Eukaryotic Initiation Factors 4A (eIF4A) and 4G (eIF4G) Mutually Interact in a 1:1 Ratio in Vivo*

Wei Li, Graham J. Belshaw, and Christopher G. Proud†‡

From the *Division of Molecular Physiology, School of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, United Kingdom and the ‡Biotechnology and Biological Sciences Research Council Institute for Animal Health, Pirbright Laboratory, Ash Road, Woking, Surrey GU24 0NF, United Kingdom

mRNA translation in eukaryotic cells involves a set of proteins termed translation initiation factors (eIFs), several of which are involved in the binding of ribosomes to mRNA. These include eIF4G, a modular scaffolding protein, and eIF4A, an RNA helicase, of which two closely related forms are known in mammals, eIF4AI and eIF4AII. In mammals, eIF4G possesses two independent sites for binding eIF4A, whereas in other eukaryotes (e.g. yeast) only one site appears to be present, thus raising the issue of the stoichiometry of eIF4G-eIF4A complexes in different eukaryotes. We show that in human embryonic kidney cells eIF4G is associated with eIF4AI or eIF4AII but not with both simultaneously, suggesting a stoichiometry of 1:1 rather than 1:2. To confirm this, eIF4AI or eIF4AII was expressed in a tagged form in these cells, and complexes with eIF4G were again isolated. Complexes containing tagged eIF4AI or eIF4AII contained no endogenous eIF4A, supporting the notion that eIF4G binds only one molecule of eIF4A. Each binding site in eIF4G can bind either eIF4AI or eIF4AII. The data imply that the second binding site in mammalian eIF4A does not bind an additional eIF4A molecule and that initiation factor complexes in different eukaryotes contain one eIF4A per eIF4G.

Translation of mRNA in mammalian cells involves a set of proteins termed “translation factors.” A number of the proteins are involved in the initiation phase of translation, the main control point in this process, and are accordingly termed “initiation factors” (eIFs).† Several of the eIFs play a role in the recruitment of mRNAs to ribosomes and in the events leading to the recognition of the initiation codon. One such protein is eIF4A, an ATP-dependent RNA helicase of the “DEAD-box” family (1, 2). It is thought to act to remove regions of secondary structure within the 5′-untranslated region of the mRNA to facilitate ribosome binding and scanning (1, 3). As revealed by the use of dominant negative mutants (4), eIF4A appears to be essential for cap-dependent and also for cap-independent initiation directed by picornavirus internal ribosome entry sites.

eIF4A interacts with a scaffold protein, eIF4G, to form complexes that also contain the cap-binding protein eIF4E, which binds the cap structure (m7GpppN . . . ) at the 5′-end of the mRNA (for reviews see Refs. 1 and 5). These complexes are termed “eIF4F.” eIF4A shows much higher helicase activity as part of such complexes than as free eIF4A (6).

Three related proteins have been designated as distinct forms of eIF4A, eIF4AI and eIF4AII are closely related and are both known to be cytoplasmic and to interact with eIF4G (7). Some evidence for a preferential association of eIF4G with eIF4AI as compared with eIF4AII as presented (7). More recently, a further protein termed eIF4AIII has been identified and reported to inhibit translation (8). However, this protein is more distantly related and appears to be primarily nuclear (9). Its involvement in translation initiation has therefore been questioned (8, 9).

Recently, two independent binding sites were identified within mammalian eIF4G for eIF4A (10). One lies in the central region of eIF4G whereas the other lies toward its C terminus (10–12). In contrast, both species of yeast eIF4G lack the C-terminal domain that interacts with eIF4A and thus appear to contain only one site for interaction with this factor within the central region of the yeast eIF4G polypeptide (13, 14). Mammalian eIF4A can interact with the remaining site in the central region of eIF4G (15) but cannot functionally replace the yeast eIF4A gene (16). Recent data from Morino et al. (12) suggest that the C-terminal region of mammalian eIF4G may function as a modulatory domain.

The existence of two eIF4A binding sites in eIF4G suggested that mammalian eIF4F may contain two copies of eIF4A, and a recent report from Korneeva et al. (17) using surface plasmon resonance to explore eIF4G/eIF4A interactions in vitro offered support to this idea. If true in vivo, this could reflect a fundamental difference in the composition and function of eIF4F complexes in mammals compared to eukaryotes, given that yeast eIF4G only possesses one site for binding eIF4A (13, 14). We therefore considered it important to study the interactions between mammalian eIF4G and eIF4A in vivo, and our data indicate that in fact eIF4G can only bind one molecule of eIF4A at a time under in vivo conditions. Thus, eIF4F complexes in yeast and mammals do not appear to differ in their eIF4A content, despite the presence in mammalian eIF4G of the second eIF4A binding site.

MATERIALS AND METHODS

Chemicals and Biochemicals—General laboratory chemicals were from Sigma-Aldrich (Gillingham, United Kingdom) or BDH Merck (Dorset, UK). Materials for cell culture were from Life Technologies, Inc. m7GTP-Sepharose was from Amersham Pharmacia Biotech. Oligonucleotides were synthesized from MWG Biotech (Milton Keynes, UK). Restriction enzymes were purchased from either Promega (Southampton, UK) or New England Biolabs (Hitchin, UK).

*This work was supported by Project Grant 94/C11079 (to G. J. B and C. G. P.) from the Biotechnology and Biological Sciences Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed. Tel.: 44 1382 344919; Fax: 44 1382 322424; E-mail: c.g.proud@dundee.ac.uk.

‡The abbreviations used are: eIF, eukaryotic initiation factor; His, hexahistidine; m7GTP, 7-methylguanosine triphosphate. Ni-NTA, nickel-nitrilotriacetic acid; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

Received for publication, May 30, 2001, and in revised form, June 13, 2001
Published, JBC Papers in Press, June 14, 2001, DOI 10.1074/jbc.C100284200

This paper is available online at http://www.jbc.org

Vol. 276, No. 31, Issue of August 3, pp. 29111–29115, 2001
Printed in U.S.A.
Production of Antisera—Polyclonal antibodies were generated by immunizing sheep (Diagnostix Scotland) with the appropriate peptide immunogen coupled (via the C-terminal cysteiny1 residue) to keyhole limpet hemocyanin. Antibodies were purified over columns consisting of the relevant peptide coupled to Affi-Gel (Bio-Rad). Peptides used were as follows: the C-terminal Cys being used for coupling: ASQDSSRSRDNGPDAC (residues 3–16 of human eIF4AI); GGSADYNREHGGRFGRKC (sequence common to eIF4A II); GATCCAGAGGATCTGGGATCCATGTCTGGTGTCGCGG (residues 354–371). The anti-eIF4AI antisera were raised in a similar manner using the following peptides as immunogens: for the middle domain of eIF4A I, KLEEELEARDIARRC (corresponding to residues 841–854). For expression in human embryonic kidney (HEK) 293 cells, the anti-eIF4AII antibody is roughly twice as sensitive as the antibody to eIF4AI but showed no reaction with eIF4A II, whereas the converse was true for the anti-eIF4A II antibody (Fig. 1).

Vectors—pETHM-eIF4AI and pETHM-eIF4AII are vectors encoding His- and Myc-tagged eIF4AI and eIF4AII, respectively. pETHM-eIF4AI was made by ligating an NheI-KpnI-digested PCR fragment encoding the N terminus of eIF4AI and a Km-IstI fragment from pGemZfA into the BamHI-Sacl sites of pETHM. pETHM is a modified version of the expression vector pET28, by replacing the BamHI-Sacl-digested PCR fragment of N-terminal eIF4AI, and a SpeI-Sacl fragment from pA4Inns (a kind gift from P. Nielsen (18)) into the BamHI-Sacl sites of pETHM. pETHM is a modified version of the expression vector pET28, containing a Myc-tag instead of the T7 tag found in pET28. It was made by replacing the Spbl-BamHI region of pET28 with the Spbl-BamHI fragment from pETHM-eIF4AI. The primers used for amplification of the N terminus of eIF4AI, 3'-ACGGATTCATGTCTGGTGGCTC-9 and the m7GTP-Sepharose beads were then washed twice with extraction buffer containing 200 mM mGDP to elute the bound proteins. Samples of eluate were incubated with anti-eIF4AI or anti-eIF4AII, proteins were recognized by a third antibody raised against a peptide sequence common to the C termini of both proteins. The antisera were calibrated using differing amounts of recombinant eIF4AI and eIF4AII (Fig. 1). The anti-eIF4AI antibody recognized eIF4AI but showed no reaction with eIF4AII, whereas the converse was true for the anti-eIF4AII antibody (Fig. 1A).

Cell Culture, Transfection, and Extraction—HEK 293 cells were maintained and transfected (20–30 μg of DNA/10-cm dish) as described earlier (19). The extractions were performed using our standard buffer (19), except that for Ni-NTA-agarose pulldown experiments the EDTA concentration was decreased from 1 to 0.2 mM.

RESULTS

The main aim of this study was to investigate the composition of eIF4F complexes in vivo in mammalian cells. To determine whether HEK 293 cells express both eIF4AI and eIF4AII, we made use of antisera that specifically recognize these proteins. The antisera were raised against peptides corresponding to residues within the N termini of eIF4AI and eIF4AII, which are completely different between the two polypeptides (see “Materials and Methods”). To verify that the antisera were indeed specific for each protein and to compare their potencies, we studied their abilities to recognize recombinant eIF4AI and eIF4AII in Western blots. The anti-eIF4AI antibody recognized eIF4AI but showed no reaction with eIF4AII, whereas the converse was true for the anti-eIF4AII antibody (Fig. 1A). Both proteins were recognized by a third antibody raised against a peptide sequence common to the C termini of both proteins. The antisera were calibrated using differing amounts of recombinant eIF4AI and eIF4AII (Fig. 1B). The anti-eIF4AI antibody should detect each form equally, and this was borne out by the data in the upper part of Fig. 1B, where a signal of similar strength was seen for the same amounts of the two proteins. In the lower part of Fig. 1B it can be seen that similar signals were seen for 150 ng of eIF4AI and 75 ng of eIF4AII (and so on for the three amounts of protein tested) indicating that the anti-eIF4AI antibody is roughly twice as sensitive as the antibody to eIF4AI.

Western blot analysis using the specific antibodies revealed that both eIF4AI and eIF4AII are present in HEK 293 cells (Fig. 1C). In the cell extracts, the strengths of the signals for eIF4AI and eIF4AII were similar suggesting (given the data in Fig. 1B) that the level of eIF4AI is probably slightly (about 2-fold) higher than that of eIF4AII. Cells were treated with insulin for 20 min prior to extraction to enhance the formation of eIF4F complexes (21). Samples of cell extract were also subjected to chromatography on mGTP-Sepharose. This resin binds eIF4E, and thus eIF4A that is part of eIF4F complexes is also retained on the resin through the interaction of eIF4A with eIF4G. Western blotting with the above antibodies revealed that both...
that contained only one eIF4A binding site (Fig. 1). It is possible that the eIF4G had been degraded to produce fragments that contained full-length eIF4G, thus ruling out the possibility that other species of eIF4A was absent (Fig. 1). Immunoprecipitation of eIF4A against which the antibody was raised and that the resulting immunoprecipitates contained only the isoform of eIF4A (10), it was possible that eIF4F complexes could contain both forms of eIF4A but these must both be either eIF4AI or eIF4AII. To distinguish between these possibilities, HEK 293 cells were transfected with vectors encoding the middle (4G M) and C-terminal (4G C) domains of eIF4G, each containing a His6 tag, or with empty vector (−). A expression was verified by analysis of samples of the cell extracts by SDS-PAGE/Western blotting with anti-His6 antibody. Samples of extract were applied to Ni-NTA-agarose (as indicated) to purify the tagged eIF4G fragments and associated proteins; the fragments were retained on this resin as shown by Western blotting with the anti-His6 antibody. The positions of eIF4GM and eIF4GC are shown. B and C, samples of the material retained on Ni-NTA-agarose were also subjected to SDS-PAGE/Western blotting using the anti-eIF4Ac antibody (B, position of eIF4A is indicated) or with the anti-eIF4AI or anti-eIF4AII antibodies, as indicated (C).

The above data support the idea that eIF4F just binds a single eIF4A molecule, either eIF4AI or eIF4AII. They therefore imply that each of the two binding sites for eIF4A on eIF4G can bind only to either eIF4AI or eIF4AII and that a single eIF4A molecule might bind simultaneously to both sites. It has, however, not previously been shown that eIF4F binds both sites in eIF4G. To test this, HEK 293 cells were transfected with plasmids encoding the middle (eIF4GM) and C-terminal (eIF4GC) fragments of eIF4G. These polypeptides include His6 and Myc tags and were efficiently expressed in HEK 293 cells (Fig. 3A). Samples of extract from these cells were subjected to chromatography on Ni-NTA-agarose, and the bound material was analyzed by SDS-PAGE/immunoblotting. The tagged eIF4G fragments were clearly retained on the resin (Fig. 3A), whereas full-length, untagged eIF4G was not (data not shown). Analysis of the Ni-NTA-agarose-bound material showed that eIF4AT was retained on this resin when either of the tagged eIF4G fragments was expressed but not in the absence of these fragments. eIF4A does not therefore bind nonspecifically to the resin (Fig. 3B). To determine whether both forms of eIF4A were bound by the eIF4G fragments, we used polyclonal antibodies recognizing eIF4AI and eIF4AII, which were each retained on the resin when samples from cells transfected with either the middle or C-terminal fragment of eIF4G were analyzed, implying that each protein can bind to both sites in eIF4G (Fig. 3C). This experiment also demonstrates that the interactions between eIF4A and eIF4G are relatively stable, thus ruling out the possibility that our observation that only the eIF4A molecule is found in each eIF4F complex is an artifact because of dissociation of eIF4A from one of the binding sites in eIF4G. If eIF4A can bind simultaneously to both sites in eIF4G, it might be able to act as a bridge between two different eIF4G molecules effectively bringing about the dimerization of eIF4G. To test this, we transfected HEK 293 cells with plasmids encoding the His6-tagged eIF4G3M or eIF4G3C polypeptides. These fragments of eIF4G do not contain the binding site for eIF4E that is present in native eIF4G and should therefore only be
In Vivo Interactions between Mammalian eIF4G and eIF4A

These data suggest that no such bridging between eIF4G molecules (mediated, e.g. by eIF4A) occurs. This could reflect either (i) a strong “preference” for eIF4A to interact in cis with the two binding sites on a single eIF4G molecule rather than in trans with sites on two different eIF4G molecules or (ii) that only a single eIF4A binding site can actually be occupied at one time on a given eIF4G molecule. Our experiments do not allow us to distinguish between these two possibilities.

DISCUSSION

The present study provides strong evidence that the mammalian eIF4F complex contains only one molecule of eIF4A, despite the finding that mammalian eIF4G actually possesses two independent sites for interaction with eIF4A (10). Two possible explanations for the 1:1 stoichiometry are (i) that eIF4A cannot bind simultaneously to the two sites, so that it is associated with one or the other, giving rise to the 1:1 stoichiometry indicated by our results or (ii) that eIF4A does bind to both sites on eIF4G at the same time, through different binding sites in the eIF4A protein, so that it is “sandwiched” between these sites as originally proposed by Imataka and Sonenberg (10). Earlier data (10, 12) indicate that the C-terminal binding site enhances cap-dependent translation and the binding of ribosomes to mRNA in vitro. However, these studies and those of de Gregorio et al. (22) (who showed that the central “core” of eIF4G could support translation, albeit rather inefficiently) indicate that this site is not essential for mRNA translation. In contrast, binding of eIF4A to the central region is both essential and sufficient for translation (10). Based on these considerations, Morino et al. (12) have suggested that the C-terminal domain of eIF4G plays a modulatory role.

Our data lend support to the ideas initially put forward by Sonenberg and coworkers (10, 12) that eIF4G binds only one molecule of eIF4A. Thus, the eIF4F complex of mammals does not appear to differ in overall composition from the likely situation in yeast, where both isoforms of eIF4G each contain only one binding site for eIF4A (23). The residues within the C terminus of eIF4G identified as being required for eIF4A binding (Phe-Val-Arg (10)) are not conserved in the orthologs from wheat (24) or Drosophila melanogaster (25). As noted before (17), the existence of two binding sites in mammalian eIF4G may result in more stable binding of eIF4A than for the forms of eIF4G that only have one such site. This could explain why eIF4A does not co-purify with eIF4G from yeast (26) or Drosophila (27), even though an interaction between eIF4G and eIF4A can be demonstrated in vitro for these species (8, 14, 15). Mutation of just one of the sites in mammalian eIF4G was enough to reduce by about 10-fold the association of eIF4A with eIF4G in toe-printing experiments (12) although there is ample data showing that eIF4A can interact stably with fragments of eIF4G containing only one binding site (see, for example, Refs. 10, 12, and 17 and this study).

Recent studies from Korneeva et al. (17) used surface plasmon resonance techniques, with purified proteins, to determine the binding affinities of the middle and C-terminal domains of eIF4G for eIF4A and the association and dissociation constants for these interactions. It was found that the middle domain of eIF4G binds eIF4A with a 20-fold higher affinity than the C-terminal fragment. Each domain of eIF4G could bind one molecule of eIF4A, and they reported that the region of eIF4G containing residues 642–1560 (with both binding sites) could bind two molecules of eIF4A. This last conclusion is in disagreement with our own data showing a 1:1 interaction of eIF4A with endogenous full-length eIF4G in vivo. It could be argued that the our approach involving immunoprecipitation may miss weak protein/protein interactions, which dissociate during the work-up of the samples, and that this could account for the
difference between our studies. However, it should be noted that in our studies (see Fig. 3) both domains of eIF4GIII and eIF4GC did appear to interact with similar efficiencies with eIF4AI and eIF4AII. Thus, we do not appear to be “losing” eIF4A that is bound to one of the sites during the isolation of complexes from the cell extracts. This would be consistent with the finding of Korneeva et al. (17) that although the Kd values for the two interactions do markedly differ, this is due to differences in the association rate constants rather than in the dissociation constants. The lack of bridging between two fragments of eIF4G by eIF4A observed by Korneeva et al. (17) in solution is also entirely consistent with our own findings. The implication of our study that eIF4G only binds one eIF4A molecule at a time is also in agreement with the data of Li et al. (8) who showed that, when endogenous eIF4G is bound to eIF4AIII (which binds only to the middle domain of eIF4G), eIF4AI cannot bind to this eIF4G molecule. If eIF4G could simultaneously bind to two eIF4A molecules, both species of eIF4A should be found.

Our data show further that both eIF4AI and eIF4AII can each bind to both domains of mammalian eIF4G independently. Early data (7) suggested that eIF4AI might be preferentially incorporated into eIF4F complexes; our data reveal no gross difference between the binding of eIF4AI and eIF4AII to the two domains of eIF4G. The possibility that eIF4A is sandwiched between the two binding sites in eIF4G requires that eIF4A possesses two different sites for interaction with eIF4G rather than binding through a single site to both regions of eIF4G. To date, the two binding sites in eIF4G have been partially defined. The PVR motif in the C-terminal domain has been noted above, and the key residues in the central region of eIF4G that are required for the binding of eIF4A are hydrophobic ones (10). An important goal of further studies will be to define the binding sites within eIF4A for the two domains of eIF4G. This may be aided by the recent determinations of the three-dimensional structure of yeast eIF4A (28–30).

REFERENCES
1. Gingras, A.-C., Raught, B., and Sonenberg, N. (1999) Annu. Rev. Biochem. 68, 913–963
2. de la Cruz, J., Kressler, D., and Linder, P. (2000) Trends Biochem. Sci. 25, 192–198
3. Svitkin, Y. V., Pause, A., Haghighat, A., Pyronnet, S., Witherell, G., Belsham, G. J., and Sonenberg, N. (2001) RNA (N.Y.) 7, 382–394
4. Pause, A., Methot, N., Svitkin, Y., Merrick, W. C., and Sonenberg, N. (1994) EMBO J. 13, 1205–1215
5. Pain, V. M. (1996) Eur. J. Biochem. 236, 747–771
6. Rozen, F., Edery, I., Mevorich, K., Dever, T. E., Merrick, W. C., and Sonenberg, N. (1990) Mol. Cell. Biol. 10, 1134–1144
7. Conroy, S. C., Dever, T. E., Owens, C. L., and Merrick, W. C. (1990) Arch. Biochem. Biophys. 282, 363–371
8. Li, Q., Imataka, H., Morino, S., Rogers, G. W., Richter-Cook, N. J., Merrick, W. C., and Sonenberg, N. (1999) Mol. Cell. Biol. 19, 7336–7346
9. Holzmann, K., Gerner, C., Pottl, A., Schäfer, R., Ohrist, P., Ensinger, C., Grimm, R., and Sauermann, G. (2000) Biochem. Biophys. Res. Commun. 267, 339–344
10. Imataka, H., and Sonenberg, N. (1997) Mol. Cell. Biol. 17, 6940–6947
11. Lamphear, B. J., Kirchweiger, R., Skern, T., and Rhoads, R. E. (1995) J. Biol. Chem. 270, 21975–21983
12. Morino, S., Imataka, H., Svitkin, Y. V., Pestova, T. V., and Sonenberg, N. (2000) Mol. Cell. Biol. 20, 468–477
13. Neff, C. L., and Sachs, A. B. (1999) Mol. Cell. Biol. 19, 5557–5564
14. Domínguez, D., Altmann, M., Benz, J., Baumann, U., and Trachsel, H. (1999) J. Biol. Chem. 274, 26720–26726
15. Domínguez, D., Kidig, E., Altmann, M., and Trachsel, H. (2001) Biochem. J. 355, 223–230
16. Prat, A., Schmid, S. R., Buser, P., Blum, S., Trachsel, H., Nielsen, P. J., and Linder, P. (1990) Biochim. Biophys. Acta 1050, 140–145
17. Korneeva, N. L., Lamphear, B. J., Hennigan, F. L. C., Merrick, W. C., and Rhoads, R. E. (2001) J. Biol. Chem. 276, 28722–28728
18. Nielsen, P. J., and Trachsel, H. (1988) EMBO J. 7, 2997–29720
19. Scheper, G. C., Morrice, N., and Proud, C. G. (2001) Mol. Cell. Biol. 21, 741–754
20. Price, N. T., Nakielny, S. F., Clark, S. J., and Proud, C. G. (1989) Biochim. Biophys. Acta 1098, 177–182
21. Herbert, T. P., Kilhams, G. R., Batty, I. H., and Proud, C. G. (2000) Biochim. Biophys. Acta 1775, 11249–11256
22. de Gregorio, E., Preiss, T., and Hentze, M. W. (1999) EMBO J. 18, 4865–4874
23. Goyer, C., Altmann, M., Lee, H. S., Blum, S., Trachsel, H., Nielsen, P. J., and Linder, P. (1990) Biochim. Biophys. Acta 1050, 140–145
24. Allen, M. L., Metz, A. M., Timmer, R. T., Rhoads, R. E., and Browning, K. S. (1992) J. Biol. Chem. 267, 23232–23236
25. Hernandez, G., del mar Castellano, M., Aguado, M., and Sierra, J. G. (1998) Eur. J. Biochem. 253, 27–35
26. Goyer, C., Altmann, M., Trachsel, H., and Sonenberg, N. (1989) J. Biol. Chem. 264, 7603–7610
27. Zapata, J. M., Martinez, M. A., and Sierra, J. M. (1994) J. Biol. Chem. 269, 18047–18052
28. Caruthers, J. M., Johnson, E. R., and McKay, D. B. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 13080–13085
29. Benz, J., Trachsel, H., and Baumann, U. (1999) Structure 7, 671–679
30. Johnson, E. R., and McKay, D. B. (1999) RNA (N.Y.) 5, 1526–1534
Eukaryotic Initiation Factors 4A (eIF4A) and 4G (eIF4G) Mutually Interact in a 1:1 Ratio in Vivo
Wei Li, Graham J. Belsham and Christopher G. Proud

J. Biol. Chem. 2001, 276:29111-29115.
doi: 10.1074/jbc.C100284200 originally published online June 14, 2001

Access the most updated version of this article at doi: 10.1074/jbc.C100284200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 18 of which can be accessed free at http://www.jbc.org/content/276/31/29111.full.html#ref-list-1