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Human eIF4E promotes mRNA restructuring by stimulating eIF4A helicase activity

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Elevated eukaryotic initiation factor 4E (eIF4E) levels frequently occur in a variety of human cancers. Overexpression of eIF4E promotes cellular transformation by selectively increasing the translation of proliferative and prosurvival mRNAs. These mRNAs possess highly structured 5′-UTRs that impede ribosome recruitment and scanning, yet the mechanism for how eIF4E abundance elevates their translation is not easily explained by its cap-binding activity. Here, we show that eIF4E possesses an unexpected second function in translation initiation by strongly stimulating eukaryotic initiation factor 4A (eIF4A) helicase activity. Importantly, we demonstrate that this activity promotes mRNA restructuring in a manner that is independent of its cap-binding function. To explain these findings, we show that the eIF4E-binding site in eukaryotic initiation factor 4G (eIF4G) functions as an autoinhibitory domain to modulate its ability to stimulate eIF4A helicase activity. Binding of eIF4E counteracts this autoinhibition, enabling eIF4G to stimulate eIF4A helicase activity. Finally, we have successfully separated the two functions of eIF4E to show that its helicase promoting activity increases the rate of translation by a mechanism that is distinct from its cap-binding function. Based on our results, we propose that maintaining a connection between eIF4E and eIF4G throughout scanning provides a plausible mechanism to explain how eIF4E abundance selectively stimulates the translation of highly structured proliferation and tumor-promoting mRNAs.

protein synthesis | DEAD-box | ATPase

Recruitment of mRNAs to the ribosome must be tightly controlled in human cells because the dysregulation of protein synthesis has a direct impact on cancer development and progression (1, 2). Eukaryotic initiation factor 4F (eIF4F) is the protein complex that binds the 5′ 7-methyl guanosine cap found on all cellular mRNAs and is comprised of the cap-binding protein, eIF4E, the DEAD-box helicase, Eukaryotic initiation factor 4A (eIF4A), and the scaffold protein Eukaryotic initiation factor 4F (eIF4G) (3). The eIF4E component of eIF4F is generally considered to be the rate-limiting factor in translation initiation (4). Consistent with this, eIF4E availability is tightly controlled through regulated interaction with eIF4E-binding proteins (4E-BPs). These proteins function as competitive inhibitors of eIF4E binding to eIF4G and are regulated through phosphorylation events coordinated by the PI3K–AKT–mammalian target of rapamycin (mTOR) signaling pathway (5, 6). Activation of mTOR complex 1 (mTORC1) releases eIF4E from 4E-BPs, leading to an increase in the translation of a pool of mRNAs often referred to as "eIF4E-sensitive" (2, 5). These mRNAs possess regulatory elements in their 5′-UTRs that somehow confer their sensitivity to eIF4E levels. The overwhelming majority of these mRNAs possess long structured 5′-UTRs that must be unwound to allow ribosome recruitment and scanning (2, 5, 7, 8). Accordingly, overexpression of eIF4E selectively increases the translation of highly structured proliferative and prosurvival mRNAs that can transform immortalized cells and form tumors in mice (9–12). However, the mechanism by which the availability of eIF4E selectively controls translation initiation of mRNAs containing structured 5′-UTRs is unknown.

Unwinding of secondary structure in the mRNA 5′-UTR involves the activity of the eIF4A helicase component of eIF4F (13, 14). Human eIF4A functions as an RNA-dependent ATPase that bidirectionally unwinds RNA duplexes (15–17). Consistent with other DEAD-box proteins, strand separation is promoted through well-defined conformational rearrangements of its RecA homology domains (18–20). Although eIF4A is a relatively poor helicase on its own, its ATPase and duplex unwinding activities are stimulated by eIF4G and the helicase accessory protein, eIF4B (15, 17, 21–24). In addition, the interaction of the poly(A) binding protein (PABP) with eIF4G also stimulates the ATPase and duplex unwinding activity of eIF4A (25). Despite the fact that an increase in eIF4E availability stimulates the rate of translation initiation on highly structured mRNAs, no evidence exists to link the presence of eIF4E in the eIF4F complex with the activity of the eIF4A (15, 17). Previous attempts to study the helicase activity of eIF4A in the eIF4F complex did not control for eIF4E abundance, raising a fundamental question regarding the possible role of eIF4E in controlling the helicase activity of the eIF4F complex.

Here, we have used a real-time fluorescence assay to reveal the kinetic parameters of duplex unwinding by the human eIF4F complex. Our data demonstrate that, in addition to its role in cap-binding, eIF4E stimulates eIF4A duplex unwinding activity in the eIF4F complex. We further show that this unexpected activity of eIF4E increases the rate of translation initiation by a mechanism that is distinct from its role in cap-binding. This regulatory function of eIF4E provides a plausible mechanism to explain how eIF4E can selectively stimulate the translation of mRNAs that possess structured 5′-UTRs.

Results

eIF4E Stimulates the Rate of Duplex Unwinding by eIF4A. To determine the kinetic framework of duplex unwinding by the human eIF4F complex, we generated a modified version of our previously described fluorescence unwinding assay that uses an uncapped RNA duplex substrate (Fig. 1*A*) (21). This assay design provides greater flexibility compared with our previous assay because it does not require a fluorescently modified RNA loading strand. The assay can be used to accurately measure the kinetics of RNA strand separation by monitoring an increase in total fluorescence in real time by using highly purified initiation factors (Fig. S1). Consistent with our previous results, efficient strand separation by eIF4A is observed in the presence of fixed amounts of eIF4B and an eIF4G truncation that spans amino acids 682 to 1105 ($eIF4G_{682–1105}$; Fig. 1*B* and Table S1) (21). Although $eIF4G_{682–1105}$ constitutes a conserved region of eIF4G that is able to stimulate eIF4A duplex unwinding, we wanted to determine if

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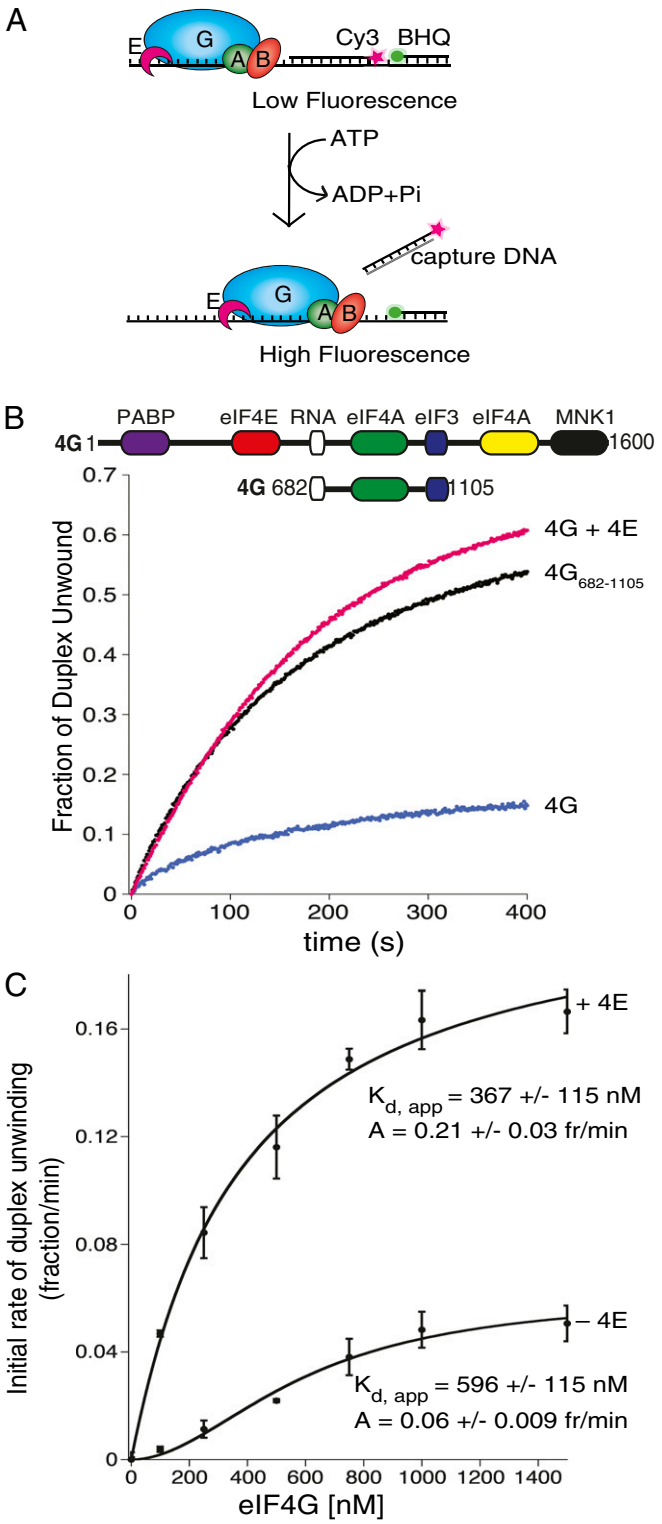

Fig. 1. eIF4E stimulates eIF4A helicase activity. (*A*) Unwinding assay: a Cy3-labeled reporter RNA is annealed to an uncapped RNA loading strand with a 20-nt overhang. A BHQ-labeled RNA is annealed to the loading strand to quench the reporter fluorescence. ATP-dependent helicase activity dissociates the reporter, resulting in increased Cy3 fluorescence. A DNA capture strand prevents reannealing. Each assay contains 50 nM RNA substrate, 2 mM ATP-Mg, and 1 μM each protein component unless otherwise stated (*Materials and Methods*). (*B*) Representative unwinding time course of helicase reactions containing eIF4A, eIF4B, and eIF4G$_{682–1105}$ (black) or eIF4G in the absence (blue) or presence of eIF4E (magenta). A cartoon depicts human eIF4G domains. (*C*) Initial rate of duplex unwinding (fraction per minute) for eIF4A, eIF4B, and varied concentrations of eIF4G in the absence or presence of eIF4E. Data are fit to the Hill equation as described in *SI Materials and Methods*. The $K_{d,app}$ and maximum initial rates of unwinding (i.e., "A") are means of three independent experiments ± SEM.

other domains of eIF4G might affect eIF4A activity. To this end, we tested the ability of purified full-length human eIF4G to stimulate eIF4A duplex unwinding in the presence of eIF4B. In contrast to eIF4G$_{682–1105}$, full-length eIF4G is much less efficient at stimulating the helicase activity of eIF4A (Fig. 1*B* and Table S1). Surprisingly, efficient duplex unwinding comparable to that of eIF4G$_{682–1105}$ is achieved when eIF4E is added to the reaction (Fig. 1*B* and Table S1). This activity is observed even without a cap structure present on the mRNA loading strand, implying that eIF4E has a role in stimulating eIF4A duplex unwinding activity in addition to its cap-binding function.

To reveal the molecular basis by which eIF4E promotes eIF4A duplex unwinding, we tested whether eIF4E stimulates the rate of duplex unwinding by the eIF4F complex and/or increases the apparent affinity of the complex to the substrate. To distinguish between these possibilities, duplex unwinding was measured at a fixed concentration of eIF4B and increasing concentrations of full-length eIF4G in the absence or presence of eIF4E (Fig. 1*C*). The initial rate of duplex unwinding for each reaction is calculated and the data are used to estimate the apparent affinity ($K_{d,app}$, in nanomolars]) and the maximum initial rate of duplex unwinding at initiation factor saturation (A; fraction duplex unwound per minute), as described in *Materials and Methods*. Our data reveal very little change in the apparent affinity of the eIF4F complex for the duplex substrate upon the addition of eIF4E [596 ± 115 nM (–4E) vs. 367 ± 115 nM (+4E); Fig. 1*C*]. Most notable, however, is that the presence of eIF4E in the eIF4F complex increases the rate of duplex unwinding by approximately 3.5-fold from 6×10^{-2} (SEM 0.9×10^{-2}) fraction duplex unwound per minute to 21×10^{-2} (SEM 3×10^{-2}) fraction duplex unwound per min (Fig. 1*C*). Thus, eIF4E stimulates eIF4A helicase activity and not the affinity of the eIF4F complex for RNA in a substrate lacking the cap structure.

eIF4E Stimulates eIF4A Helicase Activity Independent of Its Cap-Binding Function. To establish that the increase in the rate of RNA duplex unwinding in our assay is eIF4A-dependent, we used a well-characterized small molecule, hippuristanol (13), to specifically inhibit eIF4A helicase activity in the presence of eIF4E. Addition of 3 μM hippuristanol results in a 70% inhibition in the initial rate of duplex unwinding for helicase reactions containing 1 μM of eIF4A, eIF4B, eIF4G, and eIF4E (Fig. 2*A* and Table S1). In addition, to test if binding of the cap to eIF4E changes its ability to stimulate eIF4A helicase activity, the cap-binding pocket of eIF4E was bound with the m^{7}GTP cap-analog. Duplex unwinding was then monitored in an assay containing eIF4A, a fixed concentration of eIF4B, and a concentration of eIF4G close to the $K_{d,app}$ value of the complex for RNA (500 nM). Addition of an equimolar (1 μM) or 20-fold molar excess (20 μM) of cap-analog to eIF4E does not alter the initial rate of duplex unwinding (Fig. 2*B* and Table S1). Importantly, translation of a capped mRNA reporter using a reticulocyte lysate system is severely inhibited at concentrations of cap-analog above 10 μM (Fig. S2). In addition, published equilibrium dissociation constants for eIF4E binding to m^{7}GTPpppG range from 80 nM (26) to 450 nM (27) at 100 mM KCl. If we assume the lowest affinity, our experiments use a concentration of m^{7}GTP (20 μM) that is ~45-fold higher than the published K_d. Assuming that m^{7}GTP possesses a similar affinity as m^{7}GTPpppG for eIF4E, the amount of added m^{7}GTP will therefore saturate the cap-binding pocket of eIF4E. Taken together, these data imply that the function of eIF4E in duplex unwinding is not affected by its cap-binding

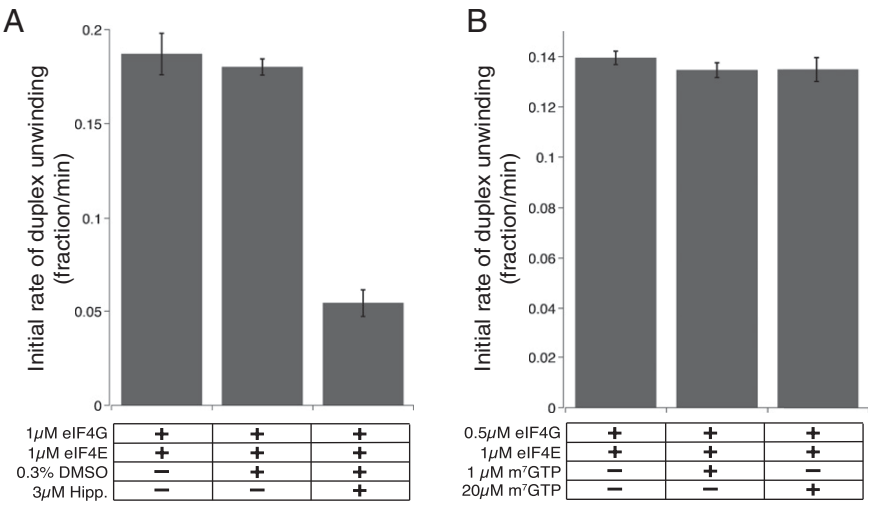

Fig. 2. eIF4E stimulation of eIF4F helicase activity is eIF4A-dependent and cap-independent. (*A*) Initial rates of duplex unwinding for helicase reactions containing 1 μM eIF4A, eIF4B, eIF4E, and eIF4G in the absence or presence of 0.3% DMSO (final concentration) ± 3 μM hippuristanol. (*B*) Initial rates of duplex unwinding for helicase reactions containing 1 μM eIF4A, eIF4B, eIF4E, and 0.5 μM eIF4G in the absence or presence of m^7GTP (1 μM or 20 μM). Data are presented as means of three independent experiments ± SEM.

function. Thus, eIF4E is able to stimulate eIF4A helicase activity independent of cap-binding.

The eIF4E-Binding Region in eIF4G Functions as an Autoinhibitory Domain That Modulates eIF4A Helicase Activity. To determine which domain of eIF4G is responsible for eIF4E-dependent regulation of eIF4A helicase activity, we generated two additional eIF4G truncations. One truncation removes the N-terminal 556 aa ($eIF4G_{557–1600}$), whereas the second truncation removes an additional 463 aa from the C terminus ($eIF4G_{557–1137}$). For each truncation, duplex unwinding is measured in the presence of eIF4A, eIF4B, and eIF4G in the absence or presence of eIF4E. In keeping with full-length eIF4G, eIF4E stimulates the rate of eIF4A duplex unwinding for $eIF4G_{557–1600}$ (Fig. S3*A*) and $eIF4G_{557–1137}$ (Fig. S3*B*). For each eIF4G truncation, we tested multiple duplex unwinding reactions at a fixed concentration of eIF4B and increasing concentrations of each eIF4G truncation in the absence or presence of eIF4E. Consistent with full-length eIF4G, the addition of eIF4E to these reactions increases the rate of duplex unwinding between three and five fold without an appreciable change in apparent substrate affinity (Fig. 3 and Table S1). In contrast, the addition of eIF4E to $eIF4G_{682–1105}$ (which lacks the eIF4E binding domain) does not further stimulate duplex unwinding (Fig. 3*C* and Table S1). Taken together, this implies that eIF4E stimulates eIF4A helicase activity indirectly through both proteins binding to eIF4G. It should be noted that our eIF4G truncation constructs vary slightly on the C terminus by 32 aa ($eIF4G_{557–1137}$ vs. $eIF4G_{557–1105}$). This region is not conserved between yeast and humans, and we have no reason to believe it contributes to the autoinhibitory function of eIF4G. Nevertheless, we cannot rule out a possible role of this region in autoinhibition at this time.

eIF4E Can Stimulate Translation Independent of Its Cap-Binding Function. Our biophysical data imply a mechanism whereby eIF4E stimulates eIF4A duplex unwinding activity in addition to its role in cap binding. To test if this eIF4E activity regulates translation initiation in a more physiological context, we used a translation assay that recruits ribosomes independently of the cap structure. This assay recruits a ribosome to a mRNA via a boxB hairpin structure that binds specifically to the 22-aa sequence of the bacteriophage λ-transcription anti-terminator protein N (λN-amino acids 1–22) (28). Previous work has shown that conjugating the λ-domain to an eIF4G truncation successfully recruits ribosomes that are able to translate a luciferase reporter in vivo by a cap-independent mechanism (28). We modified this assay for use in a reticulocyte lysate system so that we can more precisely manipulate eIF4G forms and eIF4E abundance. To this end, we generated a luciferase reporter construct that possesses a boxB hairpin and a moderately structured 5′-UTR (Fig. 4*A*). Addition of this RNA construct to a nuclease treated lysate results in very low translation activity, indicating that little 5′-end–dependent translation occurs in the absence of λ-eIF4G (Fig. 4*B*). Upon addition of purified λ-$eIF4G_{557–1600}$ to the lysate, we observe a ∼6.5-fold increase in luciferase translation (Fig. 4*B* and Table S2). We further supplemented the lysate with purified eIF4E to ensure that eIF4E is not limiting for translation in this system. This stimulates translation by more than twofold, resulting in a ∼15-fold total increase in luciferase translation vs. background (Fig. 4*B*). Importantly, untagged $eIF4G_{557–1600}$ does not appreciably promote luciferase translation in the absence or presence of eIF4E (Fig. S4). To verify that this increase in translation is caused by the interaction between eIF4E and λ-$eIF4G_{557–1600}$, we added to the lysate a five-fold molar excess of purified 4E-BP1 compared with added eIF4E. As expected, 4E-BP1 reduces translation to levels similar to that observed before addition of eIF4E (Fig. 4*B*). To confirm that the eIF4E-binding domain in eIF4G is responsible for this activity, we also tested the ability of λ-$eIF4G_{682–1105}$ to stimulate translation. The addition of λ-$eIF4G_{682–1105}$ stimulates translation approximately 23-fold greater than background, with no further change in translation observed upon the addition of eIF4E or 4E-BP1 (Fig. S5). Interestingly, the addition of 4E-BP1 in the absence of added eIF4E does not inhibit λ-$eIF4G_{557–1600}$-stimulated luciferase translation (Fig. S6). The reason for this is not clear, but may simply reflect on a very low concentration of available eIF4E in the reticulocyte lysate compared with 1 μM of added λ-$eIF4G_{557–1600}$ (*Discussion*). Upon addition of 20 μM m^7GTP, we still observe a ∼12-fold stimulation of λ-$eIF4G_{557–1600}$-dependent translation in the presence of eIF4E (Fig. 4*B*). This further demonstrates that eIF4E stimulation of eIF4A duplex unwinding and cap-binding events are independent functions that together promote ribosome recruitment, scanning, and translation initiation.

Discussion

Since its discovery more than 30 years ago, eIF4E has been demonstrated to play a critical role in controlling capped mRNA translation via its interactions with the cap structure and eIF4G. It has been proposed that eIF4E phosphorylation on Ser(209) reduces its affinity for the cap (29), whereas eIF4E binding to eIF4G is precisely regulated by the 4E-BPs (5). Moderate overexpression of eIF4E protein by 2.5 fold is sufficient to dramatically increase translation of eIF4E-sensitive growth-promoting mRNAs (30), resulting in malignant transformation

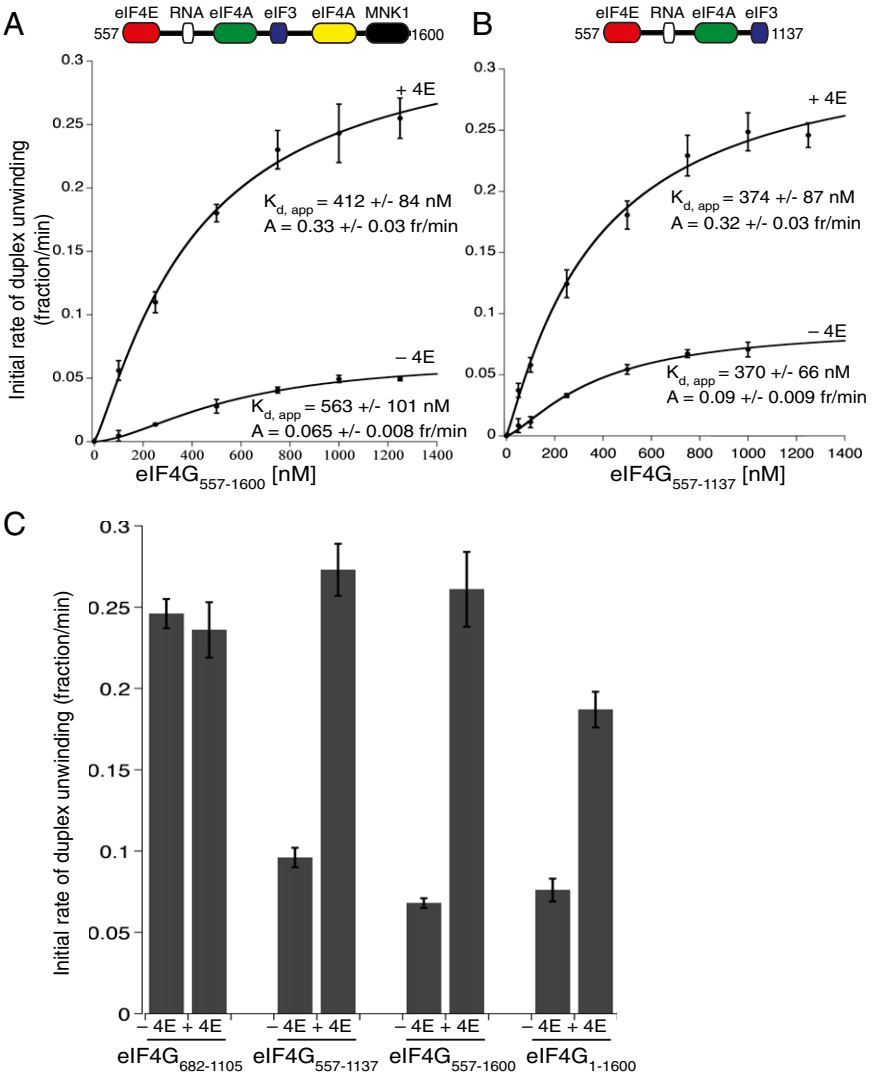

Fig. 3. The eIF4E-binding domain in eIF4G regulates eIF4A activity. Initial rates of duplex unwinding (fraction per minute) for each reaction containing 1 μM eIF4A and eIF4B are plotted vs. increasing concentrations of eIF4G$_{557–1600}$ (*A*) or eIF4G$_{557–1137}$ (*B*) in the absence or presence of 1 μM eIF4E. Data are fit to the Hill equation (*SI Materials and Methods*). The $K_{d,app}$ and maximum initial rates of unwinding (i.e., "A") are shown as means of three independent experiments ± SEM. Cartoons are shown to depict human eIF4G domains in the constructs used. (*C*) Initial rates of duplex unwinding for different truncations of eIF4G ± eIF4E. Each reaction contains 1 μM eIF4G, eIF4A, eIF4B, and eIF4E.

of immortalized cells and tumor formation in mice (9, 10, 12). Consistent with this, 30% of human cancers show similar elevated eIF4E levels, underscoring the importance of eIF4E overexpression in cancer progression (31). Despite this, a mechanism to explain why a subset of cellular mRNAs requires higher levels of available eIF4E for their translation had not been identified. One can speculate that some mRNAs may have their cap structure occluded by 5′-UTR secondary structure, causing them to be poor cap-binding substrates for eIF4E. Thus, an increase in the total amount of eIF4F complex would preferentially stimulate this type of mRNA pool. This potential mechanism, however, has not been rigorously demonstrated for eIF4E-sensitive mRNAs, and awaits further investigation.

Unexpectedly, we have uncovered a second function of eIF4E: the stimulation of eIF4A helicase activity in the eIF4F complex. In addition to increasing the total amount of eIF4F, our data now show that the resulting complex is in fact more active with regard to mRNA restructuring (Fig. 1). Importantly, we show that the ability of eIF4E to stimulate eIF4A helicase activity is independent of its cap-binding function (Fig. 2). Interestingly, the stimulation of eIF4A helicase activity occurs despite a relatively small effect of eIF4E on the affinity of the eIF4G/4A complex for RNA substrate. However, as our calculated affinities represent apparent equilibrium dissociation constants, it will be important in the future to determine the direct equilibrium dissociation constant for eIF4G/4A binding to RNA substrate in the absence and presence of eIF4E. This information will be necessary to determine if eIF4E, in addition to stimulating eIF4A helicase activity, also increases the direct affinity of eIF4G/4A for RNA independently of cap binding. Moreover, by using a tethered-RNA assay to bypass the need for the cap structure in ribosome recruitment, we further show that cap-analog does not change the ability of eIF4E to stimulate the rate of protein synthesis (Fig. 4). It is worth noting that we observe a small (~20%) inhibition of λ-eIF4G$_{557–1600}$-dependent translation upon m^{7}GTP addition in the presence of eIF4E (Fig. 4*B*). As we do not observe a change in eIF4E stimulated eIF4A helicase activity in the presence of m^{7}GTP (Fig. 2), this may indicate that cap binding may influence another translation initiation step that is yet to be determined. Interestingly, we find that the addition of 4E-BP1 does not inhibit the stimulation of translation by 1 μM λ-eIF4G$_{557–1600}$ in the absence of exogenously added eIF4E (Fig. S6). The reported concentration of eIF4E in reticulocyte lysate preparations varies between 8 nM (32) and 400 nM (33). Even at the highest eIF4E concentration reported, 4E-BP1 is present at a ratio of 1:1 with eIF4E (33). Thus, the failure of added 4E-BP1 to inhibit λ-eIF4G$_{557–1600}$-dependent translation might simply reflect the presence of very little free endogenous eIF4E to bind the exogenously added λ-eIF4G$_{557–1600}$. Alternatively, this finding may imply that the

stimulation of λ-eIF4G$_{557–1600}$-dependent translation by exogenous eIF4E is only relevant to situations in which eIF4E is overexpressed (e.g., in certain tumors). Future work will hopefully distinguish between these two possible models.

We have used eIF4G truncations in our unwinding assay to demonstrate that the eIF4E-binding site in eIF4G functions as a classic autoinhibitory domain (Fig. 3). In the absence of eIF4E binding, this domain reduces the ability of eIF4G to stimulate eIF4A helicase activity. The binding of eIF4E to this inhibitory domain counteracts the autoinhibition, enabling eIF4G to stimulate eIF4A helicase activity (Figs. 1 and 3). Consistent with this, removal of this domain results in an eIF4G truncation (eIF4G$_{682–1105}$) that is constitutively active with regard to stimulating eIF4A helicase activity (Fig. 1 and ref. 21). Interestingly, it has previously been shown that eIF4E binding induces a structural change in eIF4G, as revealed by increased sensitivity to viral protease cleavage (34–36). Therefore, it is likely that eIF4E induces an eIF4G conformation that stimulates eIF4A helicase activity, as depicted in Fig. 5. In light of this model, maintaining an eIF4E/eIF4G interaction throughout scanning provides a plausible mechanism to explain how eIF4E abundance promotes translation of highly structured mRNAs. Moreover, this activity of eIF4E further explains why uncapped mRNA translation is sensitive to eIF4E availability (37).

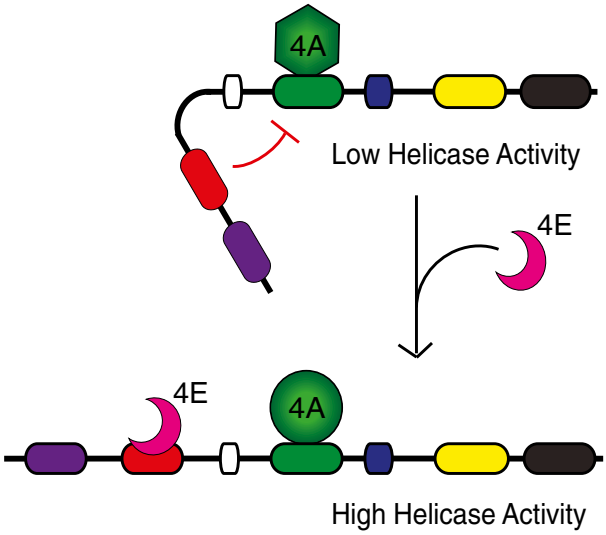

Fig. 5. Proposed mechanism by which eIF4E enhances translation of structured mRNAs. In the absence of eIF4E, the eIF4E-binding domain maintains a conformation of eIF4G that possesses low eIF4A helicase stimulating activity. Upon eIF4E binding, a conformation of eIF4G is induced that possesses a high eIF4A helicase stimulating activity.

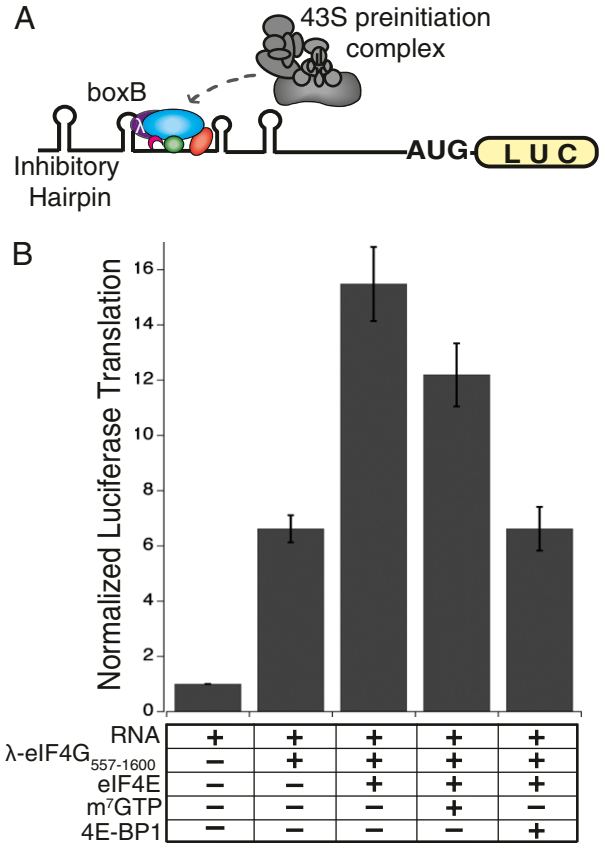

Fig. 4. eIF4E stimulates translation independent of cap-binding. (*A*) Schematic of the luciferase translation assay: the boxB RNA element recruits λ-eIF4G$_{557–1600}$ to the reporter RNA construct. The mRNA contains two additional stem loops between the boxB hairpin and the luciferase reporter gene as described in *Materials and Methods*. An inhibitory hairpin is located upstream of the boxB element to prevent any 5′ end-dependent ribosome loading. The binding of λ-eIF4G$_{557–1600}$ enables eIF4F and eIF4B to unwind any secondary structure so that the 43S preinitiation complex (gray) can be recruited to the mRNA independent of cap-binding. (*B*) Bar graph of relative luciferase translation rates measured for 30 min at 30 °C for reactions containing 250 nM RNA and 1 μM λ-eIF4G$_{557–1600}$ with or without 2 μM eIF4E, 10 μM 4E-BP1, and 20 μM m^{7}GTP. Each reaction is normalized to basal nonspecific levels of luciferase translation in the absence of λ-eIF4G$_{557–1600}$.

Recently, the translation of 5′-terminal oligopyrimidine tract (5′TOP) containing messages has been shown to be sensitive to eIF4E levels, despite not possessing obvious predicted secondary structure (38, 39). Our data imply that these mRNAs are strongly dependent on efficient eIF4A activity for their translation, perhaps through removal of 5′TOP-specific *trans*-acting factors (40). It should be noted, however, the degree to which 5′TOP mRNA translation requires elevated eIF4E levels is not clear, as previous work found no change in their translation rate upon overexpression of eIF4E (41). Chemotherapeutic drugs are currently being developed to target eIF4E and mTOR as a means to control tumor formation (1). It has recently been shown that cancer cells can acquire resistance to mTOR inhibitors by downregulating 4E-BPs so that eIF4E availability increases (42). By revealing an additional activity of eIF4E we describe here, we anticipate that our work may aid in the development of more effective cancer therapeutic agents that can target the independent functions of this central component of the translation machinery.

Materials and Methods

Purified Components. Detailed sample purification protocols are described in *SI Materials and Methods*. Briefly, recombinant eIF4A isoform I (eIF4AI), 4E-BP1, eIF4G$_{682–1105}$, and PABP proteins are expressed in bacteria as maltose-binding protein fusion constructs, cleaved by using recombinant tobacco etch virus (TEV) protease, and purified by using established procedures to generate untagged proteins (21, 43). Human eIF4E is expressed in bacteria as a protein G fusion construct, cleaved by using TEV protease, and purified by ion-exchange chromatography to yield untagged protein. Human eIF4B is expressed as a 6-histidine–tagged construct in insect cells (sf9) and purified as described previously (21). Human eIF4G$_{557–1137}$ and eIF4G$_{557–1600}$ are expressed as 6-histidine–tagged constructs in sf9 cells and purified by immobilized metal ion affinity chromatography (IMAC) and ion-exchange and size-exclusion chromatography. Following elution from Ni-NTA Superflow resin (Qiagen), purified human eIF4AI is added and incubated overnight at 4 °C to form a human eIF4G/eIF4A heterodimer. All λ-tagged eIF4G constructs are generated as N-terminal 6-histidine–λ fusion constructs and purified as described for the non–λ-tagged proteins. Endogenous eIF4F is purified from HeLa cell cytoplasmic extract by using established procedures (44), and stripped of its eIF4E component by using size-exclusion chromatography.

Duplex Substrates. Fluorescent reporter RNA oligonucleotides are chemically synthesized, modified, and HPLC-purified by Integrated DNA Technologies (IDT). The reporter strand is modified with cyanine 3 (Cy3) on its 5′-end, and the quenching strand is modified with a spectrally paired black hole quencher (BHQ) on its 3′-end. The loading RNA oligonucleotide is in vitro-transcribed from annealed DNA oligonucleotides as described in *SI Materials and Methods*. The sequences of Cy3-labeled RNA (ΔG = –21.3 kcal/mol), BHQ-labeled RNA (ΔG = –49.5 kcal/mol), loading RNA, and "competitor" DNA are shown, respectively, with underlined base pairs signifying a duplex region: reporter strand (5′-Cy3-GUUUUUUAAUUUUUUAAUUUUUUC–3′); quenching strand (5′-GGCCCCACCGGCCCCUCCG-BHQ-3′); loading strand (5′-GAACAACAACAACAACAACAGAAAAAAUUAAAAAAUUAAAAAACUCGGAGGGGCCGGUGGGGCC-3′); and DNA capture strand (5′-GAAAAAATTAAAAAATTAAAAAAC-3′).

Helicase Assay. Unwinding reactions are performed in a 50-μL cuvette (Starna) by using a Fluorolog-3 spectrofluorometer (Horiba) as previously described (21). Cy3 and BHQ-labeled RNA oligos (IDT oligo) are annealed to an uncapped RNA loading strand, forming a duplex region with a 5′ extension that possesses low fluorescence. Duplex substrate is incubated with different combinations of protein components, and the unwinding reaction is initiated by the addition of 2 mM ATP-Mg at 25 °C. The change in fluorescence is calibrated to the fraction of duplex unwound over time as described previously (21).

Translation Assay. Translation assays are carried out in a messenger-dependent reticulocyte lysate system (Promega) with the following final concentrations [0.5 U/μL rRNasin (Promega), 20 μM amino acid mixture minus methionine, 20 μM amino acid mixture minus leucine, 2 mM magnesium acetate, 150 mM potassium acetate, 45 mM sodium chloride, and 250 nM RNA, with or without 1 μM λ-eIF4G, 2 μM eIF4E, 10 μM 4E-BP1, and 20 μM m^7GTP, as indicated]. A Renilla luciferase reporter mRNA construct possesses an inhibitory hairpin, a boxB hairpin followed by a 5′-UTR containing two additional stem loops. For each reaction, proteins and rabbit reticulocyte lysate mixture are preincubated for 15 min at 30 °C in the absence of mRNA. The Luciferase reporter mRNA is then added to each reaction and further incubated at 30 °C for 30 min. Luminescence is measured for 10 s by using a Victor X5 Multilabel Plate Reader (Perkin-Elmer), and counts per second are used to compare the levels of Renilla luciferase translation.

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