Communication

eIF4G Dramatically Enhances the Binding of eIF4E to the mRNA 5’-Cap Structure*

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The cap structure, m7GpppN, is present at the 5’-end of all eukaryotic cellular (except organellar) mRNAs. Initiation of translation is mediated by the multisubunit initiation factor eIF4F, which binds the cap structure via its eIF4E subunit and facilitates the binding of mRNA to ribosomes. Here, we used recombinant proteins to reconstitute the cap recognition activity of eIF4F \textit{in vitro}. We demonstrate that the interaction of eIF4E with the mRNA 5’-cap structure is dramatically enhanced by eIF4G, as determined by a UV-induced cross-linking assay. Furthermore, assembly of the eIF4F complex at the cap structure, as well as ATP hydrolysis, is shown to be a requisite for the cross-linking of another initiation factor, eIF4B, to the cap structure. In addition, the stimulatory effect of eIF4G on the cap recognition of eIF4E is inhibited by the translational repressor, 4E-BP1. These results suggest that eIF4E initially interacts with the mRNA cap structure as part of the eIF4F complex.

Cap-dependent binding of ribosomes to mRNA is mediated by several initiation factors, eIF4F, eIF4A, and eIF4B, and requires energy derived from ATP hydrolysis (1). eIF4F is a three-subunit complex composed of (i) eIF4E, (ii) eIF4A, and (iii) eIF4G. eIF4E is a 24-kDa polypeptide that specifically interacts with the 5’-cap structure (m7GpppN; where N is any nucleotide) (2). eIF4A is a 50-kDa protein that exhibits RNA-dependent ATPase activity and, in conjunction with eIF4B, RNA helicase activity (3, 4). eIF4G is a 154-kDa polypeptide that binds to both eIF4E and eIF4A (5, 6). eIF4G also exhibits sequence-nonspecific RNA binding activity that is most probably responsible for the RNA binding activity of eIF4F (7\textsuperscript{1}).

eIF4E activity is regulated by two proteins, termed 4E-BP1 and 4E-BP2 (8, 9). Interaction of 4E-BP1 with eIF4E inhibits specifically cap-dependent translation (9). 4E-BPs are rapidly hyperphosphorylated in cells following treatment with insulin and growth factors (10, 11). The phosphorylation of 4E-BPs decreases the association of 4E-BP1 with eIF4E (9). Consequently, phosphorylation of 4E-BPs leads to stimulation of translation. 4E-BP1 competes with eIF4G for binding to eIF4E through similar sequence motifs (12). Furthermore, the association of 4E-BP1 with eIF4E prevents the \textit{in vitro} phosphorylation of eIF4E by protein kinase C, raising the possibility of a temporal relationship between eIF4E binding to 4E-BPs and eIF4E phosphorylation (13).

Two models were proposed for the pathway of eIF4F assembly and subsequent ribosome binding. One model posits that the first step of ribosome binding is the interaction between eIF4F and the mRNA cap structure (1). According to this model, eIF4F in combination with eIF4B and eIF4A, unwinds secondary structure in the 5’-untranslated region of the mRNA, to create a single-stranded region of RNA, which serves as a binding site for the 43 S preinitiation complex. eIF4B and eIF4A were shown to cross-link to the cap structure only in the presence of eIF4F in a process that requires ATP hydrolysis (14–16). Joining of the 43 S ribosomal complex is thought to be mediated through an interaction of the eIF4G subunit and eIF3, the latter being part of the 43 S preinitiation complex. An alternative model for cap recognition postulates that eIF4E alone binds first the cap structure, which is then complexed with eIF4G that is already associated with the ribosome (17). This model is based on the finding that \textit{in vitro} translated eIF4G is bound to the 43 S preinitiation complex (17). Support for the first model stems from the observation that eIF4F cross-linked much more efficiently to the cap structure than did eIF4E alone (18). Furthermore, eIF4F in extracts prepared from poliovirus-infected cells, where the eIF4G subunit is cleaved and as a result eIF4E is associated with the NH\textsubscript{2}-terminal fragment of eIF4G, cross-links extremely inefficiently to the cap structure (19, 20). These results suggest an important function played by eIF4G in the cap recognition process.

In this report we reconstituted the eIF4F cap recognition activity \textit{in vitro} using recombinant components. In a photochemical cross-linking assay, we demonstrate directly that eIF4G increases the affinity of eIF4E for the cap structure. Binding of the eIF4F complex to the cap structure, as well as ATP hydrolysis, is shown to be a requisite for the cross-linking of eIF4B to the cap structure. In addition, 4E-BP1 is shown to inhibit cap binding activity of the eIF4E-eIF4G complex.

MATERIALS AND METHODS

\textbf{Protein Factors—}Murine eIF4E protein was expressed in \textit{Escherichia coli} K38 and purified as described previously (21). Recombinant flag-eIF4G was expressed in Sf9 insect cells and purified as described previously (22). Glutathione S-transferase (GST)\textsuperscript{2} fusion proteins of HMK-4E-BP1 and HMK-4E-BP1\textsuperscript{1} were expressed in \textit{E. coli} BL21 and purified as described previously (6). Recombinant eIF4B was expressed in Sf9 insect cells as follows: for the construction of the baculovirus transfer vector, eIF4B cDNA was excised from the plasmid pGEM3-eIF4B (23) with BamHI and subcloned blunt into the \textit{NheI} site of the p10 transfer vector (24). Recombinant baculovirus was generated by cationic liposome transfection of pOeIF4B construct with the linearized genomic AcMNPV DNA according to the manufacturer's instructions (Invitrogen). Recombinant virus was isolated (25), and eIF4B was purified as described previously (26).

\textbf{UV-induced Cross-linking Assay—}Uncapped RNA encoding chloramphenicol acetyltransferase was capped and methylated with 6 units of vaccinia virus guanylyltransferase (Life Technologies, Inc.) in the presence of sheared chromatin from 1000 cells. 

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\textsuperscript{1}H. Lee, unpublished data.

\textsuperscript{2}The abbreviation used is: GST, glutathione S-transferase.
RESULTS AND DISCUSSION

Analysis of the Interaction of Cap-binding Proteins with mRNA—To study the requirements for the interaction of eIF4E with the mRNA cap structure, purified recombinant initiation factors (Fig. 1) were used in a photochemical cross-linking assay (20). mRNA labeled with $^{32}$P in the cap structure was incubated with protein factors, irradiated with UV light, and RNase-digested. Labeled proteins were then analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. In the photochemical cross-linking experiments, RNA is the limiting component in the reaction mixtures. No detectable signal was observed in the absence of protein (Fig. 2A, lane 1). Similarly, no cross-linking of eIF4E to the cap-labeled mRNA was observed with 10 and 50 ng of purified eIF4E (lanes 2 and 3, respectively). Cross-linking was observed with 100 ng of eIF4E, albeit very inefficient (lanes 4), consistent with previous data (18, 20, 27). We next examined the effect of eIF4G on the cross-linking of eIF4E to the cap structure. No cross-linking of eIF4G to the cap structure was observed (lane 5). Cross-linking of eIF4G to the cap structure was dramatically enhanced in the presence of eIF4E (lanes 6–8). As little as 10 ng of eIF4E was efficiently cross-linked to the cap structure in the presence of flag-eIF4G. Comparison of lanes 4 and 8 revealed a 7-fold increase in the cross-linking of eIF4E to the cap structure in the presence of eIF4G. The cross-linking of eIF4E was cap-specific as the interaction was inhibited with 0.6 mM GDP (lane 9). To determine the stoichiometry between eIF4E and eIF4G required for efficient cap binding, 10 ng of eIF4E was preincubated with increasing amounts of flag-eIF4G before the addition of the other components (Fig. 2B). While eIF4E alone did not cross-link to the cap structure (lane 1), addition of increasing amounts of flag-eIF4G enhanced eIF4E cross-linking to the cap structure in a dose-dependent fashion (lanes 2–6). Under these conditions, ~10 ng of flag-eIF4G enhanced significantly the cross-linking of eIF4E to the cap (lane 2), with optimum binding occurring at a stoichiometry of 1:1 (lane 4). Cross-linking was inhibited by m$^3$GDP (lane 7), but not by GDP (lane 8).

We next analyzed the effect of other initiation factors on the cross-linking of eIF4E to the cap structure (Fig. 3). eIF4E alone did not cross-link to the cap structure (Fig. 3, lane 1), as observed above. The interaction of eIF4E with the cap structure was not affected by the presence of eIF4A (lane 5), eIF4B (lane 6), or a combination of eIF4B and eIF4A (lane 7). Cross-linking of eIF4A alone was not observed either (lane 3). Cross-linking of eIF4A to the cap structure was detected only when using the chemical cross-linking assay, where periodate oxidized mRNA is used (14–16, 20). Similarly, eIF4B failed to cross-link to the cap structure under these conditions (lanes 2, 6, and 7). As expected, cross-linking of eIF4E to the cap struc-
ture was dramatically enhanced in the presence of flag-eIF4G (lane 8). Flag-eIF4G did not promote, however, the cross-linking of either eIF4A or eIF4B when present alone (lanes 9 and 10, respectively), or in combination (lane 11), to the mRNA cap structure. Furthermore, a combination of flag-eIF4G and eIF4E failed to promote cross-linking of eIF4B (lane 12) or eIF4A (lane 13) to the cap structure. Cross-linking of eIF4B was observed only in the presence of all the subunits of the eIF4F complex (eIF4A, eIF4E, and flag-eIF4G; lane 14), as shown earlier (18). As expected, the specific interaction of eIF4E and eIF4B with the cap structure was insensitive to 0.6 mM GDP (lane 15) and was inhibited by 0.6 mM m7GDP (lane 16). This confirms earlier findings that cross-linking of eIF4B to the cap structure is dependent on eIF4F and ATP hydrolysis (16, 19, 20). Taken together, these results provide direct evidence for the stimulatory effect of eIF4G on the interaction of eIF4E with the cap structure.

4E-BP1 Prevents the Stimulatory Effect of eIF4G on the Cap Binding Activity of eIF4E—The activity of eIF4E is inhibited by 4E-BP1, which binds to eIF4E and prevents its interaction with eIF4G to form the eIF4F cap-binding protein complex (8, 9, 12). Earlier reports showed that binding of 4E-BP1 to eIF4E did not prevent the interaction of eIF4E with a cap-bound matrix (9, 12). However, the effect of 4E-BP1 on eIF4E binding to the cap structure as part of the mRNA has not been determined. As we have shown above, the interaction of eIF4E with the mRNA cap structure is dramatically enhanced when it is bound to eIF4G. This is consistent with the finding that following poliovirus infection, which leads to the cleavage of eIF4G, the cross-linking of eIF4E to the mRNA cap structure is drastically reduced (18, 19, 20). Under these conditions, eIF4E complexed to the amino-terminal cleavage product of eIF4G binds a cap-bound matrix (18). Similarly, eIF4E complexed to 4E-BP1 can efficiently bind the cap affinity column (9).

Based on the above observations, it is predicted that the cross-linking of eIF4E in an extract (where it binds tightly as part of eIF4F) should be diminished in the presence of 4E-BP1 (12). To examine this, photochemical cross-linking to the mRNA cap structure was performed in a rabbit reticulocyte lysate. Cross-linking was done in the presence of Mg2+-ATP to detect also eIF4B binding. UV irradiation induced cross-linking of polypeptides of 24, 65, and 80 kDa (Fig. 4A, lane 1), as shown previously (20). The 24- and 80-kDa polypeptides correspond to eIF4F and eIF4B, respectively, while the identity of the 65-kDa polypeptide is not known (20). The cross-linking of eIF4E and eIF4B was insensitive to 0.6 mM GDP (lane 2), but was inhibited by the same concentration of m7GDP (lane 3). In contrast, the cross-linking of the 65-kDa polypeptide was not affected by either nucleotide. Strikingly, preincubation of the reticulocyte lysate with GST-4E-BP1 drastically reduced the cross-linking of eIF4E and eIF4B to the cap structure (lane 4). Since eIF4B cross-linking is dependent on eIF4F, it is inhibited in the presence of 4E-BP1, which prevents eIF4F complex formation. These results indicate that eIF4E as a complex with 4E-BP1 interacts weakly with the cap structure, as compared with eIF4E as a subunit of eIF4F.

To further substantiate these conclusions, the UV-induced cross-linking assay was performed using purified components as in Figs. 2 and 3. eIF4E alone did not cross-link to the cap structure (Fig. 4B, lane 1). To examine the effect of 4E-BP1 on the cross-linking of eIF4E to the mRNA cap structure in this reconstituted system, 4E-BP1 was preincubated with eIF4E before the addition of the other components. 4E-BP1 prevented the efficient eIF4E cross-linking that occurs in the presence of flag-eIF4G (lane 2). To verify that the inhibitory effect was a result of a direct interaction between GST-4E-BP1 and eIF4E, a mutant of 4E-BP1 (GST-4E-BP1D) was used. GST-4E-BP1D contains a deletion of the 4E binding domain and does not repress translation (6, 12). Preincubation of eIF4E with GST-4E-BP1D had no effect on the stimulatory effect of eIF4G on the cross-linking of eIF4E to the cap structure (lane 3). The cross-linking of eIF4E to the cap structure in the presence of flag-eIF4G was sensitive to inhibition by 0.6 mM m7GDP (lanes 5). Taken together, these results demonstrate that 4E-BP1 prevents the facilitative effect of eIF4G on the interaction of eIF4E with the mRNA cap structure.

In summary, we have reconstituted the cap recognition step of eukaryotic translation initiation in vitro using purified components. We have demonstrated that eIF4G significantly enhances the cap recognition activity of eIF4E, suggesting that eIF4G plays an important role in the mechanism of mRNA cap recognition during eukaryotic translation initiation. Furthermore, 4E-BP1 inhibited the stimulatory effect of eIF4G on the cap binding activity of eIF4E. However, a 4E-BP1-eIF4E complex can be isolated by a cap affinity column (9, 12). It is conceivable that eIF4G, because of its RNA binding activity (28), interacts with the RNA in the vicinity of the cap structure and facilitates a stable association between eIF4E and the mRNA 5′-cap struc-

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3 H. Lee and C. Goyer, unpublished data.
ture. Indeed, eIF4F binds much more avidly to RNA than either eIF4E or eIF4A (7). Our results also indicate that eIF4B can gain access to the cap structure only in the presence of an intact eIF4F complex and ATP hydrolysis. In support of this conclusion, disruption of eIF4F complex by 4E-BP1 interfered with the efficient cross-linking of eIF4B to the cap structure. Taken together, our results indicate that eIF4E interacts with the cap structure as a subunit of eIF4F.

Several other observations support the hypothesis that eIF4F complex assembly occurs prior to the cap recognition step of translational initiation (18–20). The equilibrium constant ($k_{eq}$) of m$_7$GpppG:eIF4E (mammalian) complex formation has been determined by spectroscopic studies to be $4.8 \times 10^{-5}$ M (29, 30). This indicates a weak interaction between eIF4E and the cap structure that is unlikely to be favored in vivo. eIF4E as a subunit of eIF4F cross-links 20-fold better to the cap structure than eIF4E alone (31). Furthermore, Pelletier and Sonenberg (20, 32) showed that insertion of secondary structures 38 nucleotides downstream from the cap structure had no effect on UV cross-linking of initiation factors to the cap structure, whereas binding of ribosomes to mRNA was impaired, suggesting that eIF4F interaction with the cap structure precedes ribosome binding to mRNA. Based on our results and the studies cited above (19, 20, 32), we favor the model where some localized unwinding of the 5'-untranslated region of mRNA precedes ribosome attachment.

Other important parameters have also been implicated in effecting the interaction between eIF4E and the cap structure. Phosphorylation of both eIF4E and eIF4F is enhanced following treatment of cells with growth factors and insulin (33). eIF4E is more phosphorylated as a subunit of eIF4F (34, 35), and phosphorylated eIF4E forms a more stable complex with eIF4G (36). Furthermore, phosphorylated eIF4E was reported to bind better to the cap structure relative to its unphosphorylated form (37), and only the phosphorylated form of eIF4E is present in the 48 S preinitiation complex (38). In addition, the association of 4E-BP1 with eIF4E in vitro prevents the phosphorylation of eIF4E (13). Taken together these findings lend support to a model where prior assembly of eIF4F complex is a requisite for the cap recognition and subsequent ribosome binding steps of translation initiation in eukaryotes.

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REFERENCES

1. Sonenberg, N. (ed) (1996) in Translational Control (Hershey, J. W. B., Mathews, M. B., and Sonenberg, N., eds) pp. 245–270, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
2. Sonenberg, N., Morgan, M. A., Merrick, W. C., and Shatkin, A. J. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 6843–6847
3. Ray, B. K., Lawson, T. G., Kramer, J. C., Cladaras, M. H., Grifo, J. A., Abrahamson, R. D., Merrick, W. C., and Thach, R. E. (1985) J. Biol. Chem. 260, 7651–7658
4. Rozen, F., Edery, I., Meervooritch, K., Dever, T. E., Merrick, W. C., and Sonenberg, N. (1990) Mol. Cell. Biol. 10, 1134–1144
5. Lamphear, B. J., Kirchwever, R., Skern, T., and Rhoades, R. E. (1995) J. Biol. Chem. 270, 21975–21983
6. Mader, S., Lee, H., Pause, A., and Sonenberg, N. (1995) Mol. Cell. Biol. 15, 4990–4997
7. Jaramillo, M., Dever, T. E., Merrick, W. C., and Sonenberg, N. (1991) Mol. Cell. Biol. 11, 5992–5997
8. Lin, T. A., Kung, X., Haystead, T. A., Pause, A., Belsham, G., Sonenberg, N., and Lawrence, J., Jr. (1994) Science 266, 655–656
9. Pause, A., Belsham, G. J., Gingras, A. C., Denze, O., Lin, T. A., Lawrence, J., Jr., and Sonenberg, N. (1994) Nature 371, 762–767
10. Belenky, G. J. and Denvee, S. M. (1980) Biochem. Soc. Trans. 8, 382–383
11. Haystead, T. A., Haystead, C. M., Hu, C., Lin, T. A., and Lawrence, J., Jr. (1994) J. Biol. Chem. 269, 23183–23191
12. Haghighat, A., Mader, S., Pause, A., and Sonenberg, N. (1995) EMBO J. 14, 5701–5709
13. Whalen, S. G., Gingras, A. C., Amankwa, L., Mader, S., Branton, P. E., Aebersold, R., and Sonenberg, N. (1996) J. Biol. Chem. 271, 11831–11837
14. Sonenberg, N. (1981) Nucleic Acids Res. 9, 1643–1656
15. Grifo, J. A., Tahara, S. M., Morgan, M. A., Shatkin, A. J., and Merrick, W. C. (1983) J. Biol. Chem. 258, 5804–5810
16. Edery, I., Hummelin, M., Darvean, A., Lee, K. A., Milburn, S., Hershey, J. W., Trachsel, H., and Sonenberg, N. (1983) J. Biol. Chem. 258, 11388–11403
17. Joshi, B., Yan, R., and Rhoades, R. E. (1994) J. Biol. Chem. 269, 2048–2055
18. Lee, K. A., Edery, I., and Sonenberg, N. (1985) J. Virol. 54, 515–523
19. Lee, K. A., and Sonenberg, N. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 5447–5451
20. Pelletier, J., and Sonenberg, N. (1985) Mol. Cell. Biol. 5, 3222–3230
21. Edery, I., Altman, M., and Sonenberg, N. (1988) Gene (Amst.) 74, 517–525
22. Haghighat, A., Svitkin, Y., Novoa, I., Kuechler, E., Skern, T., and Sonenberg, N. (1996) J. Virol. 70, 8444–8450
23. Methot, N., Pause, A., Hershey, J. W., and Sonenberg, N. (1994) Mol. Cell. Biol. 14, 2307–2316
24. Vialard, J., Labumieri, M., Vernet, T., Breidis, D., Alkhaiti, G., Henning, D., Levin, D., and Richardson, C. (1990) J. Virol. 64, 37–50
25. Summers, M. D., and Smith, G. E. (1987) A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas Agricultural Experiment Station and Texas A & M University, College Station, TX
26. Pause, A., and Sonenberg, N. (1992) EMBO J. 11, 2643–2654
27. Sonenberg, N., Rupprech, K. M., Hecht, S. M., and Shatkin, A. J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4345–4349
28. Pestova, T. V., Shatkin, J. I., and Hellen, C. U. T. (1996) Mol. Cell. Biol. 16, 6870–6878
29. Carberry, S. E., Rhoads, R. E., and Goss, D. J. (1989) Biochemistry 28, 8078–8083
30. Ueda, H., Maruyama, H., Oue, M., Isida, T., Mioka, H., Tanaka, T., Nishikawa, S., and Uesugi, S. (1991) J. Biochem. (Tokyo) 109, 882–889
31. Edery, I., Lee, K. A. W., and Sonenberg, N. (eds) (1987) Translational Regulation of Gene Expression (Ilan, I., ed) pp. 335–366, Plenum Press, New York
32. Pelletier, J., and Sonenberg, N. (1985) Cell 40, 515–526
33. Morley, S. J., and Traugh, J. A. (1990) J. Biol. Chem. 265, 10611–10616
34. Lampheur, B. R., and Panniers, R. (1990) J. Biol. Chem. 265, 5333–5336
35. Tuzon, P. T., Morley, S. J., Dever, T. E., Merrick, W. C., Rhoads, R. E., and Traugh, J. A. (1990) J. Biol. Chem. 265, 10617–10621
36. Bu, X., Haas, D. W., and Hagedorn, C. H. (1993) J. Biol. Chem. 268, 4975–4978
37. Minich, W. B., Balasta, M. L., Goss, D. J., and Rhoads, R. E. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7668–7672
38. Joshi-Barve, S., Rychlik, W., and Rhoades, R. E. (1990) J. Biol. Chem. 265, 2979–2983