Stabilization of Eukaryotic Initiation Factor 4E Binding to the mRNA 5'-Cap by Domains of eIF4G*

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The eukaryotic cap-binding complex eIF4F is an essential component of the translational machinery. Recognition of the mRNA cap structure through its subunit eIF4E is a requirement for the recruitment of other translation initiation factors to the mRNA 5'-end and thereby for the attachment of the 40 S ribosomal subunit. In this study, we have investigated the mechanistic basis of the observation that eIF4E binding to the cap is enhanced in the presence of the large eIF4F subunit, eIF4G. We show that eIF4E requires access to both the mRNA 5'-cap and eIF4G to form stable complexes with short RNAs. This stabilization can be achieved using fragments of eIF4G that contain the eIF4E binding site but not the RNA recognition motifs. Full-length eIF4G is shown to induce increased eIF4E binding to cap analogues that do not contain an RNA body. Both results show that interaction of eIF4G with the mRNA is not necessary to enhance cap binding by eIF4E. Moreover, we show that the effect of binding of full-length eIF4G on the cap affinity of eIF4E can be further modulated through binding of Pab1 to eIF4G. These data are consistent with a model in which heterotrophic cooperativity underlies eIF4E function.

One of the first steps during the initiation of translation on eukaryotic mRNAs is the recruitment of a translationally competent 40 S ribosomal subunit to the mRNA 5'-end. This is mediated by the cap-binding complex known as eukaryotic initiation factor eIF4F. The minimal cap-binding complex that can be isolated from all organisms investigated so far comprises two proteins, eIF4E and eIF4G. Whereas eIF4E manifests direct affinity for the cap structure (1), eIF4G serves as a multipurpose adaptor capable of recruiting a number of necessary activities to the mRNA 5'-end (2). The binding of eIF4G to the ribosome-associated initiation factor eIF3 is thought to establish physical contact between the mRNA and the 40 S ribosomal subunit (3), whereas binding to the RNA helicase eIF4F was proposed to be necessary for the disruption of secondary structure in the 5'-untranslated region (4). Interactions between eIF4G and the poly(A)-binding protein Pab1 have been shown to be capable of promoting circularization of mRNA (5) and to be responsible for the synergistic effect of the 5'-cap and the 3'-poly(A) tail of mRNAs on translation initiation (6, 7). In addition, eIF4G contains two putative RNA recognition motifs (8) and, at least in mammalian cells, binds to the eIF4E-phosphorylating kinase Mnk1 (9).

The interaction between eIF4E and eIF4G involves a conserved motif with the consensus sequence Tyr-X-X-X-Leu-Φ (where Φ is a hydrophobic amino acid) in eIF4G (10–12); although it has been suggested that intermolecular contacts involving further amino acids outside this consensus sequence contribute to the stability of eIF4F (13). In the case of eIF4E, a necessary but possibly not fully sufficient binding motif has been identified corresponding to the sequence Val-Glu-X-X-Trp (14), which is situated on the cap distal surface of the protein in both yeast and mammalian eIF4E (15, 16). Because the interaction between these two motifs is required for coupling the cap-binding activity to the activities of other initiation factors, its disruption or reinforcement may be utilized by cells to control specifically the translation of capped messages (see e.g. Ref. 17).

It is therefore of interest that binding of the cap-binding complex to other initiation factors affects the stability of the eIF4E-cap interaction. So far, there is experimental evidence for a mutual reinforcement of the majority of interactions in the chain 5'-cap-eIF4F:eIF4G:Pab1:poly(A)-3' for initiation factors purified from wheat germ (18, 19). For yeast and mammalian eIF4E, the binding of different ligands (namely the eIF4E binding domain of eIF4G (4G-BD4E), p20, and 4E-BP2) has been shown to enhance (to differing degrees) the affinity of eIF4E for immobilized cap analogues and short RNAs (14, 20). In contrast, a fluorescence study determining the affinity of eIF4E in the presence of a 17-mer peptide comprising the minimal binding motif of eIF4G found no significant change in the equilibrium binding constant with soluble cap analogues (21). The binding of this peptide to eIF4E does not alter the crystal structure of this protein (12).

The interpretation of these, at first sight contradictory, findings is further complicated by the fact that mammalian eIF4E shows strong differences in its binding to several cap analogues and short RNAs, and that there is currently considerable uncertainty as to the affinity values for these interactions. Thus, one study found that the binding constant with cap analogues was two times smaller than with RNAs (K_{d} values are 2 × 10^{-6} M for m7GTP and m7GpppG and 1 × 10^{-6} M for capped RNAs, (22)). On the other hand, a very recent study found equilibrium binding constants of 1 × 10^{-5} M for m7GTP and 2 × 10^{-5} M for the larger analogue, m8GpppG (21).

Cooperativity effects in factors such as eIF4E could play a key role in the mechanism and control of translation (17). It has been proposed that, contrary to the cooperative model for eIF4E function (14), eIF4G enhances the eIF4E-cap interaction solely by virtue of the stabilizing influence of RNA binding to the eIF4G RRM regions (23). We have therefore designed a series of experiments to investigate further the proposed coop-
erative behavior of eIF4E, using a range of different techniques as well as different domains of eIF4E. The strategy was designed specifically to clarify the confusion arising from apparently contradictory results reported in previous work.

MATERIALS AND METHODS

Preparation of Proteins—Purification of recombinant yeast eIF4E from inclusion bodies was performed as described previously (24), but 20 mM GTP was used to elute the protein from m7GTP-Sepharose instead of 0.1 mM m7GTP. The nucleotide was removed from protein-containing fractions by dialysis overnight against 2000 volumes of buffer B (20 mM HEPES, pH 7.5, 1 M KCl) followed by dialysis for 4 h against 2000 volumes of buffer A.

His6-tagged fragments of yeast eIF4G were purified as described earlier (14). Full-length, His6-eIF4G1 was expressed in SF9 insect cells and purified via heparin chromatography (application of the cleared extract in 20 mM HEPES, pH 7.5, 30 mM KCl, 2 mM MgCl2, and elution with a gradient of 30–500 mM KCl in the same buffer), followed by standard nickel-chelate chromatography. His6-tagged Pab1 was expressed in Escherichia coli BL21(DE3) (hsdS gal (Acha585 ind1 Sin7 min5 lacUV5-T7 gene 1)) using the expression plasmid pET2a at a growth temperature of 28 °C. Cells were lysed by sonication, and the extract was subjected to nickel-chelate chromatography. PAB1-containing fractions were pooled and subjected to heparin chromatography as described for eIF4E.

RNA Transcription in Vitro—Synthetic DNA primers with the sequences 5'-GAATTGTAATACGACTCACTATAG-3' and 5'-TGATGTTTGGTGTCTATAGTGAGTCGTATTACAA TTC-3' were used to generate capped or uncapped RNAs as described previously (20, 24).

SPR Assays—Surface Plasmon Resonance (SPR) experiments were performed on a BIA3000 (BIACore) essentially as described earlier (20). The eluent buffer for all experiments was 20 mM HEPES, pH 7.5, 100 mM KCl, 100 μg/ml wheat germ tRNAs (Roche Molecular Biochemicals), and 0.005% surfactant p20 (BIACore). For estimation of the equilibrium binding constant, 10−7 to 10−15 M eIF4E in eluent buffer was injected over the chip, and the equilibrium responses were plotted against protein concentration. BIACore evaluation software (version 3.1; BIACore) was used to calculate a value corresponding to the curve.

RNA Gel Shifts—2 μg of eIF4E were mixed with equimolar amounts of the respective binding partners, plus 1 μg/ml TRNAs and 20 units RNasin in a final volume of 20 μl of buffer (20 mM HEPES, pH 7.5, 100 mM KCl, 2 mM MgCl2). The mixture was left for 5 min at room temperature. The radiolabeled probe was added in a volume of 2 μl, and the mixture again left for 5 min at room temperature. The samples were then separated on a 10% polyacrylamide gel (19:1 acrylamide:bisacrylamide) at 2000 volts. After electrophoresis, the bands were visualized through Coomassie Blue staining and quantified with a Bio-Rad GS-700 imaging densitometer and Bio-Rad molecular analyst software. eIF4E-binding proteins used in this study. 5 μg of each protein were loaded onto a SDS-polyacrylamide gel electrophoresis gel and stained with Coomassie Blue. C, removal of GTP from eIF4E. eIF4E was mixed with [γ-32P]GTP and subjected to the procedure used for the removal of GTP after elution of eIF4E from cap analogue Sepharose. 3 μg of eIF4E were cross-linked to the nucleotide immediately after mixing and after each dialysis step. The samples were then run on a 12.5% SDS-polyacrylamide gel electrophoresis gel and subjected to autoradiography. The numbers shown were determined by excision of the eIF4E bands from a Coomassie Blue-stained gel and scintillation counting. wt, wild type.

RESULTS

Preparation of Cap Analogue-free eIF4E—The commonly used method for the purification of recombinant eIF4E employs m7GTP immobilized on Sepharose as a highly specific affinity matrix for the binding of eIF4E. The elution of eIF4E from this resin is usually performed using buffer containing 0.1 mM soluble m7GTP. The cap analogue is then removed by means of either dialysis or further chromatographic methods (25).

However, the efficiency of removal of the cap analogue during the purification of eIF4E has been questioned (21). It is important that eIF4E utilized in mRNA cap binding studies is essentially cap analogue-free, because any presence of a strongly binding cap analogue would reduce the effective concentration of active eIF4E. In earlier studies, mammalian eIF4E was shown to bind to nonmethylated nucleotides with 20–30-fold lower affinity than to their methylated counterparts (26). We therefore modified the standard method of elution of eIF4E from the cap resin, utilizing nonmethylated GTP instead of m7GTP, because the former should be more readily removed from the cap-binding protein. We found that 20 mM GTP elutes yeast eIF4E from m7GTP-Sepharose with similar efficiency to 0.1 mM m7GTP (data not shown). The eluted protein was then dialyzed overnight against 2000 volumes of buffer containing 1 M KCl, followed by dialysis for a further 4 h against the same volume of low salt buffer.

To show that dialysis efficiently removes the nucleotide, a small aliquot of eIF4E was mixed with [γ-32P]GTP and subjected to the same procedure. Samples were taken after the initial mixing as well as after each dialysis step, and cross-linking was induced by irradiation with UV light. The resulting preparations were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 1C). In addition, the eIF4E bands were visualized through Coomassie Blue staining and excised from the gel, and the associated radioactivity was determined via scintillation counting. Both results show that GTP can be removed from the protein preparation with high efficiency, with the final protein containing less than 0.5% of GTP-attached eIF4E. eIF4E associated radioactivity was determined via scintillation counting.
that is characterized by rapid binding and release, as was previously observed for the human protein (20). Injections in the presence of 0.1 mM GTP or m7GTP, respectively, showed that the methylated cap analogue completely prevents the association of eIF4E with the chip, whereas the nonmethylated nucleotide has only a minor effect (Fig. 2C). This indicates that the observed sensorgrams were generated solely by cap binding and not by nonspecific interactions of the protein with the negatively charged mRNA matrix.

We do not at present know the origin of the slower binding and release phases following the initial rapid interaction. However, we found that the maximum response ($R_{max}$) for these events is independent of the amount of immobilized mRNA. This suggests that this phase does not arise directly from the eIF4E-cap interaction. Part of it may stem from eIF4E molecules that are trapped in the dextran and/or mRNA matrix after release from the mRNA caps.

In contrast, the rapid phases of the sensorgrams were dependent on both the amount of immobilized mRNA and the concentration of injected eIF4E. Inspection of the end points of the rapid phases at different protein concentrations yielded an estimated equilibrium binding constant of 3.6 × 10^{-6} M (data not shown). This is in good accordance with the majority of published results derived from fluorescence studies for capped RNAs and eIF4Es from other species (e.g., 1 × 10^{-6} M for human eIF4E (22) and 0.8 × 10^{-6} M for wheat eIF4E (27)).

The dissociation rates observed with this method could not be subjected to standard analysis procedures using the BIAevaluation curve-fitting software, because the short time from the end of injection to complete release of eIF4E did not yield sufficient data points for a detailed analysis. However, manual comparison of the actual curves with simulated release curves for off rates between 0.1 and 1 s^{-1} indicated that the release constant for the eIF4E-mRNA interaction lies between 0.5 and 1 s^{-1}, corresponding to an association rate constant of 0.5–1 × 10^{-6} s^{-1} M^{-1}.

Interaction of eIF4E with Both the 5'-Cap and eIF4G Is Necessary for Tight Binding to mRNA—In a further procedure designed to yield information about the interaction between eIF4E and the cap structure, gel mobility shift experiments were conducted with radiolabeled mRNA identical to that used in the SPR experiments, but containing no biotinylated residue. As has already been observed (5), yeast eIF4E alone is not sufficient to generate detectable signals of shifted RNA in this type of assay (Fig. 3, lane 1). This is understandable in light of the results obtained in the SPR experiments, because the complex characterized by the observed low affinity and fast rate constants would not remain stably bound to the cap structures. However, the presence of a 17-kDa fragment of eIF4G comprising the eIF4E binding site flanked by ~70 amino acids on each side (termed here 4G-BD4E) leads to a clearly detectable shift, whereas the eIF4G fragment alone does not interact detectably with the capped mRNA (lanes 2 and 3). The ability of the eIF4G fragment to induce the shift is likely to be linked solely to the increased cap-binding affinity of the complex with eIF4E, because this fragment lacks the RRM domains of eIF4G. Moreover, the presence of the cap analogue m7GTP completely prevents retardation of the RNAs (lane 4), and no shift can be induced under identical conditions when uncapped mRNA is used (lane 9).

We next investigated the properties of a mutant form of eIF4E with the substitution W75R. The apo-form of this protein binds the cap structure with an affinity similar to the wild type, but can no longer interact with eIF4G (14). This mutant protein was unable to cause the band-shift (lane 12). A slight smear of radioactivity above the free probe in this lane stems mostly likely from residual interactions between the eIF4G fragment and the mutated eIF4E. In conclusion, both the availability of a cap structure and the eIF4G interaction are necessary for eIF4E to be able to form a stable complex with mRNA.

In previous work, a fragment of eIF4G comprising only the 17-amino acid region covering the eIF4E binding site has been shown to bind stably to eIF4E (12). However, this peptide did not have the ability to strengthen the interaction with the cap sufficiently to cause a mobility shift (lanes 5 and 6).

m7GTP-Sepharose Binding Studies Using Larger Fragments of eIF4G—The first indications of enhanced cap binding in the presence of fragments of eIF4G were obtained using equilibrium studies with purified proteins and m7GTP-Sepharose (14). The use of immobilized cap analogues is particularly suited for the determination of cooperative effects during cap binding, because the absence of an RNA body excludes additive effects arising from the presence of RNA-binding activities in the eIF4E binding partners. Briefly, a small amount of eIF4E is incubated with a small amount of immobilized cap analogue,
and an equilibrium between cap-bound and -free eIF4E is established through incubation in a large buffer volume. The amount of eIF4E that can subsequently be eluted from the resin is then taken as an indicator for the affinity of the protein for the cap.

We used this method to obtain data on the influence of larger fragments of eIF4G on the cap affinity of eIF4E (data summarized in Fig. 4). As was shown previously, the presence of 4G-BD4E significantly displaces the equilibrium between soluble and resin-associated eIF4E toward the cap-bound state. To our surprise, we found that the complete N-terminal half of eIF4G (4G-Nt, comprising both the eIF4E and Pab1 binding site, Fig. 1A) had a much weaker effect on the recovery of eIF4E from the resin compared with the 17-kDa fragment. This is consistent with observations that larger fragments of eIF4G show a weaker interaction with eIF4E in yeast two-hybrid experiments.\(^2\) Interestingly, the addition of Pab1 to this reaction restored the ability of eIF4G to increase the association of eIF4E with the cap analogue. The effect of 4G-Nt in a complex with Pab1 slightly surpassed that of 4G-BD4E alone. The same effect was observed when complete eIF4G1 purified from insect cells was used. Indeed, the combination of complete eIF4G1 and Pab1 produced the strongest effect in this assay, leading to a 3-fold increase in the amount of eIF4E recovered from the column.

It was noted that the greatest increase in recovery observed in this assay was less dramatic than the increase in the signal observed in the gel shift experiments. We assume that this reflects differences in the interactions between eIF4E and cap analogues as compared with those between eIF4E and mRNAs. Recent fluorescence studies found that the binding of free eIF4E to mGTP, which was used in the affinity resin experiments described here, is almost one order of magnitude stronger than the binding to the larger cap analogue, m\(^7\)GpppG, which more closely resembles a capped mRNA. However, we assume that the more dramatic results from our gel shift experiments using capped mRNAs more closely reflect affinity changes in vivo. Finally, we observed that, in contrast to the larger eIF4G fragments, the 17-amino acid peptide comprising the minimal eIF4E binding motif had only a very small effect on the recovery of eIF4E, again confirming the results obtained in the gel shift experiments.

CD Spectral Analysis Reveals Conformational Changes in eIF4E upon Cap Binding—The data presented here suggest that binding of eIF4E to eIF4G leads to an effective increase of the former protein’s affinity for mRNA cap structures. Nothing is known, however, about the structural basis for this effect. Inspection of the sequences of eIF4E from different organisms revealed an area of high conservation in the internal parts of eIF4E. It was suggested that this area forms an allosteric tract, which might be involved in structural changes that eIF4E undergoes upon interaction with its ligands (20).

To investigate whether such changes do indeed take place, CD experiments were performed with free and cap-bound eIF4E. We compared the CD spectrum of buffer containing 10 \(\mu\)M eIF4E, recorded between 198 and 250 nm in the presence and absence of cap analogue (Fig. 5A). Upon the addition of 20 \(\mu\)M m\(^7\)GpppG, the basic spectrum of eIF4E undergoes changes between 220 and 235 nm. Consistent with earlier studies (28), we did not find any absorbance of the free cap analogue at these wavelengths. We conclude, therefore, that the observed changes in the CD spectrum arise from conformational rearrangements within eIF4E.

As expected, the eIF4E-eIF4G-BD4E complex shows a spectrum that is significantly different from that of free eIF4E. However, upon addition of the cap analogue this complex shows changes in the CD spectrum very similar to those observed with eIF4E alone (Fig. 5B). This suggests, but does not prove, that the conformational changes are limited to a large extent to the eIF4E part of the cap complex and do not extend into the 17-kDa domain of eIF4G used here. Taken together with the data on the increased cap affinity of the eIF4E complex, these results suggest that the eIF4E binding domain stabilizes conformational rearrangements within eIF4E. Our data do not allow us to draw an unequivocal conclusion regarding the influence of eIF4E-BD4E binding on the conformation of apo-eIF4E.

**DISCUSSION**

The solving of the three-dimensional structure of eIF4E (15) has recently provided a new basis for understanding how eIF4E interacts with the cap structure. However, the initially

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\(^2\) M. Ptushkina and J. E. G. McCarthy, unpublished results.
assumed “static” binding model, in which eIF4E interacts with the cap only in the conformation deduced from the crystallographic data (12, 15), seems unlikely to provide an adequate explanation of the functional characteristics of this protein as part of eIF4F.

Most studies concerning the nature of cap binding by eIF4E assume a relatively low affinity for capped mRNAs, with a $K_{d}$ of $\sim 10^{-6}$ M. Such a low affinity appears problematic with respect to the many functions that eIF4G is thought to fulfill at the mRNA 5′-end, which may include removal of secondary structure from the message by virtue of the recruitment of eIF4A/4B, attachment of the ribosome via interaction between eIF4G and eIF3, and according to a very recent study, involvement in decapping of the message through recruitment of Dcp1 (29). These functions require a sequence of events to occur that are firmly anchored at the 5′-end of the RNA, suggesting that a sufficiently tight interaction between eIF4F and the mRNA is important. The potential problem is further highlighted if one assumes the operation of a very rapid binding cycle, especially with the rapid release of eIF4E from capped RNA, as reported in this study. It seems unlikely that a sequence of events as complex as the recruitment of the 40 S subunit could be tied to the RNA through an interaction that is determined by the rate constants and affinity manifested by the eIF4E-cap interaction alone.

One explanation for this apparent discrepancy could be provided by the fact that eIF4E itself shows affinity for mRNA through the action of two weakly conserved RRMs in the C terminus of the protein (8). Thus, eIF4F might be tied to the message through the combined action of eIF4E and eIF4G. Indeed, increased cross-linking of human eIF4E to a capped RNA in the presence of full-length eIF4G has been shown (30), and it has been proposed that this increase is merely attributable to the additional nonspecific eIF4G-mRNA interaction (23). In this context, it is important to note that the binding of eIF4G to RNAs is relatively weak, with an affinity measured for wheat eIF4G of $\sim 10^{-6}$ M (27).

The results reported here indicate that a different mechanism is involved in the increased association with mRNAs when eIF4E is part of eIF4F. The clearly observed increase in cap binding with fragments of eIF4G that bind to eIF4E, but not to RNAs, as well as the increased observed in binding to cap analogues that are not attached to an RNA body, are fully consistent with a model in which binding of ligands to the dorsal site on eIF4E modulates the affinity for the cap of this protein. Of the fragments tested here, the minimal portion of eIF4G capable of inducing this effect comprises the eIF4E binding site plus ~70 amino acids on either side. This explains earlier, apparently contradictory results, which showed that no enhancement of cap binding can be observed when a peptide corresponding only to the known eIF4E binding motif is used (21). We have now seen that the corresponding peptide fails to produce any clear effect using the techniques described in this paper.

With larger portions of eIF4G, or the full-length protein, the simultaneous presence of PAB1 was found to be necessary to achieve maximum reinforcement of the eIF4E-cap interaction. A comparable effect has been described for wheat eIF4F, where the presence of the poly(A)-binding protein PAB1 was found to enhance the affinity of both eIF4F and eIF(iso)F for cap analogues (19).

The data presented in this study, together with results derived from earlier studies (Table I), form a considerable body of evidence supporting the notion that cooperative effects play a role in the assembly and function of the cap-binding complex eIF4F. The finding of cooperative effects is significant for understanding how eIF4F can function during translation initiation despite the potentially transient nature of the basic eIF4E-cap interaction. Further work will have to address the role that cooperativity plays during translation initiation in vivo, and the way in which this might affect the overall process of translation and its regulation.

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