not dependent on the cap-binding function of eIF4E.

To determine whether eIF4G cleavage by 2Apro regulates eIF4A-dependent duplex unwinding activity on the HAV and PV IRESs, we generated an eIF4G truncation mimicking the C-terminal fragment following 2Apro cleavage (eIF4G682–1599; Fig. 2B). At a concentration approaching saturation (1 μM), eIF4G682–1599 stimulated eIF4A duplex unwinding activity on the HAV and PV IRESs to approximately the same degree as eIF4G557–1599 in the presence of eIF4E (Fig. 2 C–F and SI Appendix, Table S1). This demonstrates that cleavage of eIF4G by 2Apro relieves the requirement of eIF4E for optimal unwinding activity on both IRESs. It also explains why eIF4E availability does not regulate IRES-mediated translation following eIF4G cleavage (SI Appendix, Fig. S5B).

The direct interaction of eIF4G with the PV IRES has been mapped to dV (6). To verify that efficient PV IRES-mediated duplex unwinding requires the specific interaction between eIF4G and dV, we monitored the rate of duplex unwinding on a mutant PV IRES with dV deleted. We found a 50% reduction in the rate of unwinding by eIF4E in the presence of eIF4G682–1599 and eIF4B (Fig. 2F and SI Appendix, Fig. S7E and Table S1). This confirms that dV plays an important role in directing eIF4A-dependent restructuring of the PV IRES, and that our assay accurately monitors IRES-dependent duplex unwinding.

eIF4E Increases the Affinity and Rate of IRES-Mediated Duplex Unwinding by eIF4G/4A/4B. To elucidate the molecular basis by which eIF4E stimulates IRES-mediated duplex unwinding, we examined whether eIF4E increases the rate of duplex unwinding

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and/or increases the apparent affinity of eIF4G/4A/4B to the HAV and PV IRESs. To this end, we measured duplex unwinding on both IRES constructs at fixed concentrations of eIF4A and eIF4B (1 μM each) and increasing concentrations of eIF4G557-1,599 in the absence or presence of 1 μM eIF4E. In the presence of eIF4E, the apparent affinity (KD,app) of the unwinding complex for the HAV IRES was increased by approximately fourfold [407 ± 55 nM (±4E) vs. 94 ± 3 nM (+4E)] (Fig. 3 and SI Appendix, Fig. S9A and Table S2). An even greater effect of eIF4E was observed on the PV IRES, where the KD,app of the unwinding complex for the PV IRES was increased by eightfold [477 ± 118 nM (±4E) vs. 61 ± 3 nM (+4E)] (Fig. 3B and SI Appendix, Fig. S9B and Table S2). Interestingly, the maximum rate of duplex unwinding at saturating concentrations of eIF4G557-1,599 was also increased on both IRES constructs upon addition of eIF4E. The maximum rate of duplex unwinding on the HAV IRES was stimulated by fivefold, whereas that on the PV IRES was stimulated by threefold (Fig. 3 and SI Appendix, Table S2). This clearly indicates that eIF4E increases both the apparent affinity of the unwinding complex for these IRESs and stimulates the rate of eIF4A-dependent duplex unwinding.

We next wanted to determine how eIF4G cleavage by 2Apro alters the interaction of the unwinding complex with the HAV and PV IRESs. Compared with eIF4G557-1,599 in the absence of eIF4E, the apparent affinity of eIF4G682-1,599 for the HAV and PV IRESs was increased by 1.7-fold (239 ± 10 nM) and 4.5-fold (98 ± 7 nM), respectively (Fig. 3 and SI Appendix, Fig. S9A and Table S2). However, with eIF4G682-1,599, the maximum rate of duplex unwinding at saturation on both IRESs was essentially the same as eIF4E/eIF4G557-1,599 (Fig. 3 and SI Appendix, Table S2). In contrast, a complex containing eIF4G682-1,599 unwound a previously characterized non-IRES-containing duplex substrate at an appreciably slower rate at saturation compared with eIF4E/eIF4G557-1,599 (28) (SI Appendix, Fig. S10 and Table S2). This unexpected finding suggests that the PV and HAV IRESs bind and activate eIF4G682-1,599 to stimulate the helicase activity of eIF4A.

eIF4E Enhances the Binding Affinity of eIF4G to dV of the PV IRES. To examine the molecular basis by which eIF4E increases the apparent affinity of eIF4G/4A/4B to the PV IRES, we directly measured the affinity of eIF4G for the PV IRES using a quantitative, fluorescent anisotropy equilibrium binding assay. Although the anisotropy assays were performed in a slightly different buffer than used in our unwinding assays, we did not observe a substantial change in affinity due to buffer composition (SI Appendix, Fig. S1A). Thus, we used optimized FP buffer for the binding experiments owing to the observed higher change in anisotropy signal (SI Appendix, Table S3). We found a modest KD value for eIF4G557-1,599 to PV dV-FI of 276 ± 21 nM (Fig. 4). On the addition of eIF4E, the KD value was reduced by a factor of 5, to 49 ± 2 nM (Fig. 4). This clearly demonstrates positive cooperativity between binding of eIF4G557-1,599 and eIF4E on the PV IRES. To substantiate this finding, we found that a competition-binding assay using an unlabeled PV dV generated a similar affinity, albeit with a slightly more modest threefold reduction (SI Appendix, Fig. S12 and Table S3). Furthermore, filling the cap-binding pocket of eIF4E with m’GTP had only a modest effect on the affinity of eIF4G557-1,599 to PV dV-FI (KD = 96 ± 10; SI Appendix, Fig. S11B and Table S3).

We also examined whether a 2Apro cleavage mimic of eIF4G can overcome the dependency of eIF4E for a high PV IRES binding affinity. Our data for eIF4G682-1,599 show a KD value of 75 ± 4 nM for PV dV-FI (Fig. 4). This is very similar to the KD value of 49 ± 2 nM for eIF4G557-1,599 in the presence of eIF4E, indicating that the cleavage of eIF4G by 2Apro substantially increases the affinity of eIF4E in the absence of eIF4E for the PV IRES. It should be noted that recombinant eIF4G557-1,599 copurifies with an equimolar amount of eIF4A (28). Interestingly, the interaction of eIF4A with eIF4G682-1,599 did not change the KD of eIF4G682-1,599 for PV dV-FI (76 ± 10 nM; Fig. 4B and SI Appendix, Table S3). This indicates that eIF4A does not contribute to the interaction between eIF4G682-1,599 and dV of the PV IRES. It should be noted that these binding experiments were carried out in the absence of ATP to prevent the duplex unwinding activity of eIF4A. Nevertheless, including AMP-PNP did not change the affinity of eIF4E/eIF4G557-1,599 for PV dV-FI (SI Appendix, Fig. S11B and Table S3). Taken together, our data support a model in which eIF4E binding to eIF4G enhances the affinity of eIF4E to the PV IRES before cleavage of eIF4G by 2Apro. Subsequent cleavage of eIF4G generates a high-affinity binding state of eIF4G/4A that binds to the PV IRES independently of eIF4E.

Discussion

A typical feature of picornavirus translation is the requirement for a direct interaction between the viral IRES and eIF4G. This interaction plays two fundamental roles in directing IRES-mediated translation. First, eIF4G acts as a bridge between the IRES and the 43S preinitiation complex. Second, eIF4G functions to recruit eIF4A to restructure the IRES and enable ribosome recruitment. The eIF4E component of eIF4F is an additional regulator of IRES-mediated translation, but the mechanism by which it performs this function is unknown.

Our data show that eIF4E regulates picornavirus IRES-mediated translation by two distinct mechanisms. First, the binding of eIF4E to eIF4G increases the apparent affinity of the entire unwinding complex (including eIF4B) for the HAV and
PV IRESs by fourfold and eightfold, respectively. Consistently, the equilibrium binding affinity of eIF4G for dV of the PV IRES is increased by fivefold in the presence of eIF4E. Second, our data show that eIF4E binding to eIF4G increases the rate of eIF4A-dependent duplex unwinding on the HAV and PV IRESs by fivefold and threefold, respectively (Fig. 3). These observations likely reflect the cap-independent function of eIF4E, which is to reverse the inhibition caused by an autoinhibitory domain in eIF4G (28). This suggests that the autoinhibitory domain in eIF4G regulates the interaction of eIF4F with picornavirus IRESs and the ability of eIF4A to restructure these IRESs to promote ribosome recruitment and translation. Although the HAV and PV IRESs do not share any obvious sequence or structural similarity, our data suggest that these diverse IRESs share apparent commonality in the way in which they bind and use the helicase activity of eIF4F to restructure their IRES domains. This may reflect a fundamental mechanism through which all viral RNAs interact with eIF4G, and we anticipate that future atomic resolution structural models will help determine this.

Curiously, the cap-binding pocket of eIF4E is required for efficient HAV IRES-mediated translation, yet we observed no defect in unwinding on the HAV IRES in the presence of m’GTP cap analog, or with the use of an eIF4E W56L mutant in place of WT eIF4E (Fig. 2D). These findings suggest that the cap-binding ability of eIF4E is likely required for a step independent of eIF4F recruitment and IRES restructuring. It is important to note that the positive role of eIF4E on HAV IRES-mediated translation has not been verified during infection of cells with HAV. This verification has proven to be difficult because HAV barely grows in cell culture and accumulates attenuating mutations when passaged (30).

Two previous reports have shown that the addition of 4E-BP1 inhibits EMCV IRES-mediated translation in a nucleasetrated cell-free extract (3, 5). However, no titration was made to establish the extent of inhibition, and no model has been proposed to explain how 4E-BP1 could inhibit IRES-mediated translation. To precisely determine the extent to which eIF4E availability can regulate HAV, PV, and EMCV IRES-mediated viral translation, we used reporter genes containing these IRESs in a nuclease-treated cell-free extract. This system was used to avoid the complication of needing to account for the effects of competing between the IRES and endogenous mRNAs. In contrast to cap-dependent translation, the translation from these IRES-containing reporters continued at an appreciably lower efficiency (~50%) in the absence of free eIF4E (Fig. 1C and D and SI Appendix, Fig. S2B). This is consistent with a regulatory role of eIF4E in the translation of these different IRESs. To further strengthen these findings, we also monitored the translation of a PV replicon RNA that is competent for translation, viral polypeptide processing, and RNA replication (25). Consistent with the findings for the PV replicon, the addition of eIF4E and eIF4E W56L stimulated the rate of PV replicon translation by more than twofold compared with the addition of 4E-BP1 (Fig. 1E). Unfortunately, replication of PV was found to be inefficient in our lysate system, preventing us from determining whether the increased translation of the replicon also promotes viral replication. Nevertheless, these data show that eIF4E can have a positive effect on the translation of a PV replicon.

To understand the mechanism of eIF4G binding to the PV IRES during late viral infection, we investigated the interaction of eIF4G with this IRES following eIF4G cleavage. Our data show that the affinity (apparent and direct) of eIF4G82–1,599 for the PV IRES is very similar to that of eIF4G557–1,599 in the presence of eIF4E (SI Appendix, Table S2 and S3). Consistent with this, eIF4G82–1,599 increased the rate of eIF4A-dependent duplex unwinding on the PV IRES to a similar level as eIF4G557–1,599 in the presence of eIF4E (Fig. 3B and SI Appendix, Table S2). In contrast, eIF4G82–1,599 could only partially stimulate eIF4A helicase activity on a non–IRES-containing duplex substrate compared with unwinding activation by eIF4E/4G557–1,599 (28) (SI Appendix, Table S2). This unexpected result suggests that the PV IRES has an RNA-based activation domain that enables eIF4G82–1,599 to fully stimulate the helicase activity of eIF4A. This activation mechanism appears to function only after eIF4G cleavage by 2Apro, while the eIF4E-dependent activation of eIF4G is required for maximum duplex unwinding before cleavage. Thus, our data reveal a plausible mechanism by which viral-mediated cleavage of eIF4G obviates the need for eIF4E during translation in the later stages of PV infection (Fig. 5).

Interestingly, eIF4E is required for rapid cleavage of eIF4G when using recombinant picornaviral proteases in cell-free extracts (26, 27). Our data strengthen this model by showing that a virally encoded 2Apro is dependent on available eIF4E for cleaving eIF4G in a nuclease-treated lysate (Fig. 1F and SI Appendix, Fig. S6). However, we cannot rule out the possibility that some of the enhanced eIF4G cleavage that we observed might be due to the increased production of the 2Apro in the presence of eIF4E. Nevertheless, since eIF4G cleavage is almost abolished in the presence of 4E-BP1, this is unlikely to completely explain this observation. It thus appears that eIF4E can positively function during PV infection by promoting PV translation and eIF4G cleavage by 2Apro (Fig. 5). These positive effects of eIF4E on the PV lifecycle are puzzling given the fact that high eIF4E concentrations inhibit PV and EMCV translation and replication in the presence of endogenous mRNAs in cells and cell-free extracts (5). This is presumably due to competition between capped mRNAs and the viral IRES for free eIF4G. Thus, it will be important in the future to understand the degree to which the positive and negative effects of eIF4E can regulate picornavirus IRESs and the ability of eIF4A to restructure these IRESs to promote ribosome recruitment and translation. Although the HAV and PV IRESs do not share any obvious sequence or structural similarity, our data suggest that these diverse IRESs share apparent commonality in the way in which they bind and use the helicase activity of eIF4F to restructure their IRES domains. This may reflect a fundamental mechanism through which all viral RNAs interact with eIF4G, and we anticipate that future atomic resolution structural models will help determine this.
translation in response to physiological conditions that alter the amount of competition between mRNAs. Interestingly, the production of infectious PV particles in single cells is very heterogeneous and generally independent of the number of viruses used in the infection (31). The precise molecular basis for this heterogeneity is not clear, but it is entirely possible that variation in eIF4E availability between cells could contribute to this phenomenon through its positive and negative effects on PV translation.

Materials and Methods

Purified Components. Protein expression, purification, and transcription protocols are described in detail in SI Appendix, Materials and Methods. Recombinant eIF4AI, eIF4E, eIF4E W56L, 4E-BP1, eIF4G557–1599, eIF4G2–21–1599, and Lb protease were prepared as described previously (20, 28, 52, 63–64, 240). In vitro translation reactions were performed as described previously (29, 20, 75) with the following final concentrations: 90 mM KOAc, 45 mM KCl, 2 mM Mg(OAc)2, 0.5 μg/mL RNasin (Promega), a 20 μM amino acid mixture (–methionine), and a 20 μM amino acid mixture (–leucine). Translation reactions with the PV-Fluc reporter and PV-Luc replicon also included 12% Hela extract. Proteins were incubated in lysate for 7–10 min at 30 °C in the absence of RNA. Then, 5 μg of rV, PV, or EMVC RNAs for translation, HAV, and PV RNAs for helicase assays, and PV dV RNA for fluorescence anisotropy assays were prepared as described previously (29) and in SI Appendix, Materials and Methods. A plasmid containing the PV-Luc replicon [prib(+)-Luc-WT] was a kind gift from Raul Andino (25). PV dV RNA for fluorescence anisotropy was 3′-labeled with fluorescein-5-thiosemicarbazide (Thermo Fisher Scientific) as described previously (33) and in SI Appendix, Materials and Methods.

In Vitro Translation. Translation assays were carried out in 50% nuclease-treated RRL (Promega) with the following final concentrations: 90 mM KOAc, 45 mM KCl, 2 mM Mg(OAc)2, 0.5 μg/mL RNasin (Promega), a 20 μM amino acid mixture (–methionine), and a 20 μM amino acid mixture (–leucine). Translation reactions with the PV-Fluc reporter and PV-Luc replicon also included 12% Hela extract. Proteins were incubated in lysate for 7–10 min at 30 °C in the absence of RNA. Then, 5 μg of rV, PV, or EMVC RNAs for translation, HAV, or 20 μg/ml PV-Luc replicon RNA was added, and the reactions were incubated for another 30 min at 30 °C. Firefly or Renilla luciferase assay substrate (Promega) was added, and luminescence was measured for 10 s with a VICTOR XS multilabel plate reader (PerkinElmer). For PV-Luc replicon reactions, 1/10 volume was removed at each time point for measurement of luciferase production.

eIF4G Cleavage Assay. In vitro translation reactions were performed as described for the PV-Luc replicon but supplemented with 10% of a 10x NTPr/energy mix (10 mM ATP, 2.5 mM GTP, 2.5 mM CTP, 2.5 mM UTP, 300 mM creatine phosphate, 4 mg/ml creatine kinase, and 155 mM Hepes-KOH pH 7.4). At 4, 7, 9, and 12 h, a 1/5 volume of the reaction was analyzed by SDS-PAGE, and eIF4G was detected by immunoblotting using anti-eIF4G antibody (Proteintech; 15704-1-AP) as described in detail in SI Appendix, Materials and Methods.

Helicase Assay. Unwinding reactions were performed as described previously with minor modifications (29). Unwinding reactions were assayed with a Fluorolog-3 spectrofluorometer (Horiba), and data were analyzed as described previously (28, 29) and in SI Appendix, Materials and Methods.

Fluorescence Anisotropy Binding Assay. Fluorescence anisotropy was measured using a VICTOR XS plate reader (PerkinElmer) and analyzed as described previously (33). Reactions containing 20 nM dV-dFl were incubated with varying concentrations of eIF4G in binding buffer (20 mM Hepes-KOH pH 7.5, 80 mM KCl, 2 mM MgCl2, 1 mM DTT, and 0.1 mg/ml BSA) for 4 min at 37 °C, followed by 20 min at 25 °C.

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