Interaction between the NH$_2$-terminal Domain of eIF4A and the Central Domain of eIF4G Modulates RNA-stimulated ATPase Activity*

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The eukaryotic translation factor 4A (eIF4A) is a member of DEAd/DEM-box RNA helicase family, a diverse group of proteins that couples ATP hydrolysis to RNA binding and duplex separation. eIF4A participates in the initiation of translation by unwinding secondary structure in the 5′-untranslated region of mRNAs and facilitating scanning by the 40 S ribosomal subunit for the initiation codon. eIF4A alone has only weak ATPase and helicase activities, but these are stimulated by eIF4G, eIF4B, and eIF4H. eIF4G has two eIF4A-binding sites, one in the central domain (cp$_{c3}$) and one in the COOH-terminal domain (cp$_{c2}$). In the current work, we demonstrate that these two eIF4G domains have different effects on the RNA-stimulated ATPase activity of eIF4A. cp$_{c3}$ stimulates ATP-hydrolytic efficiency by about 40-fold through two mechanisms: lowering $K_m$ by 10-fold and raising $k_{cat}$ by 4-fold. cp$_{c2}$ also stimulates RNA cross-linking to eIF4A in an ATP-independent manner. Studies with eIF4G and eIF4A variants suggest a model by which cp$_{c3}$ alters the conformation of the catalytic site to favor RNA binding. cp$_{c2}$ does not stimulate ATPase activity and furthermore increases both $K_m$ and $k_{cat}$ at saturating RNA concentrations. Both cp$_{c2}$ and cp$_{c3}$ directly interact with the NH$_2$-terminal domain of eIF4A, which possesses conserved ATP- and oligonucleotide-binding motifs, but not with the COOH-terminal domain.

Initiation of translation is the most studied step for regulation of protein synthesis (1, 2). It involves the sequential assembly of initiation complex (43, 48, and 80 S), each step catalyzed by a different class of initiation factors (eIF1–eIF6)$^1$ (3). The rate-limiting step under normal conditions (i.e. absence of viral infection, amino acid starvation, chemical poisoning, etc.) is the recruitment of mRNA to the 43 S complex to form the 48 S complex, which is catalyzed by the eIF4 class of factors. The mammalian eIF4 proteins consist of eIF4A, a 46-kDa ATP-dependent RNA helicase; eIF4B, an 80-kDa protein that stimulates the processivity of eIF4A; eIF4H, a 25-kDa protein that enhances the stimulatory activity of eIF4B; eIF4E, a 25-kDa cap-binding protein; and eIF4G, a 185-kDa protein that binds most factors involved in mRNA recruitment and anchors them to the 40 S subunit through binding to eIF3. These factors are required both to recognize mRNA and to unwind secondary structure in the 5′-untranslated region, which is necessary for 48 S initiation complex formation and scanning for the initiation codon.

Helicases unwind duplex DNA or RNA at the expense of energy derived from NTP hydrolysis (4). Sequence comparisons have classified the helicases into three superfamilies, SFI, SFII, and SFIII (5). There are striking similarities in the tertiary structure of two core domains called 1A and 2A (6, 7). These domains have homology with each other and also with the central region of the RecA protein (8). Domain 1A contains motifs I, Ia, Ic, II, and III, whereas domain 2A contains motifs IV, V, and VI. The most highly conserved motifs are Walker A and B, which possess sequences characteristic of ATPases (9). The ATP-binding site is situated in a cleft between domains 1A and 2A. The bound NTP acts as a cross-bridge pulling NH$_2$- and COOH-terminal domains closer together. Conserved amino acid residues in both domains lining the active site cleft interact with the metal ion and the γ-phosphate group of the bound NTP, thus stabilizing the transition state. Binding and hydrolysis of ATP at this site affect the relative positions of domains 1A and 2A.

eIF4A is a bidirectional ATP-dependent helicase (10). It is the prototype for the DEAd/DEM-box protein family, which falls into SFII (4), and contains eight motifs conserved in nucleic acid helicases (11, 12). Crystallographic analysis of eIF4A shows a two-domain structure with a similar fold to PcrA domains 1A and 2A as well as to the RNA helicase NS3 of hepatitis C virus (13–16), but with connectivity more like adenylate kinase than RecA (6). Extensive mutational analysis has identified motifs involved in substrate binding and coupling of ATP hydrolysis to nucleic acid unwinding (17–20).

Initial studies of eIF4A showed that it is a poor helicase on its own (21–23). However, during initiation of translation, eIF4A acts in concert with other proteins. A complex containing eIF4A, eIF4E, and eIF4G can be isolated by high salt treatment of mammalian ribosomes and is termed eIF4F (24, 25). eIF4A exhibits maximal RNA-unwinding activity in the presence of eIF4F, eIF4B, and eIF4H (26). eIF4B and eIF4H stimulate ATP hydrolysis and processivity of duplex unwinding by promoting RNA binding to the complex (27, 28). eIF4F may affect the helicase activity of eIF4A by increasing its affinity for RNA and by changing its substrate specificity (29, 26). A kinetic model for the function of eIF4A has been proposed in which the enzyme undergoes a cycle of ligand-induced conformational changes (30, 31).
eIF4A has two binding sites for eIF4A (32–34). One is located in the middle portion of eIF4G (cp-c3), which also possesses the RNA- and eIF3-binding sites (Fig. 1A) (32). The other is located in the COOH-terminal portion of eIF4G (cp-c2), which also binds the eIF4E kinases Mnk1 and Mnk2 (35, 36). cp-c3 is sufficient to catalyze cap-independent translation in vivo and in vitro (37, 38). Intriguingly, eIF4G constructs in which either of the two eIF4A-binding sites is eliminated by site-directed mutagenesis are less active than the unmodified construct, suggesting a modulatory role for cp-c3 (39).

In the current study, we investigated the mechanism by which eIF4G stimulates eIF4A activity. In particular, we wished to determine whether both of the eIF4A-binding sites on eIF4G could affect ATPase activity. Furthermore, we examined the effect of eIF4G on the various steps in the catalytic cycle of RNA-dependent ATP hydrolysis. Finally, we sought to determine the functional domains of eIF4A that bind eIF4G.

EXPERIMENTAL PROCEDURES

Materials—mRNA, Sepharose, heparin-Sepharose CL-6B, and a Mono-Q HR 5/5 column were obtained from Amersham Biosciences. Econo-Pac® 10 DG disposable chromatography columns and the Bio-Rad Protein Assay Kit were obtained from Bio-Rad. RNasin was obtained from Promega (Madison, WI). S-Protein-agarose was obtained from Novagen (Madison, WI). Ni²⁺-nitrilotriacetic acid-agarose was obtained from Qiagen (Chatsworth, CA). Bovine serum albumin was obtained from Pierce. Poly(A), ATP, and ADP were obtained from Sigma. The average length of poly(A) was ~500 nucleotides based on size exclusion column chromatography and PAGE. The concentration of all RNAs is given in terms of nucleotide residues.

Construction of Plasmids—Plasmids pTS4G(653–1600), pTS4G(653–1313), pTS4G(917–1118), and pTS4G(917–1118) have been described previously (34). Vectors for the expression of EMCV RNA (termed pTE1 in Ref. 40) were made by inserting a restriction fragment containing the Renilla luciferase gene into pET4A(1–109) and pET4A(110–294) was constructed by inserting a restriction fragment containing the ATPase domain of eIF4A between the XhoI sites of pET-28b (Novagen). pET-4A(917–1118), and S-eIF4G(1118–1600), and the T7-tagged eIF4G fragment T7-eIF4G(653–1600), are a gift from Dr. Hans Trachsel (Bern, Switzerland). Anti-T7 antibody conjugated with alkaline phosphatase were purchased from Novagen.

MALDI TOF MS Analysis of eIF4A Fragments—Mass spectrometric analysis was performed in the LSUHSC-S Research Core Facility on a PerSeptive Biosystems Voyager-DE PRO Biospectrometry work station. Peptides were prepared for MALDI-TOF MS prior to proteolytic digestion by extraction from gel pieces twice with 50-μl portions of 50% acetonitrile and 5% trifluoroacetic acid. Peptides in the extract were dried, dissolved in 15 μl of 50% acetonitrile, 0.1% trifluoroacetic acid and purified on a ZipTip (Millipore Corp., Bedford, MA). They were eluted with 2 μl of 0.1 mg/ml solution of a-cyano-4-hydroxy-trans-cinnamic acid (Sigma) in 50% acetonitrile, 0.1% trifluoroacetic acid and spotted on a MALDI plate. Data were summed over 50–100 acquisitions in delayed extraction mode. Data analysis was performed using the Data Explorer software, version 3.5–4.0. Spectra were subjected to algorithms for base-line correction and a linear fit and post-processed. Calculated and empirical masses were determined using the Peptide Mass tool at the ExPASy Proteomics site on the World Wide Web (www.expasy.org/tools/). Peaks seen in both the sample of interest and also in a blank gel treated identically were eliminated from further consideration. We considered that peptides matched if their masses were within 1 Da for the range 800–10,000 Da.

Immunologic Procedures—Mouse monoclonal anti-eIF4A antibody was a gift from Dr. Hans Trachsel (Bern, Switzerland). Anti-T7 antibody conjugated with alkaline phosphatase were purchased from Novagen. For Western blotting, proteins were transferred after SDS-PAGE to an Immobilon-P membrane (Millipore) using a Mini Trans-Blot cell (Bio-Rad). The membrane was incubated with 1:1000 dilution of anti-T7 antibody in 5% milk in TBS (20 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20, pH 7.5) for 1 h at room temperature, washed three times for 10 min with buffer C, and incubated for 1 h at room temperature with secondary anti-mouse antibody conjugated with alkaline phosphatase (Vector Laboratories, Inc., Burlingame, CA) at a dilution of 1:2000 in 5% milk proteins in buffer C. Blots were developed with the nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate color development reagents (Promega, Madison, WI). eIF4A(1–109) and eIF4A(110–294) were identified by incubating membrane with anti-T7 antibody conjugated with alkaline phosphatase at 1:3000 dilution. Blots were developed as described for eIF4A Western blots.

Protein Binding Assays on S-Protein-Agarose—Binding of S-eIF4G fragments with eIF4A fragments was performed using S-Protein-agarose. After 40 min of preincubation of S-eIF4G fragments with eIF4A on ice, proteins were mixed with at least a 10-fold molar excess of S-Protein-agarose and incubated for 1 h in buffer B, containing 0.3 mg/ml bovine serum albumin at 4 °C. The resin was washed four times with 200-μl aliquots of buffer B. Proteins were eluted in SDS-electrophoresis buffer and analyzed by SDS-PAGE with detection by Coomassie Blue staining or Western blotting.

Results

eIF4A, eIF4A(3R62Q), eIF4A(1–109), and eIF4A(110–294) were expressed in BL21(DE3)pLysS and purified by Ni²⁺-nitrilotriacetic acid-agarose chromatography as described for eIF4G. eIF4A and eIF4A(3R62Q) were further purified by Mono Q chromatography. Prior to ATPase assays, eIF4A samples were diluted with buffer B2, to adjust the final KCl concentration to 100 mM.

RNA Cross-linking—eIF4G fragments with or without eIF4A were incubated in the presence of 32P-labeled EMCV RNA. Reactions (15 μl) containing various recombinant eIF4A fragments (1 μM), various forms of eIF4A (1 μM), 32P-labeled RNA (0.3 mM), RNAs (10 units), buffer B100, 2 mM MgCl₂, 0.25 mM spermidine, and 1 mM ATP were preincubated for 10 min at 37 °C. Reactions were irradiated on ice in a GS Gene Linker UV chamber (Bio-Rad) for 99 s. Then reactions were incubated for 15 min at 37 °C in the presence of RNase A (25 μg/ml) and RNase T1 (0.5 units/μl). Samples were analyzed by SDS-PAGE followed by PhosphorImager detection (Storm; Amersham Biosciences).

Limited Proteolysis—Reactions containing 100 μM eIF4A(1–109) and 500 μM trypsin in buffer B2, were incubated at 25 °C. Reactions were initiated by the addition of protease. Aliquots (5 μl) were removed at various times and made 1 μM in trypsin inhibitor where indicated, and the reaction was quenched in 2% SDS, 0.1% bromophen blue, 10% glycerol, 50 mM Tris-HCl, pH 6.8, and 100 mM dithiothreitol.

Conclusions

In summary, our results suggest that the eIF4A/h eIF4G interaction stimulates ATP hydrolysis, but that this effect is indirect. In further support of this conclusion, we find that eIF4A(1–109) cannot stimulate ATP hydrolysis on its own, but that eIF4G stimulates both eIF4A(1–109) and eIF4A(110–294). These results suggest that the eIF4A/h eIF4G interaction is necessary for optimal stimulation of ATPase activity, but that additional components are required for stimulation of AT.

ATPase Assays—ADP and ATP were titrated to pH 7.0 with KOH and were added to reactions in stoichiometric amounts with MgCl₂. Reactions (20 μl) contained 20 mM Tris-HCl, pH 7.5, 0.5 mM MgCl₂, 100 mM KCl, 2 mM dithiothreitol, 0.1% (v/v) Tween 20, and 10% (v/v) glycerol, pH 7.5. Before buffer exchange, the concentrations of recombinant proteins were determined using the Bio-Rad Protein Assay Kit, using bovine serum albumin as a standard.
cause this homopolymer was found to be the most stimulatory for ATPase activation in previous studies (22). In some experiments, reactions also contained eIF4F, eIF4F fragments, an eIF4A variant, or ADP, as noted in the figure legends. Reactions were incubated at 37 °C, and 2-μl aliquots were quenched with an equal volume of 25 mM EDTA, pH 8.0. Products were analyzed by thin layer chromatography on polyethyleneimine-cellulose sheets using 0.8 M LiCl, 0.8 M acetic acid. Sheets were air-dried, cut, and analyzed by Cherenkov radiation in a Beckman LS 6500 scintillation counter. \(^{32}P\) was calculated as a fraction of total radioactivity (\(^{32}P\) + \(^{32}P\)ATP). The value of \(P_i\) in reactions without enzyme was subtracted as background (usually about 1%).

**Kinetic Measurements**—Steady-state ATPase assays were performed using an excess of ATP and RNA (i.e. poly(A)) over enzyme. Reactions were followed only during the linear range of time (generally 10–15 min). Values for \(P_i\) release were used to determine kinetic constants only if <20% of the ATP was hydrolyzed at saturating ATP concentrations and <10% at saturating ATP concentrations. In the current study, the term saturating substrate concentrations is defined as being \(>2 \times K_m\). The term saturating substrate concentrations is defined as being \(>4 \times K_{\text{ATP}}\), \(\geq10 \times K_m\) for eIF4A + cpC, and for eIF4A + cpC and \(\geq3 \times K_{\text{RNA}}\) for eIF4A + cpC. Under these conditions, eIF4A is in either the unliganded form (at saturating substrate concentrations) or in the full liganded form (at saturating substrate concentrations). When eIF4A is present in the unliganded form, the rate is given by the equation \(v = \left(k_{\text{cat}}[\text{RNA}]/[\text{ATP}] + K_m^{\text{RNA}}K_m^{\text{ATP}}[\text{RNA}] + ([\text{RNA}]/K_m^{\text{RNA}})ight)^{-1}\), where \(K_m\) indicates the \(K_m\) at saturating concentrations of the other substrate. In this situation, a plot of \(v\) versus [RNA] can be fit to a Michaelis-Menten equation. A detailed derivation can be found in Ref. 46. In the case of saturating substrate concentrations, the dependence of ATP hydrolysis on the concentration of RNA (or ATP) follows classical pseudo-first-order kinetics, allowing the data to be fit to a Michaelis-Menten equation. The \(K_{\text{ATP}}\) for reactions with free enzyme (E) or with the E/RNA complex were determined by measuring ATP hydrolysis with various concentrations of ATP at either saturating or saturating concentrations of RNA. Reciprocal experiments were performed to measure \(K_m^{\text{RNA}}\) at saturating or saturating concentrations of ATP. Inhibition constants (\(K_i\) for ADP) were measured at 10 μM ATP. Each ATPase assay was repeated at least three times. Kinetic analysis was performed using KaleidaGraph software (version 3.06; Synergy Software, Reading, PA). The data were fit to the Michaelis-Menten equation with iterative least squares minimizations.

**RESULTS**

**Effect of eIF4G Domains on ATPase Activity of eIF4A**—eIF4F stimulates the RNA-activated ATPase and ATP-dependent RNA helicase activities of eIF4A (47). eIF4F consists of three proteins, eIF4F, eIF4E, and eIF4A, that co-purify as a complex (43). We tested recombinant cpC3, cpC2, and cpC in the eIF4A-driven ATPase assay (Fig. 2B). Increasing amounts of either cpC or cpC2 significantly stimulated \(^{32}P\) release (compare lane 2 with lanes 3–8 and 15–20). The maximal stimulation was observed at an eIF4A/eIF4G molar ratio of \(\sim 1:1\) for both cpC and cpC2 (Fig. 2C). Surprisingly, the maximal stimulation by cpC was less than that of cpC2, although cpC contains cpC3. On the other hand, cpC2 failed to stimulate ATP hydrolysis (Fig. 2, B (lanes 9–14) and C), even at a 5-fold molar excess over eIF4A (data not shown). For subsequent experiments, we used a 1–2-fold molar excess of eIF4G over eIF4A.

The RNA-stimulated ATPase activity of eIF4A has been analyzed in terms of individual kinetic steps (Fig. 3) (30). To determine which step is affected, we measured steady-state kinetic parameters for poly(A)-activated ATP hydrolysis using either eIF4A alone or eIF4A in combination with eIF4F fragments (Fig. 4). \(K_m^{\text{RNA}}\) was measured at both saturating (Fig. 4A) and saturating (Fig. 4B) concentrations of poly(A). These correspond to the steps labeled a and b, respectively, in Fig. 3. Similarly, \(K_m^{\text{RNA}}\) was measured at both saturating (Fig. 4C) and saturating (Fig. 4D) concentrations of ATP. \(K_m^{\text{ADP}}\) was measured at both saturating (Fig. 4E) and saturating (Fig. 4F) concentrations of poly(A). Lorsch and Herschlag (30) have previously shown that ADP has little effect on \(K_m^{\text{RNA}}\) under the conditions used in the present study, so this parameter (step g in Fig. 3) was not determined. Fig. 4 shows typical experiments used for measurement of kinetic parameters. The

**FIG. 1. Proteins used in this study.** A, schematic representation of human eIF4G-1. Binding sites for proteins are shown in gray, and binding sites for RNA are shown in cross-hatching. B, SDS-PAGE of proteins used in this study stained with Coomassie Blue.

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The nomenclature system recommended by an ad hoc committee appointed by the IUBMB is used here (58). However, since this work concerns only eIF4G-1, the term "eIF4G" will subsequently refer to eIF4G-1.
average kinetic values are collected in Table I. The panels in Fig. 4 (A, B, etc.) correspond to columns in Table I (A, B, etc.) and kinetic steps in Fig. 3 (a, b, etc.), respectively.

The $K_m^{\text{ATP}}$ for eIF4A alone is significantly lower at saturating (Table I, kinetic step B) compared with subsaturating (kinetic step A) RNA (79 versus 250 $\mu$M). The $K_m^{\text{RNA}}$ for eIF4A alone does not change significantly at saturating (kinetic step D) compared with subsaturating (kinetic step C) ATP (45 versus 60 $\mu$M). Also, the $K_i^{\text{ADP}}$ of eIF4A alone is similar for saturating (kinetic step F) and subsaturating (kinetic step E) concentrations of RNA (17 versus 27 $\mu$M). Finally, $k_{\text{cat}}$ for eIF4A alone is $-3$ min$^{-1}$ (kinetic step H), similar to the $k_{\text{cat}}$ reported for eIF4A in earlier studies (30, 48).

The addition of cpC2 causes little change in $K_m^{\text{ATP}}$ at subsaturating RNA compared with eIF4A alone (240 versus 250 $\mu$M; kinetic step A). At saturating RNA, cpC2 increases $K_m^{\text{ATP}}$ by 2.5-fold (193 versus 79 $\mu$M; kinetic step B). The most dramatic effect of cpC2 is on $K_m^{\text{RNA}}$ at subsaturating ATP, a 3-fold increase (190 versus 60 $\mu$M; kinetic step C). At saturating ATP, cpC2 does not affect $K_m^{\text{RNA}}$ (37 versus 45 $\mu$M; kinetic step D). This suggests that excess ATP overcomes the inhibitory effect of cpC2 on RNA binding to eIF4A (compare “eIF4A + cpC2” with “eIF4A” in kinetic step D versus kinetic step C). The addition of cpC2 increases $K_i^{\text{ADP}}$ by 2.6-fold at saturating RNA (kinetic step F) but has little effect at subsaturating RNA (kinetic step E). Finally, $k_{\text{cat}}$ is similar for eIF4A alone and eIF4A-cpC2 (kinetic step H).

The addition of cpC3 does not affect $K_m^{\text{ATP}}$ at either saturating (kinetic step F) and subsaturating (kinetic step E) concentrations of RNA (17 versus 27 $\mu$M). Finally, $k_{\text{cat}}$ for eIF4A alone is $-3$ min$^{-1}$ (kinetic step H), similar to the $k_{\text{cat}}$ reported for eIF4A in earlier studies (30, 48).

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ing (kinetic step B) or subsaturating (kinetic step A) concentrations of RNA. Similar to eIF4A alone, reactions containing cpC2 show a significant decrease in $K_m^{\text{ATP}}$ upon saturation with RNA (98 versus 170 μM; compare “eIF4A + cpC3” in kinetic step B versus A). The addition of cpC3 does not significantly affect the $K_A^{\text{ATP}}$ or $K_A^{\text{RNA}}$ at either saturating or subsaturating concentrations of RNA (kinetic steps F and E). The most dramatic effect of cpC3 is on $K_m^{\text{RNA}}$. cpC3 decreases $K_m^{\text{RNA}}$ ~10-fold at both subsaturating (kinetic step C) and saturating (kinetic step D) ATP. For eIF4A plus cpC3, $K_m^{\text{RNA}}$ is unaffected by ATP concentration (kinetic step D versus C), as was noted above for eIF4A alone.

The second major effect of cpC3 is an increase of $k_{cat}$ by ~4-fold (kinetic step H). Thus, cpC3 increases both of the specificity factors, $k_{cat}/K_m^{\text{ATP}}$ and $k_{cat}/K_m^{\text{RNA}}$ (Table I, last two columns). The increase of $k_{cat}/K_m^{\text{ATP}}$ is primarily due to stimulation of the catalytic step, whereas the increase of $k_{cat}/K_m^{\text{RNA}}$ is due to both reduction of $K_m^{\text{RNA}}$ and stimulation of the catalytic step.

The effects of cpC on ATP hydrolysis by eIF4A are similar to those of cpC3, the only difference being that whereas cpC3 significantly decreases $K_m^{\text{ATP}}$ at saturating RNA concentrations, cpC fails to cause any decrease (Table I, kinetic step A). Similar to cpC3, cpC dramatically decreases $K_m^{\text{RNA}}$ at both saturating (kinetic step D) and subsaturating (kinetic step C) ATP concentrations and also stimulates the turnover rate (kinetic step H).

Summarizing the effect of the eIF4G fragments on seven partial reactions occurring during RNA-stimulated ATP hydrolysis, we can conclude that cpC2 decreases affinity of both ATP (at saturating RNA concentrations) and RNA (at subsaturating ATP concentrations) but has no effect on the catalytic step. By contrast, cpC3 does not affect affinity of ATP (at saturating RNA concentrations) but greatly increase both RNA affinity and the catalytic step. cpC has properties intermediate between cpC2 and cpC3 with respect to both ATP binding at saturating RNA and RNA binding at subsaturating ATP.

cpC2 and cpC Stabilize RNA Binding to eIF4A—The foregoing conclusions were based solely on the kinetics of ATP hydrolysis. We wished to confirm them by an independent method, UV cross-linking of $^{32}$P-labeled RNA to eIF4A. Both eIF4G (Fig. 1A) and eIF4A (Fig. 5A) have RNA-binding sites and can be UV-cross-linked to RNA (19, 43). $^{32}$P-Labeled EMCV RNA was preincubated with eIF4A in the presence of eIF4G fragments or bovine serum albumin, both with and without ATP. The samples were irradiated with UV light and digested with RNAse A and T1, and proteins were separated by SDS-PAGE. In the presence of ATP, UV-cross-linking to eIF4A alone is low (Fig. 6A, lane 1). This cross-linking is markedly increased by cpC3 (lane 2 and cpC, lane 6). Cross-linking is not changed by cpC2 (lane 4). Conversely, there is little if any effect of eIF4A on cross-linking to the eIF4G fragments themselves (cpC3, lane 3 versus lane 2; cpC, lane 7 versus lane 6). These results corroborate the conclusions drawn from kinetic data that cpC2 and cpC reduce $K_m^{\text{RNA}}$ whereas cpC3 fails to change $K_m^{\text{RNA}}$ at saturating ATP.

Similar, but not identical, cross-linking results are obtained in the absence of ATP (Fig. 6B). In particular, eIF4A labeling in the presence of cpC3 is similar regardless of the ATP concentration. (The eIF4A band appears stronger in Fig. 6B than in Fig. 6A, but so do most other bands; we attribute this to UV shielding caused by 1 mM ATP.) This confirms the kinetic data indicating that ATP does not alter $K_m^{\text{RNA}}$ in the presence of cpC3 (i.e., RNA and nucleotide binding to the eIF4A-cpC3 complex are uncoupled). Interestingly, cpC3 has a greater effect than cpC on both the labeling of eIF4A (Fig. 6, A and B, lane 2

![Fig. 4. Determination of kinetic constants for RNA-stimulated ATP hydrolysis by eIF4A in the presence of eIF4G fragments.](http://www.jbc.org/)

**Table I**

**Kinetic parameters for ATP hydrolysis by eIF4A alone or in the presence of eIF4G**

Kinetic constants were derived from several experiments similar to Fig. 4. The panels in Fig. 4 (A, B, etc.) correspond to columns in Table I (A, B, etc.) and kinetic steps in Fig. 3 (a, b, etc.).

| Proteins | $K_m^{\text{ATP}}$ | $K_m^{\text{RNA}}$ | $K_m^{\text{ADP}}$ | $k_{cat}$ | $k_{cat}/K_m^{\text{ATP}}$ | $k_{cat}/K_m^{\text{RNA}}$ |
|----------|-------------------|-------------------|-------------------|--------|--------------------------|--------------------------|
|          | (μM)              | (μM)              | (μM)              | (μM)   | (μM)                     | (μM)                     |
| eIF4A    | 250 ± 40          | 79 ± 22           | 60 ± 23           | 45 ± 15| 27 ± 6                   | 17 ± 4                   |
| eIF4A + cpC2 | 240 ± 49 | 193 ± 41          | 190 ± 60          | 37 ± 13| 15 ± 4                   | 45 ± 10                  |
| eIF4A + cpC3 | 170 ± 33 | 98 ± 21           | 6.2 ± 1.5         | 4.7 ± 0.9| 28 ± 1                   | 30 ± 8                   |
| eIF4A + cpC | 280 ± 48          | 140 ± 41          | 12 ± 3.8          | 5.7 ± 1.2| 30 ± 10                  | 65 ± 16                  |

Kinetic step  
A  B  C  D  E  F  G  H  I  J
versus lane 6) and decreasing $K_m$RNA (Table I, kinetic steps C and D). The cross-linking data show that cpC2 decreases labeling of eIF4A in the absence of ATP (Fig. 6B, lane 4 versus lane 1) but causes no change in the presence of ATP (Fig. 6A, lane 4 versus lane 1). This correlates with the kinetic data showing that cpC2 increases $K_m$RNA at subsaturating ATP (Table I, kinetic step C) but has little effect on $K_m$RNA at saturating ATP (Table I, kinetic step D). We also found that RNA cross-linking to cpC3 and eIF4A in the absence of nucleotide is the same either with 2 mM Mg$^{2+}$/H$_{11001}$ or with no Mg$^{2+}$/H$_{11001}$ (data not shown), suggesting that a particular RNA conformation is not required for cross-linking to either eIF4A or cpC3.

Since both eIF4A and eIF4G have RNA-binding sites, and since cpC3 binds eIF4A with high affinity (34), one can envision two models for the stimulation of cross-linking to eIF4A by cpC3. In the first, cpC3 alters the conformation of eIF4A to increase its affinity for RNA. In the second, cpC3 binds both RNA and eIF4A, effectively increasing the RNA concentration in the vicinity of eIF4A. To distinguish between these two models, we performed UV cross-linking of $^{32}$P-labeled EMCV RNA to eIF4A in the presence of an eIF4G fragment, eIF4G(737–989), that lacks part of the RNA-binding site. This fragment was shown to have a 16-fold lower affinity for EMCV RNA, but it still interacts with eIF4A, based on the observations that it is bound to RNA 7-fold more tightly in the presence of eIF4A and that it is able to promote 48 S complex formation (42). We confirmed that UV cross-linking of $^{32}$P-labeled EMCV RNA to eIF4G(737–989) is much weaker than to cpC3 (Fig. 6C, lane 3 versus lane 2). Importantly, eIF4G(737–989) is less able to stimulate RNA cross-linking to eIF4A than cpC3 (lane 3 versus lane 2).

We also measured the stimulation of ATPase activity by eIF4G(737–989). Whereas cpC3 stimulates ATPase activity 4-fold (Fig. 7, column 2), eIF4G(737–989) stimulates only 2-fold (column 3). An eIF4G fragment that does not bind eIF4A, eIF4G(917–1118) (Fig. 1A) (34), fails to stimulate ATP hydrolysis at all (Fig. 7, column 4). Thus, the full RNA-binding site contained within aa 682–916 of eIF4G (see Fig. 1A) is necessary for stimulation of both RNA cross-linking to eIF4A and ATP hydrolysis; the ability of eIF4G(737–989) merely to interact with eIF4A is not sufficient. This favors the second model, that eIF4G recruits RNA to the eIF4G-eIF4A complex by direct binding of RNA rather than by altering the conformation of eIF4A.

We also tested the opposite combination, an eIF4G fragment with a complete RNA-binding site (cpC3) and an eIF4A variant defective in RNA binding (eIF4A(R362Q)). In the well-characterized DNA helicase PcrA, the equivalent residue, Arg-610, interacts with the $\gamma$-phosphate of AMP-PNP and stabilizes ATP binding in the cleft between NH$_2$- and COOH-terminal domains (49–51). This residue is also thought to participate in oligonucleotide binding by motif IV (see Fig. 5A). In the case of eIF4A, the R362Q substitution inhibits helicase activity of eIF4F and 80 S complex formation, indicating that it still interacts with eIF4G (19, 20). We find that eIF4A(R362Q) is unable to cross-link to RNA (Fig. 6D, lane 4), but the addition of cpC3 permits a limited degree of cross-linking (lane 5). However, cpC3 cannot stimulate the ATPase activity of eIF4A(917–1118) (Fig. 7, column 6); nor can eIF4G(737–989) (column 7) or eIF4G(917–1118) (column 8). We conclude that, although eIF4G can bring RNA into close proximity to eIF4A, as indicated by cross-linking, this is not sufficient to permit RNA-stimulated ATP hydrolysis. These two experiments with eIF4G(737–989) and eIF4A(R362Q) indicate

FIG. 5. Schematic representation of human eIF4A and its proteolytic fragments. A, the conserved helicase domains and motifs (conserved amino acids are in uppercase) and their suggested functions are indicated. B, schematic representation of tryptic fragments of eIF4A. a, b, c, e, f, and g correspond to labels in Fig. 8A, lane 8. The peptides identified by MALDI-TOF MS analysis are represented inside each rectangle.
that the RNA-binding sites of both eIF4G and eIF4A must be intact for the stimulation of ATPase activity by eIF4G.

cpC3 and cpC2 Both Bind to the NH2-terminal but Not the COOH-terminal Domain of eIF4A—The catalytic center of eIF4A is formed by conserved motifs located in both NH2-terminal and COOH-terminal domains (Fig. 5A). To better understand the different effects of cpC3 and cpC2 on enzymatic activity, we investigated which domains of eIF4A bind to these eIF4G fragments. Previously, we showed that cpC2 and cpC3 compete with each other for binding to eIF4A (34), suggesting that they bind to the same or overlapping regions of eIF4A.

We first obtained a set of eIF4A fragments by limited trypsin digestion (Fig. 8A). A time course of trypsin digestion produced a number of large fragments with molecular masses estimated from SDS-PAGE to be 32, 30, 29, 28, 25, 23, and 16 kDa. From 30–120 min, the pattern changed very little. We chose to characterize the set of fragments produced at 60 min (lane 8). The structures of fragments were determined by two methods, mass spectrometry and Western blotting. We excised each of the bands in lane 8 and digested them with trypsin to completion. The peptides were subjected to MALDI-TOF MS, and masses were matched with those predicted from the eIF4A sequence (Fig. 5B). Peptides matched are shown within the boxes. A sufficient number of peptides were matched to allow identification of all bands except d. Bands a, b, c, e, and f were identified as eIF4A(147–406), eIF4A(178–406), eIF4A(178–382), eIF4A(147–334), and eIF4A(238–406), respectively.

Band g was identified as the NH2-terminal portion of eIF4A. However, based on the location of basic amino acid residues, it is probably a mixture of four peptides beginning at either aa 1 or 9 and ending at either aa 141 or 146. Unfortunately, MALDI-TOF MS detects some but not all possible peptides, either because they are too small for unambiguous assignment (<800 Da) or because they fail to ionize. The peptides we identified do not allow precise assignment of structures to the band g fragments. They will subsequently be referred to as eIF4A(9–146). Band g was further identified by immunological analysis. A monoclonal antibody against eIF4A was tested against recom-
binant fragments of eIF4A representing aa 1–109 and 110–294 (Fig. 8B). It reacted only with eIF4A(1–109), indicating that the epitope is located within the first 109 aa residues of eIF4A. This antibody also recognized the largest fragment produced after 2 min of trypsin digestion (Fig. 8C). This fragment was previously identified as eIF4A(9–406) (31). Thus, the epitope for this anti-eIF4A antibody is located between aa residues 9 and 109. When tested against the partial trypsinolysis products, this antibody reacted strongly with band g (Fig. 8C), confirming that these peptides are derived from the NH2 terminal of eIF4A.

eIF4A fragments that bound to S-tagged cpC3 and cpC3 were separated by SDS-PAGE, with detection by either Coomassie Blue staining (Fig. 8D) or Western blotting (Fig. 8E). There is no binding of eIF4A fragments to eIF4G(917–1118) (Fig. 8, D lane 7) and E (lane 6). Both cpC3 (Fig. 8D, lane 3) and cpC2 (lane 5) bind bands a and b. However, whereas cpC3 binds bands a and b approximately equally, cpC2 binds band b only ~10% as strongly as band a. This suggests that there is a binding site for cpC2 in band a that is lost upon further trypsin cleavage to band b (see Fig. 5B), which therefore must be located between aa 147 and 177. Consequently, the binding site for cpC3 in band a is located more in the COOH-terminal direction than that of cpC2 (since it is not lost upon cleavage of band a to band b). No binding of eIF4G fragments to band f, the COOH-terminal portion of eIF4A, was detected. Thus, the interaction of bands a and b with cpC3 occurs through sequences located NH2-terminal to aa 238. Similarly, the binding of band a to cpC2 occurs through sequences located NH2-terminal to aa 238. Western blot analysis (Fig. 8E) showed that cpC3 (lane 2) and cpC2 (lane 4) both bind band g. Thus, cpC3 and cpC2 recognize one or more sites between aa 9 and 237 but not between aa 238 and 406.

**DISCUSSION**

eIF4A was discovered as a factor involved in initiation complex formation (reviewed in Ref. 47). It was subsequently shown to bind and unwind mRNA in an ATP-dependent manner in concert with eIF4B, eIF4H, and eIF4F. There are several events that could be affected by these proteins during RNA unwinding: substrate binding, the catalytic step of RNA unwinding, ATP binding, the catalytic step of ATP hydrolysis, coupling between ligand and substrate binding, and coupling between ATP hydrolysis and RNA unwinding. Both eIF4B and eIF4F lower the \( K_m \) for RNA in the eIF4A-catalyzed ATPase assay (21, 23, 24, 29) and also change the selectivity for RNA substrates in the helicase assay (26). eIF4B and eIF4H make eIF4A more processive (26, 28). eIF4F does not differ in processivity from eIF4A alone but is more active per mol of eIF4A as a helicase. However, neither eIF4B nor eIF4F affect the \( V_{max} \) of eIF4A-driven ATP hydrolysis when tested separately at subsaturating concentrations of ATP, but they cause a 3-fold increase in \( V_{max} \) when used together (29). Thus, paradoxically, although eIF4F is more active per mol as a helicase, its \( V_{max} \) in an ATPase assay has been reported to be the same as that of eIF4A.

One aspect that makes interpretation of earlier work more complicated is that naturally isolated eIF4F contains variable amounts of eIF4A (52, 53). This makes it difficult to measure \( k_{cat} \) for eIF4F in ATPase or helicase reactions. Furthermore, it is not clear whether reactions are measuring the stimulation of eIF4A by eIF4F or **vice versa**. It is not clear from these studies whether eIF4A/eIF4G molar ratios greater than 1 are necessary for maximal activity or whether added eIF4A is simply producing stoichiometric quantities of eIF4E, eIF4G, and eIF4A. Another potential complication of using natural eIF4F is that it may contain small but catalytic amounts of other proteins that affect the eIF4A reaction (*e.g.* eIF4B or eIF4H). In the current study, we were able to avoid these problems by using recombinant eIF4A and eIF4G. This enabled us to ask several questions for the first time: whether eIF4G affects RNA binding to eIF4A in a direct versus an ATP-dependent manner, the effect of eIF4G on the rate of ATP hydrolysis (\( k_{cat} \)), the effect of eIF4G on RNA and ATP binding to eIF4A, and the roles of different eIF4G domains in the reaction.

Our observation that cpC3 and cpC decrease \( K_m \) by ~10-fold suggests two alternative models: 1) in the catalytic cycle, eIF4A goes through a phase with high affinity for RNA, and eIF4G stimulates that cycle at steps involving ATP binding or hydrolysis; or 2) eIF4G stabilizes RNA binding to eIF4A, which then permits ATP hydrolysis to proceed. Several experiments support the latter model. First, there is no effect of ATP concentration on \( K_m \) in the presence of either cpC or cpC2. Second, both cpC and cpC2 stimulate RNA cross-linking to eIF4A by at least 5-fold, and this can occur in the presence or absence of ATP. Third, eIF4G-stimulated RNA cross-linking to eIF4A is not affected by the presence of ADP or by the absence of Mg2+ in the reaction (data not shown), suggesting that RNA binding to eIF4A in the presence of cpC or cpC2 is not coupled to the ATPase activity. Fourth, cpC3 stimulates RNA cross-linking to eIF4A(362Q), which is defective in RNA binding and ATP hydrolysis. This second model, in which eIF4G stabilizes RNA binding on eIF4A, is attractive since eIF4A alone has very low affinity for RNA and dissociates faster than product formation (30).

Within this model, one can further ask whether both eIF4A and eIF4G must have active RNA-binding sites in order to achieve accelerated ATP hydrolysis in the complex. Two experiments support the view that this is so. First, we used eIF4G (737–989), which is missing part of the RNA-binding site but retains the ability to interact with eIF4A (42). We showed that reduced RNA-binding activity of eIF4G(737–989) correlates with reduced stimulation of RNA cross-linking to eIF4A and ATP hydrolysis. Second, we showed that cpC3 fails to stimulate
ATP hydrolysis by eIF4A(R362Q) although it stimulates RNA cross-linking to eIF4A(R362Q). This indicates that the RNA-binding site of eIF4A is required for ATP hydrolysis.

We found that cpC2 behaves in a manner opposite to cpC3, increasing $K_m^RNA$ at low ATP concentrations and inhibiting RNA cross-linking to eIF4A in the absence of ATP. Recently, it was shown that the transformation suppressor Pdcd4, which contains two MA-3 domains similar to one located in cpC2, inhibits helicase activity by direct binding to eIF4A (54). This implies that cpC2 may inhibit the helicase activity of eIF4A. (This has never been tested.) An inhibitory role for cpC2 is consistent with our finding that cpC is less active than cpC3 in stimulation of ATPase activity (the $K_m^RNA$ at subsaturating ATP is higher). Measurement of the $K_m^RNA$ at saturating ATP concentrations indicates that this inhibition by the cpC2 domain is alleviated by the binding of ATP to eIF4A, suggesting
that there is synergistic binding of the ATP and RNA substrates. Although this synergistic interaction is not observed for the binding of RNA to the eIF4AcpC2 complex, this is probably due to the low affinity of this complex for RNA, which prevented the $K_{\text{RNA}}^{\text{ATP}}$ from being measured at saturating RNA concentrations. A similar, albeit weaker, synergistic interaction is observed for the binding of the RNA and ATP substrates to eIF4A both in the presence and absence of cpC3 and cpC2.

Insight into the structural basis for eIF4G modulation of eIF4A activity is provided by analysis of eIF4A tryptic fragments bound to individual eIF4G regions. Both cpC3 and cpC2 bind the NH2-terminal domain of eIF4A, which is equivalent to PcrA domain 1A (Fig. 5A). This domain contains the Q-rich motif (55) and motifs I (Walker motif A) and II (Walker motif B), which are involved in direct interaction with ATP (9). This domain also possesses motifs Ia, Ic, and III, which are thought to be involved in substrate binding (DNA in the case of Rep helicase) (56) and also coupling of ATP hydrolysis to helicase activity (57). Our observed stabilization of RNA binding by cpC3 probably occurs through an interaction with motifs Ic and/or III of eIF4A, since both bands a and b efficiently cross-link to RNA in the presence of cpC3 (data not shown). Other RNA-binding determinants are contained in band f, which does not cross-link to RNA. Since ATP and RNA binding to enzyme require motifs in both the NH2- and COOH-terminal eIF4A domains, cpC3 may affect functions of both. By contrast, cpC2 binds only weakly to band b and also does not affect the rate of ATP hydrolysis. The fact that cpC2 efficiently binds band a means weakly to band b and also does not affect the rate of ATP hydrolysis. The main binding determinant for cpC2 on eIF4A is located probably due to the low affinity of this complex for RNA, which may affect functions of both. By contrast, cpC2 binds only in the presence of cpC3 (data not shown). Other RNA-binding/helicase (56) and also coupling of ATP hydrolysis to helicase (57).
