Human Eukaryotic Initiation Factor 4G (eIF4G) Protein Binds to eIF3c, -d, and -e to Promote mRNA Recruitment to the Ribosome*

Received for publication, September 8, 2013, and in revised form, October 1, 2013. Published, JBC Papers in Press, October 3, 2013. DOI 10.1074/jbc.M113.517011

Nancy Villa†, Angelie Do‡, John W. B. Hershey§, and Christopher S. Fraser¶1

From the †Department of Molecular and Cell Biology, College of Biological Sciences, and §Department of Biochemistry and Molecular Medicine, University of California, Davis, California 95616

Background: The interaction between eukaryotic initiation factor 4G (eIF4G) and eIF3 promotes translation initiation in mammals. Results: Human eIF3 subunits -c, -d, and -e interact with two subdomains in eIF4G. Conclusion: Multiple contacts between eIF3 and eIF4G are required for mRNA recruitment to the human ribosome. Significance: Characterizing the eIF3-eIF4G interface might reveal a new regulatory mechanism and provide novel therapeutic targets.

Recruitment of mRNA to the 40S ribosomal subunit requires the coordinated interaction of a large number of translation initiation factors. In mammals, the direct interaction between eukaryotic initiation factor 4G (eIF4G) and eIF3 is thought to act as the molecular bridge between the mRNA cap-binding complex and the 40S subunit. A discrete ~90 amino acid domain in eIF4G is responsible for binding to eIF3, but the identity of the eIF3 subunit(s) involved is less clear. The eIF3e subunit has been shown to directly bind eIF4G, but the potential role of other eIF3 subunits in stabilizing this interaction has not been investigated. It is also not clear if the eIF4A helicase plays a role in stabilizing the interaction between eIF4G and eIF3. Here, we have used a fluorescence anisotropy assay to demonstrate that eIF4G binds to eIF3 independently of eIF4A binding to the middle region of eIF4G. By using a site-specific cross-linking approach, we unexpectedly show that the eIF4G-binding surface in eIF3 is comprised of the -c, -d and -e subunits. Screening multiple cross-linker positions reveals that eIF4G contains two distinct eIF3-binding subdomains within the previously identified eIF3-binding domain. Finally, by employing an eIF4G-dependent translation assay, we establish that both of these subdomains are required for efficient mRNA recruitment to the ribosome and stimulate translation. Our study reveals unexpected complexity to the eIF3-eIF4G interaction that provides new insight into the regulation of mRNA recruitment to the human ribosome.

*This work was supported, in whole or in part, by National Institutes of Health Grant R01GM092927 and Training Grant T32 GM007377 from the NIGMS (to N.V.).

†To whom correspondence should be addressed: Dept. of Molecular and Cell Biology, College of Biological Sciences, University of California, Davis, CA 95616. Tel.: 530-752-1716; Fax: 530-752-3085; E-mail: csfraser@ucdavis.edu.

The abbreviations used are: eIF, eukaryotic initiation factor; PIC, pre-initiation complex; UTR, untranslated region.

1 The importance of this interaction for controlling translation is implied from the observation that the affinity between eIF4G and eIF3 is increased by insulin treatment in vivo (15). Specifically, the activation of mTOR complex 1 (mTORC1) is required to stabilize the interaction between these binding partners (15, 16). It has also been suggested that eIF4A and eIF3 may bind in a cooperative manner to eIF4G since a greater amount of eIF3 can be immunoprecipitated with eIF4G in the presence of eIF4A using purified components (11). However, this has not been confirmed by using a quantitative binding assay.

Human eIF4G has been shown to possess a distinct ~90 amino acid domain that forms a high affinity interaction with the eIF3 complex in vitro (11). This binding domain can compete with full-length eIF4G for binding to eIF3, implying that this region is indeed sufficient for binding eIF3 (11). The importance of this interaction for translation has been investigated by in vitro and in vivo translation assays using an eIF4G construct containing a quadruple alanine mutation in the eIF3 binding domain (17). This study showed that the mutant is less active in stimulating translation in a protease treated in vitro reticulo-
Human $eIF4G$ Binds to Multiple $eIF3$ Subunits

**Experimental Procedures**

Recombinant Expression of $eIF4G$ Constructs—The number of $eIF4G$ constructs used in this work is based on UniProt entry Q04637, although our constructs contain a glutamine modification. These mutations do not affect $eIF3$ or $eIF4A$.

To further characterize the $eIF4G$-binding surface in $eIF3$, we have employed two different cross-linking approaches that utilize a minimum binding domain in $eIF4G$ and intact purified $eIF3$. Our data reveal that three subunits of $eIF3$ (eIF3c, -d, and -e) form a binding surface for $eIF4G$. We further show that the previously identified $eIF3$-binding domain in $eIF4G$ is comprised of two distinct subdomains that interact with these $eIF3$ subunits. Finally, we use an $eIF4G$-dependent reticulocyte lysate system to show that both of these subdomains are required to stimulate translation initiation in vitro.

**Human $eIF4G$ Binds to Multiple $eIF3$ Subunits**

Researchers have employed two different cross-linking approaches that utilize a minimum binding domain in $eIF4G$ and intact purified $eIF3$. Our data reveal that three subunits of $eIF3$ (eIF3c, -d, and -e) form a binding surface for $eIF4G$. We further show that the previously identified $eIF3$-binding domain in $eIF4G$ is comprised of two distinct subdomains that interact with these $eIF3$ subunits. Finally, we use an $eIF4G$-dependent reticulocyte lysate system to show that both of these subdomains are required to stimulate translation initiation in vitro.

**Affinity Gel (30 kDa)** is washed twice in 500 μl of buffer D then added and incubated for 1 h at 4 °C. The resin is then incubated with 10 μg of recombinant $eIF4G$ for at least 1 h in 50 μl of buffer D, washed twice in 100 μl buffer E (buffer E: 20 mM Heps pH 7.5, 200 mM KCl, 10% glycerol, 0.5 mM DTT) and once in 100 μl of buffer D. Human $eIF4A$ (10 μg) or $eIF3$ (10 μg) is then incubated with the photoactivatable cross-linker Sulfo-NHS-Diazirine (SAD) (Thermo Scientific) for fluorescence polarization and cross-linking assays, respectively. All $eIF4G$ proteins are first dialyzed for 2 h at 4 °C into buffer C (buffer C: 20 mM KCl, 10% glycerol, 1 mM TCEP), $eIF4G$ (~35 μM) is then incubated with a 20-fold molar excess of fluorescein-5-maleimide and $eIF4G$ at room temperature for 30 min. Excess modification compounds are removed by dialysis or by purifying labeled proteins using HiTrap Heparin HP or SP-Sepharose columns (GE Healthcare).

**Coimmunoprecipitation—EZ view Red Anti-FLAG M2 Affinity Gel (30 μl)(Sigma-Aldrich) is washed twice in 500 μl of buffer D (buffer D: 20 mM Heps pH 7.5, 100 mM KCl, 10% glycerol, and 0.5 mM DTT).** Resin is then incubated with 10 μg of recombinant $eIF4G$ for at least 1 h in 50 μl of buffer D, washed twice in 100 μl buffer E (buffer E: 20 mM Heps pH 7.5, 200 mM KCl, 10% glycerol, 0.5 mM DTT, and 0.5% TritonX-100) and once in 100 μl of buffer D. Human $eIF4A$ (10 μg) or $eIF3$ (10 μg) is then incubated with the photoactivatable cross-linker Sulfo-NHS-Diazirine (SAD) (Thermo Scientific) for fluorescence polarization and cross-linking assays, respectively. All $eIF4G$ proteins are first dialyzed for 2 h at 4 °C into buffer C (buffer C: 20 mM KCl, 10% glycerol, 1 mM TCEP), $eIF4G$ (~35 μM) is then incubated with a 20-fold molar excess of fluorescein-5-maleimide and $eIF4G$ at room temperature for 30 min. Excess modification compounds are removed by dialysis or by purifying labeled proteins using HiTrap Heparin HP or SP-Sepharose columns (GE Healthcare).

**Fluorescence Polarization Binding Assay—**Fluorescence polarization is measured in a Victor X5 Multilabel Plate Reader (Perkin Elmer), similar to previously published studies (25–27). Each binding reaction is set up in buffer D and allowed to reach equilibrium at 30 °C for 5 min prior to fluorescence polarization measurements. Reactions contained 10 nM $eIF4G$-FL, an $eIF3$ concentration in the range of 0–1.5 μM, and were conducted in the presence or absence of 1 μM $eIF4A$, which is a saturating amount based on previously published affinity data (28). Each $K_d$ measurement is reported as the average of at least 3 replicates.
Human eIF4G Binds to Multiple eIF3 Subunits

three experiments ± S.E. The figures show representative experiments.

BoxB Tethered Translation Assay—The transcription template for the BoxB tethered assay is a derivative of our previously described template, and was purchased from GenScript (10). The 5′-UTR, which is located between XhoI and HindIII restriction sites (underlined), has been replaced with the human beta globin 5′-UTR sequence as follows: CTGCGAGCATTTGCTTTCGACAACTGTGTCTACTAGCAACCTCAACACGACACCCAGCCT. The template is transcribed in vitro and purified using phenol-chloroform extraction following standard protocols. Functional assays are carried out in 20-μl reactions containing 65% nuclease-treated rabbit reticulocyte lysate, 20 μM amino acid mixture minus leucine (Promega), 20 μM amino acid mixture minus methionine (Promega), 0.8 units/μl Recombinant RNasin Ribonuclease Inhibitor (Promega), 45 mM potassium chloride, 115 mM potassium acetate, 1.7 mM magnesium acetate, 0.5 μM uncapped RNA template, and 1 μM λ-eIF4G. Reactions are incubated at 30 °C for 30 min prior to luminescence measurement for 10 s on the Victor X5 Multilabel Plate Reader (Perkin Elmer) by addition of Renilla luciferase substrate (Promega), as described previously (10). We normalized luciferase activity to a reaction containing no λ-eIF4G, and presented the average of 4 reactions ± S.E.

Cotranslational Benzophenone Cross-linker Incorporation and Expression of eIF4G1011–1104 Using Amber Mutants—The benzophenone cross-linker, in the form of p-Benzoylphenylalanine (Bpa; BaChem), is incorporated into recombinant eIF4G1011–1104 using the pEVOL plasmid that was kindly provided by Dr. Peter Schultz (The Scripps Research Institute) (29). Single amber codon (TAG) mutations are introduced directed mutagenesis at intervals of 600 nucleotides. The template is transcribed in vitro and purified using phenol-chloroform extraction following standard protocols. Functional assays are carried out in 20-μl reactions containing 65% nuclease-treated rabbit reticulocyte lysate, 20 μM amino acid mixture minus leucine (Promega), 20 μM amino acid mixture minus methionine (Promega), 0.8 units/μl Recombinant RNasin Ribonuclease Inhibitor (Promega), 45 mM potassium chloride, 115 mM potassium acetate, 1.7 mM magnesium acetate, 0.5 μM uncapped RNA template, and 1 μM λ-eIF4G. Reactions are incubated at 30 °C for 30 min prior to luminescence measurement for 10 s on the Victor X5 Multilabel Plate Reader (Perkin Elmer) by addition of Renilla luciferase substrate (Promega), as described previously (10). We normalized luciferase activity to a reaction containing no λ-eIF4G, and presented the average of 4 reactions ± S.E.

RESULTS

Human eIF4G Binds to eIF3 Independently of eIF4A—To quantitatively determine if eIF4G binding to eIF3 is regulated by eIF4A in the absence of the 40S ribosomal subunit, we used anisotropy-based equilibrium-binding assay that utilizes highly purified components. To this end, we generated a FLAG-tagged recombinant eIF4G truncation spanning amino acids 711 to 1104 (eIF4G711–1104), which includes the central eIF4A and eIF3 binding sites (Fig. 1A). To fluorescently modify this eIF4G truncation, we genetically encoded a single cysteine (S1041C) into the protein so that it can be site-specifically modified with fluorescein. To verify that this single cysteine eIF4G construct binds to eIF4A and eIF3 we employed a coimmunoprecipitation assay, as described under “Experimental Procedures.” Both eIF4A and eIF3 interact specifically with the FLAG-tagged eIF4G711–1104 truncation (Fig. 1, B and D). Removal of the eIF3 binding domain (eIF4G711–1104-Bpa) does not prevent eIF4A binding, but completely abolishes eIF3 binding (Fig. 1, B and D). Consistent with this and previous data (11, 13), we find that a 93 amino acid region of eIF4G (eIF4G711–1104) is sufficient for binding to eIF3 in our coimmunoprecipitation assay (Fig. 1C).

To quantitatively determine the affinity of the eIF4G-eIF3 interaction, we measured the anisotropy of fluorescein-labeled eIF4G711–1104 in the presence of an increasing concentration of purified eIF3. Anisotropy is converted into the fraction of
elf4G711–1104 bound at each elf4G concentration (Fig. 1E). Binding studies were conducted in the absence or presence of a saturating amount of elf4A to determine if elf4A influences the affinity of elf4G711–1104 to elf3. Our data show that the 132 ± 12 nM equilibrium dissociation constant \(K_d\) for the elf4G711–1104-elf3 complex in the absence of elf4A is only slightly reduced upon the addition of a saturating amount of elf4A (91 ± 19 nM; Fig. 1E). This strongly implies that the elf4A-binding domain directly upstream of the elf3-binding domain does not contribute to elf3 affinity, at least in the absence of the 40S subunit. This data does not eliminate the possibility that the C-terminal elf4A-binding site of elf4G may influence elf3 binding, although this is unlikely since this secondary binding site is known to have a much lower affinity for elf4A (28).

Site-specific Cross-linking of elf4G1011–1104 to Purified Human elf3—Affinity purification of a human elf4G1011–1108 truncation to partially proteolyzed elf3 has identified the elf3e subunit as a direct binding site for elf4G (23). To circumvent the use of partially proteolyzed elf3 to study the interaction between elf4G and elf3, we have generated a site-directed cross-linking strategy that successfully cross-links recombinant elf4G1011–1104 to intact highly purified elf3 containing all 13 subunits. To this end, we modified FLAG-tagged elf4G1011–1104 with a photoactivatable cross-linker, sulfo-NHS-Diazirine (SDA; Fig. 2A), using a single cysteine engineered into the elf3-binding domain of the protein (S1041C).

Photocross-linking is carried out between SDA-labeled elf4G1011–1104 and purified elf3 (Fig. 2B), as described under “Experimental Procedures.” The optimal UV exposure time was determined by a time course experiment using purified elf3 (1 \(\mu\)M) and a 10-fold molar excess of elf4G1011–1104-SDA to ensure maximum cross-linking efficiency. Covalently cross-linked species are identified by separation on SDS-PAGE followed by Western blotting with an anti-FLAG monoclonal antibody. Specific cross-linking between elf4G and an elf3 subunit is indicated by a molecular weight shift of an elf3 subunit (Fig. 1E). We observe a time-dependent appearance of 3 major cross-linked bands that possess apparent molecular masses of ~130, 80, and 60 kDa (Fig. 2B, filled arrows). Importantly, each cross-linked product forms only in the presence of the SDA cross-linker and elf3, confirming the formation of specific elf3-elf4G-bound complexes (data not shown). To further ensure that the elf3-elf4G cross-linked products are a result of a specific interaction not influenced by the presence of the SDA cross-linker, we performed a competition assay with unmodified elf4G1011–1104. Increasing amounts of unmodified elf4G1011–1104 effectively competes for the same elf3 binding sites as elf4G1011–1104-SDA, thereby reducing elf4G1011–1104-SDA cross-linking (Fig. 2D). In addition, as shown in Figure 2C, eIF3 binds to eIF3-eIF4G711–1104 complex in the absence or presence of eIF4A. The Kd values shown are the average of at least 3 trials ± S.E.
Human eIF4G Binds to Multiple eIF3 Subunits

A - eIF3c
+ - -
- + -
- - +
- - -
- + +

B - eIF3b
+ - -
- + -
- - +
- - -
- + +

C - eIF3d
+ - -
- + -
- - +
- - -
- + +

D - eIF3l
+ - -
- + -
- - +
- - -
- + +

E - eIF3e
+ - -
- + -
- - +
- - -
- + +

F - eIF3f
+ - -
- + -
- - +
- - -
- + +

FIGURE 3. eIF4G1011–1104-SDA cross-links to eIF3 subunits -c, -d, and -e. A–F, eIF4G1011–1104 was incubated with eIF3 and exposed to UV light as described under “Experimental Procedures.” Cross-linking reactions are separated by SDS-PAGE followed by immunoblot analysis for specific eIF3 subunits as indicated. Control lanes containing only eIF3 are shown in the third lane in each blot. Filled arrows in panels A, C, D, and E indicate eIF4G1011–1104-SDA cross-linking to subunits eIF3c, -d, -e, and to a lesser extent, -l. Empty arrows denote uncross-linked eIF3 subunits. The asterisk (*) in B denotes a degradation product of eIF3b, which is likely an artifact from the eIF3 purification process.

By subtracting the ~15 kDa contributed by eIF4G1011–1104, our data imply that the largest cross-linked band corresponds to either eIF3b or eIF3c; the 80 kDa band corresponds to either eIF3d or eIF3l; and the 65 kDa band corresponds to eIF3e or eIF3f.

The eIF3 Binding Domain in eIF4G Interacts with eIF3c, -d, and -e Subunits—To unambiguously determine which eIF3 subunits specifically cross-link to eIF4G1011–1104-SDA we used a number of commercially available eIF3 subunit specific antibodies (Fig. 3). To establish the identity of the largest cross-linked band, we probed with eIF3b and eIF3c specific antisera. Although the majority of each eIF3 subunit migrates at their expected molecular weight (Fig. 3), we detect an appreciable amount of eIF3c migrating at ~S1041X (Fig. 3, lane 1, filled arrow). Since no corresponding change in eIF3b migration is detected, this indicates that eIF4G1011–1104 directly interacts with, or is positioned very close to, eIF3c upon eIF4G-eIF3 complex formation. Repeating this process with eIF3d and eIF3l specific antisera, we find that eIF3d is identified as the major ~80 kDa cross-linked subunit (Fig. 3, C and D). We also find that a very small, but reproducible, amount of eIF3l is also detected in the ~80 kDa cross-linked band. Although not as strong as the amount of eIF3d detected, this implies that one or both of eIF3d and eIF3l may be involved in binding eIF4G. Probing with eIF3e and eIF3f specific antiserum unambiguously identifies eIF3e as the ~60 kDa cross-linked species (Fig. 3, E and F).

eIF4G Contains 2 Subdomains that Interact with eIF3c and -d or eIF3e—The diazirene active group of the SDA cross-linker is situated at the end of a 3.9 Å spacer arm. While this is a relatively short distance, it is possible that some of the subunit interactions we observe may be due to the length and flexibility of the cross-linker. To address this, we employed an alternate method to confirm which of the eIF3c, -d, -l, and -e subunits interact with eIF4G. The unnatural amino acid p-benzylphenylalanine (Bpa) has been used recently to explore multi-factor interactions in vivo and in vitro in bacteria, yeast, and mammalian systems (31–36). Bpa is photoactivatable at 350–360 nm, preferentially reacts with C-H bonds versus water, and can be activated reversibly. If no suitable reaction partner is readily available following activation, Bpa can relax to its ground state (37, 38). Importantly, the Bpa cross-linker distance is ~3 Å and is less flexible than SDA (39) (Fig. 4A). We have employed a recently developed method to site-specifically incorporate Bpa into a recombinant protein using a bacterial expression system (29). This approach employs an orthogonal tRNA/tRNA-synthetase pair for Bpa incorporation into a single amber stop codon mutant site that is engineered into the gene of interest.

We generated 10 unique amber mutant constructs of eIF4G1011–1104 mRNA for Bpa incorporation, as described under “Experimental Procedures.” Six of the ten mutants efficiently express in bacteria and appropriately span the length of the eIF3-binding domain in eIF4G (eIF4G1011–1104*—Fig. 4B). Small gaps in the coverage of this span are due to constructs that were unable to express with Bpa incorporation; these constructs are expressed only as a truncation ending at the site of the amber stop codon as would be expected if Bpa is not incorporated and peptide termination occurs. Cross-linking reactions using eIF4G1011–1104-Bpa are carried out as described for the cross-linking of eIF4G1011–1104-SDA to eIF3. Immunoblot analysis of the cross-linked products unambiguously identifies the close proximity of eIF3 subunits -c, -d, and -e to eIF4G (Fig. 4D). The weak eIF3l cross-linked band observed using eIF4G1011–1104-SDA (Fig. 4D, lane 1, Fig. 4D, lane 7) is not observed when eIF4G1011–1104-Bpa is used (Fig. 4D, middle panel). This strongly implies that the eIF3l subunit is near, but does not directly contribute to the eIF4G binding surface.

By testing several Bpa incorporation sites, we are able to reveal distinct local areas of preferential binding to the different eIF3 subunits. Subunits eIF3c and eIF3d bind to the N-terminal portion of the eIF3-binding region, with peak cross-linking intensity at S1041X (Fig. 4C, top panel, lane 3). Interestingly, S1041 is the same site used for attachment of the SDA cross-linker (Fig. 4C, lane 7) and for the fluorescence polarization assays (Fig. 1E). Subunit eIF3e shows strong cross-linking intensity toward to the C-terminal end of the eIF3 binding region (K1071X; Fig. 4C). It is worth noting that the doublets of cross-linked eIF3d and eIF3e observed with eIF4G1011–1104-
FIGURE 4. Identification of 2 independent eIF3-binding subdomains in eIF4G. A, chemical structure of the p-benzoyl-phenylalanine cross-linker (Bpa, Bachem) used for genetic incorporation. B, diagram of 4G1011–1104, with sites of amber mutations used for Bpa incorporation. The regions of maximal eIF3 subunit cross-linking from panels C and D are also depicted. C and D, results of eIF3 cross-linking with 4G1011–1104-Bpa labeled at single indicated sites (lanes 1–6), 4G1011–1104-SDA (lane 7), or eIF3 alone (lane 8) are shown. The eIF3 subunit specific antibody is indicated to the right of each panel. Filled arrows indicate cross-linked eIF3 subunits, while empty arrows indicate uncross-linked subunits. Asterisk (*) in D indicates an eIF3b degradation product.

SDA (Fig. 3, C and E) are not apparent with eIF4G1011–1104-Bpa. This indicates that the Bpa cross-linker is a more precise tool than the more flexible SDA cross-linker. These experiments confirm the formation of an eIF4G-binding surface consisting of eIF3c, -d, and -e subunits. Furthermore, our data establish distinct regions within eIF4G that preferentially bind each subunit at this interface, likely contributing to the overall stabilization of this interaction.

Both Subdomains in eIF4G Interact with eIF3 for Efficient Ribosome Recruitment—To test the importance of the eIF4G-eIF3 interaction in promoting mRNA recruitment to the ribosome, we have employed a tethered-mRNA assay previously used to identify the functional “core” region of eIF4G in vivo (14). This assay employs an engineered boxB hairpin in the 5′-UTR of an mRNA to specifically recruit a 22 amino acid sequence of the bacteriophage λ transcription anti-terminator protein N (λ N-amino acids 1–22). We have modified this assay for use in a reticulocyte lysate system so that we can precisely test the role of eIF4G truncations in promoting mRNA recruitment to the ribosome (Fig. 5, A and B). This approach avoids the prerequisite of manipulating endogenous eIF4G protein activity using a viral protease, as only λ-eIF4G can be recruited to the uncapped boxB reporter used in this assay (17). Nuclease-treated rabbit reticulocyte lysate is supplemented with 1 μM of a λ-eIF4G truncation together with 0.5 μM boxB reporter mRNA followed by measuring protein synthesis for 30 min at 30 °C. The amount of synthesized luciferase for each λ-eIF4G construct is measured and normalized to lysates supplemented with boxB reporter RNA in the absence of λ-eIF4G (Fig. 5C, Neg). Our data show that a truncation of eIF4G that begins at the 2A protease site and contains both the eIF4A and eIF3 binding domains stimulates translation roughly 35-fold (λ-eIF4G682–1104; Fig. 5C).

To establish the relative contributions of the eIF3c, -d, and -e subunits in stimulating translation, we separated the eIF3-binding region into distinct eIF3c/d (eIF4G1011–1051) and eIF3e (eIF4G1052–1104) binding subdomains (Fig. 5B). To specifically

FIGURE 5. eIF3-binding subdomains in eIF4G both stimulate mRNA recruitment and translation. A, an eIF4G-dependent translation assay is depicted that mediates mRNA recruitment to the ribosome independent of cap binding. A boxB hairpin (BoxB HP) upstream of a beta globin 5′-UTR sequence specifically recruits λ-tagged eIF4G682–1104 to the Renilla luciferase reporter. A stable inhibitor hairpin at the 5′-end is positioned to prevent 5′-end dependent mRNA recruitment to the ribosome. B, diagrams of eIF4G constructs used, which are N-terminally HIS-tagged and fused with the RNA binding domain of bacteriophage λ transcription anti-terminator protein N. Amino acid numbering is shown to indicate each eIF4G truncation construct used. Line breaks in the diagrams represent the RNA and eIF4A binding domains of human eIF4G. C-terminal truncations of the eIF3-binding domain are used to define functional subdomains of this region. A summary of the relative luciferase translation over background (no λ-eIF4G added, Neg) that has been normalized to 1 is shown as the average of 4 trials ± S.E. C, plot of relative luciferase translation following incubation of the boxB Renilla luciferase reporter in nuclease treated reticulocyte lysate for 30 min at 30 °C. Each bar represents the relative luciferase activity in the absence (Neg) or presence of λ-eIF4G truncations, as indicated. Luciferase translation is shown as the average of 4 trials ± S.E.
Human eIF4G Binds to Multiple eIF3 Subunits

determine the contribution of the eIF3e subunit-binding domain, we created three separate truncations that remove 18 amino acids (Δ-eIF4G682–1006), 28 amino acids (Δ-eIF4G682–1077), or the entire eIF3e-binding subdomain (Δ-eIF4G682–1051). Partial or complete deletion of the eIF3e-binding domain retains roughly 50–65% translation activity compared with λ-eIF4G682–1104 (Fig. 5C). Further deletion of the eIF3c/d-binding domain (Δ-eIF4G682–1001) completely abolishes luciferase translation, indicating that both eIF3c/d and eIF3e-binding domains are required for efficient mRNA recruitment and translation.

**DISCUSSION**

Human eIF4G is a scaffold protein that contains binding sites for other initiation factors, including PABP, eIF4E, eIF4A, and eIF3. The importance of individual initiation factor binding sites in eIF4G on mRNA translation has been investigated *in vitro* and *in vivo* (17). The relative contribution of the eIF4G–PABP interaction appears to be relatively modest in both yeast and mammalian mRNA translation (17, 40, 41). In contrast, the interactions of eIF4E and eIF4A with eIF4G play a critical role in mRNA recruitment, restructuring and translation (10, 42, 43). More recently, it has become evident that the interaction of eIF4G with RNA is also required for efficient mRNA recruitment and translation initiation (44, 45). The exact contribution of the eIF4G-eIF3 interaction in mRNA recruitment and scanning is less clear. No direct interaction between eIF4G and eIF3 has been identified in yeast, but a direct interaction has been identified in mammals and proposed to play an important role in mRNA recruitment and translation (12). Precise truncation analysis has revealed a ~90 amino acid region of human eIF4G that constitutes the minimal eIF3-binding domain in the absence of the 40S ribosomal subunit (11, 13).

In this study, we have further characterized the direct interaction between human eIF4G and eIF3. Using a quantitative binding assay, we have established that the affinity between eIF4G and eIF3 is not altered by the presence of eIF4A, at least in the absence of the 40S ribosomal subunit (Fig. 1). This finding is in contrast to previous data using an immunoprecipitation assay that suggested mutually cooperative binding between eIF4A and eIF3 with eIF4G (11). Since it is not possible to determine the affinity between these initiation factors by using an immunoprecipitation assay, our data strongly suggest that eIF4A and eIF3 bind independently to the middle region of human eIF4G. It is worth noting that both of these studies did not include the C-terminal HEAT repeat that is known to provide a second binding site for eIF4A (46). It therefore remains to be determined if the C-terminal domain of eIF4G may in turn regulate eIF3 binding in an eIF4A-dependent manner.

Previous work employed a pull-down assay to determine that eIF4G binds directly to the eIF3e subunit of the eIF3 complex (23). However, this approach is hindered by the requirement to use partially proteolyzed eIF3 to identify eIF4G-interacting subunits. This raises a fundamental question about whether additional eIF3 subunits may help to stabilize the interaction with eIF4G. To address this, we generated two site-specific cross-linking assays to further explore which eIF3 subunits provide a binding surface for eIF4G. Importantly, these cross-linking assays use intact purified eIF3 so that a complete interaction map between these initiation factors can be obtained. Our data unambiguously show that eIF3c, eIF3d, and eIF3e together form a binding surface for eIF4G (Figs. 3 and 4). By using multiple cross-linker positions in the eIF3 binding domain of eIF4G, we are able to generate an interaction model that reveals two distinct binding subdomains in eIF4G that bind to different eIF3 subunits. One subdomain consists of a 40 amino acid region (1011–1051) that binds to eIF3c and eIF3d. An additional subdomain consists of a 52 amino acid region (1052–1104) that binds to eIF3e. Our data imply that the eIF3c and eIF3d-binding sites in eIF4G may overlap, although it should be noted that the resolution of our cross-linking approach is not able to unambiguously determine this. It is also possible that there is a mixture of either eIF3c or eIF3d binding to eIF4G in different cross-linked complexes. A subunit interaction map of human eIF3 previously identified eIF3c, eIF3d and eIF3e as interacting subunits (19). Using this model, our data provide new constraints to show the eIF4G binding interaction with eIF3c, eIF3d and eIF3e in the context of the complete eIF3 subunit interaction map (Fig. 6). It is interesting to note that eIF3f interacts with eIF3e (19, 47). This may account for the small amount of cross-linking observed between eIF4G and eIF3f when using the SDA cross-linker (Fig. 3). Interestingly, it was recently shown using a reconstituted eIF3 complex that the presence of eIF3d was necessary to provide a high affinity interaction with eIF4G (20). These data correspond well with our new interaction model that implicates eIF3d as an important component of the eIF4G binding site in the eIF3 complex.

We employed an eIF4G-dependent translation assay to establish the biological importance of our interaction model. Previous work suggested that the eIF3e subunit likely provides the highest affinity interaction with eIF4G (23). Surprisingly, deletion of the eIF3e-binding domain in eIF4G maintains 50–65% translation activity in our assay. Further removal of the eIF3c/d-binding domain in eIF4G eliminates this remaining...
activity. This implies that both of the eIF3 interacting subdomains play important roles in providing the high affinity interaction with eIF3 that is necessary to promote translation initiation. It is important to note that our translation assay directly tethers eIF4G to the mRNA independently of the cap structure. It will therefore be important in the future to determine the exact roles of these two eIF3 interacting subdomains in eIF4G using a cap-dependent translation assay. This will likely require the generation of point mutations in eIF4G that can specifically disrupt individual eIF3 subunit interactions in the context of full-length eIF4G. We anticipate that our interaction model now provides a suitable framework to aid the design of such mutations. In addition, since the interaction is regulated in an mTOR-dependent manner, it is plausible that one or more phosphorylation sites on eIF4G and/or eIF3 may be responsible for altering the affinity between these factors. Our data now suggest that as well as eIF4G, the eIF3c, -d, and -e subunits of the eIF3 complex are possible candidates to be phosphorylated in an mTOR-dependent manner.

Acknowledgments—We thank Erioch Baldwin and the Fraser laboratory for many insightful comments during this study. We gratefully acknowledge David King and Sean Studer for helpful advice regarding cross-linking protocols. We are indebted to Peter Schultz for generously providing the pEVOL plasmid for benzophenone incorporation.

REFERENCES

1. Fraser, C. S. (2009) The molecular basis of translational control. Prog. Mol. Biol. Transl. Sci. 90, 1–51
2. Gingras, A. C., Raught, B., and Sonenberg, N. (1999) eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu. Rev. Biochem. 68, 913–963
3. Hinnebusch, A. G., and Lorsch, J. R. (2012) The mechanism of eukaryotic translation initiation: new insights and challenges. Cold Spring Harb. Perspect. Biol. 4
4. Parsyan, A., Svitkin, Y., Shahbazian, D., Gkogkas, C., Lasko, P., Merrick, W. C., and Sonenberg, N. (2011) mRNA helicases: the tacticians of translational control. Nat. Rev. Mol. Cell Biol. 12, 235–245
5. Özes, A. R., Feoktistova, K., Avanzino, B. C., and Fraser, C. S. (2011) Duplex unwinding and ATPase activities of the DEAD-box helicase eIF4A are coupled by eIF4G and eIF4B. J. Mol. Biol. 412, 674–687
6. Rozen, F., Edery, I., Meerovitch, K., Dever, T. E., Merrick, W. C., and Sonenberg, N. (1990) Bidirectional RNA helicase activity of eukaryotic translation initiation factors 4A and 4F. Mol. Cell. Biol. 10, 1134–1144
7. Rogers, G. W., Jr., Richter, N. J., Lima, W. F., and Merrick, W. C. (2001) Modulation of the helicase activity of eIF4A by eIF4B, eIF4F, and eIF4G. J. Biol. Chem. 276, 30914–30922
8. Abramson, R. D., Dever, T. E., and Merrick, W. C. (1988) Biochemical evidence supporting a mechanism for cap-independent and internal initiation of eukaryotic mRNA. J. Biol. Chem. 263, 6016–6019
9. Bi, X., Ren, J., and Goss, D. J. (2000) Wheat germ translation initiation factor eIF4F affects eIF4A and eIF4F helicase activity by increasing the ATP binding affinity of eIF4A, Biochemistry 39, 5758–5765
10. Feoktistova, K., Tuvshintugs, E., Do, A., and Fraser, C. S. (2013) Human eIF4E promotes mRNA restructuring by stimulating eIF4A helicase activity. Proc. Natl. Acad. Sci. U.S.A. 110, 13339–13344
11. Korneeva, N. L., Lamphere, B. J., Hennigan, F. L., and Rhoads, R. E. (2000) Mutually cooperative binding of eukaryotic translation initiation factor (eIF) 3 and eIF4A to human eIF4G-1. J. Biol. Chem. 275, 41369–41376
12. Lamphere, B. J., Kirchwey, R., Skern, T., and Rhoads, R. E. (1995) Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. Implications for cap-dependent and cap-independent translational initiation. J. Biol. Chem. 270,
transporters of Staphylococcus aureus. J. Biol. Chem. 287, 16477–16487
33. Ozawa, K., Horan, N. P., Robinson, A., Yagi, H., Hill, F. R., Jergic, S., Xu, Z. Q., Loscha, K. V., Li, N., Tehei, M., Oakley, A. J., Otting, G., Huber, T., and Dixon, N. E. (2013) Proofreading exonuclease on a tether: the complex between the E. coli DNA polymerase III subunits alpha, epsilon, theta, and beta reveals a highly flexible arrangement of the proofreading domain. Nucleic Acids Res. 41, 5354–5367
34. Shiota, T., Mabuchi, H., Tanaka-Yamano, S., Yamano, K., and Endo, T. (2011) In vivo protein-interaction mapping of a mitochondrial translocator protein Tom22 at work. Proc. Natl. Acad. Sci. U.S.A. 108, 15179–15183
35. Grunbeck, A., Huber, T., Sachdev, P., and Sakmar, T. P. (2011) Mapping the ligand-binding site on a G protein-coupled receptor (GPCR) using genetically encoded photocrosslinkers. Biochemistry 50, 3411–3413
36. Wu, C. C., Lin, Y. C., and Chen, H. T. (2011) The TFIIF-like Rpc37/53 dimer lies at the center of a protein network to connect TFIIIC, Bdp1, and the RNA polymerase III active center. Mol. Cell. Biol. 31, 2715–2728
37. Kauer, J. C., Erickson-Viitanen, S., Wolfe, H. R., Jr., and DeGrado, W. F. (1986) p-Benzoyl-L-phenylalanine, a new photoreactive amino acid. photocrosslinking of calmodulin with a synthetic calmodulin-binding peptide. J. Biol. Chem. 261, 10695–10700
38. Dormán, G., and Prestwich, G. D. (1994) Benzophenone photophores in biochemistry. Biochemistry 33, 5661–5673
39. Farrell, I. S., Toroney, R., Hazen, J. L., Mehl, R. A., and Chin, J. W. (2005) Photo-cross-linking interacting proteins with a genetically encoded benzophenone. Nat Methods 2, 377–384
40. Searfoss, A., Dever, T. E., and Wickner, R. (2001) Linking the 3‘ poly(A) tail to the subunit joining step of translation initiation: relations of Pab1p, eukaryotic translation initiation factor 5b (Fun12p), and Ski2p-Slh1p. Mol. Cell. Biol. 21, 4900–4908
41. Park, E. H., Walker, S. E., Lee, J. M., Rothenburg, S., Lorsch, J. R., and Hinnebusch, A. G. (2011) Multiple elements in the eIF4G1 N-terminus promote assembly of eIF4G1-PABP mRNPs in vivo. EMBO J. 30, 302–316
42. Pause, A., Belsham, G. J., Gingras, A. C., Donzé, O., Lin, T. A., Lawrence, J. C., Jr., and Sonenberg, N. (1994) Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5′-cap function. Nature 371, 762–767
43. Pause, A., Méthot, N., Svitkin, Y., Merrick, W. C., and Sonenberg, N. (1994) Dominant negative mutants of mammalian translation initiation factor eIF-4A define a critical role for eIF-4F in cap-dependent and cap-independent initiation of translation. EMBO J. 13, 1205–1215
44. Prévôt, D., Decimo, D., Herbreteau, C. H., Roux, F., Garin, J., Darlix, J. L., and Ohlmann, T. (2003) Characterization of a novel RNA-binding region of eIF4G1 critical for ribosomal scanning. EMBO J. 22, 1909–1921
45. Yanagiya, A., Svitkin, Y. V., Shibata, S., Mikami, S., Imataka, H., and Sonenberg, N. (2009) Requirement of RNA binding of mammalian eukaryotic translation initiation factor 4G(eIF4G) for efficient interaction of eIF4E with the mRNA cap. Mol. Cell. Biol. 29, 1661–1669
46. Imataka, H., and Sonenberg, N. (1997) Human eukaryotic translation initiation factor 4G(eIF4G) possesses two separate and independent binding sites for eIF4A. Mol. Cell. Biol. 17, 6940–6947
47. Morris-Desbois, C., Rétý, S., Ferro, M., Garin, J., and Jalinp, P. (2001) The human protein HSFC021 interacts with Int-6 and is associated with eukaryotic translation initiation factor 3. J. Biol. Chem. 276, 45988–45995