Mutational Analysis of the Functional Domains of the Large Subunit of the Isozyme Form of Wheat Initiation Factor eIF4F*

(Received for publication, July 31, 1996, and in revised form, September 12, 1996)

Anneke M. Metz and Karen S. Browning‡
From the Department of Chemistry and Biochemistry, University of Texas, Austin, Texas 78712

The isozyme form of plant eukaryotic initiation factor 4F (eIF(iso)4F) contains two subunits: p28, a cap-binding protein, and p86. To identify the functional domains of p86, truncations of the p86 cDNA were made, and the protein was expressed in Escherichia coli and purified. The deletion mutants were tested for the ability to bind the p28 subunit by two methods. In addition, these deletion mutants were evaluated in vitro by the ability to catalyze eIF4A and RNA-dependent ATP hydrolysis and to support polypeptide synthesis. The loss of the ability to bind p28 occurs within the first 90 amino acids of the N terminus and abrogates the ability of p86 to participate in translation initiation and bind to eIF4A, but does not affect ATP hydrolysis. Up to 299 amino acid residues from the C terminus of p86 must be deleted before an effect is observed on the ATP hydrolysis activity. Thus, the p28 binding and ATP hydrolysis activities appear to lie on two separate domains and are functionally uncoupled. In addition, at least a portion of the eIF4A binding domain appears to be in close proximity to the p28 binding domain and is also uncoupled from the ATP hydrolysis activity.

The initiation of eukaryotic protein synthesis is a complicated process and involves several initiation factors (for recent reviews, see Refs. 1 and 2). The precise mechanism of binding the mRNA and bringing it to the 40 S subunits is unknown, and several models have been proposed (3–5). One of the initiation factors, eIF4F,1 contains two subunits, eIF4E, a cap-binding protein and eIF4G, a protein of largely unknown function. Another initiation factor, eIF4A, is frequently found associated with eIF4F depending upon the method of purification (6, 7).

The subunits of mammalian eIF4F are believed to be involved in regulation of protein synthesis. The large subunit of mammalian eIF4F, eIF4G, is cleaved by picornaviral proteases, severely affecting the ability of the cell to translate capped mRNAs (8). These proteases cleave mammalian eIF4G into N-terminal and C-terminal fragments (9, 10). The N-terminal fragment binds the mammalian cap-binding protein, and the C-terminal fragment binds eIF4A and eIF3 (9). The C-terminal fragment alone has been shown to promote cap-independent translation of mRNAs containing an internal ribosome binding site (10).

Phosphorylation of mammalian eIF4E is believed also to be involved in regulation of initiation. However, there is no clear difference in the ability of phosphorylated eIF4E to either bind mGTP-Sepharose or interact with eIF4G (1). In studies where the phosphorylation state of eIF4E was altered, there were also changes in the phosphorylation state of other initiation factors (11–14). Consequently, the exact mechanism of this regulation remains to be elucidated. Recently, another protein was identified that binds to mammalian eIF4E. This protein, eIF4E-BP1 or PHAS-I, is phosphorylated in response to insulin in mammalian cells (15, 16). There is now considerable evidence that this protein sequesters eIF4E and prevents eIF4E from interacting with eIF4G (17). The phosphorylated form of eIF4E-BP1 is unable to bind eIF4E and releases any bound eIF4E (15). Several kinases in the signal transduction pathways have been implicated in this process (18).

Wheat translation initiation factor eIF(iso)4F, the isozyme form of eIF4F, contains two subunits, p28 and p86 which are antigenically distinct from the two eIF4F subunits isolated from wheat germ (19). The isozyme form of eIF4F appears to be unique to higher plants and has not been identified as yet in other eukaryotes. The protein has been observed in wheat, maize, and cauliflower (20), and cDNA expressed sequence tags for the subunits have been identified for rice and Arabidopsis thaliana.2 The p28 subunit is a cap-binding protein (19). Analogous to mammalian eIF4G, no specific function(s) has been assigned to p86. The p86 subunit is considerably smaller (86 kDa) than the predicted molecular mass for human eIF4G (154 kDa (21)) or yeast eIF4G (104 kDa (22)). There are seven regions of similarity between p86 and mammalian and/or yeast eIF4G (see Fig. 1A). eIF(iso)4F has the same functional properties as wheat germ eIF4F: 1) it substitutes for eIF4F in an in vitro translation system deficient in eIF4F; 2) it substitutes for eIF4F in supporting the binding of mRNA to 40 S ribosomal subunits; 3) it exhibits RNA-dependent ATP hydrolysis activity in the presence of eIF4A; and 4) it exhibits ATP-dependent RNA unwinding activity in the presence of eIF4A (19, 23–25).

Plant eIF(iso)4F carries out the same functions as eIF4F, but with a large subunit approximately one-half the size of mammalian eIF4G. The smaller size and the ability to express a functionally active protein in Escherichia coli for p86, makes it ideal for identifying the functional domains of this protein. In the present paper, we initiate mapping of the domains of the p86 subunit to better understand its interaction with the cap-binding protein and other initiation factors. Truncation mutations of p86 were made and tested for the ability to bind to p28, to participate in ATP hydrolysis, to support polypeptide synthesis, and to interact with eIF4A.

EXPERIMENTAL PROCEDURES

Materials—Wheat germ high-salt-washed ribosomes, 40–70% ammonium sulfate fraction, and highly purified fractions of eIF3 (26), eIF4A (26), eIF4C (27), eIF(iso)4F (20), and recombinant p28 (28) were prepared as described previously. Purification of eIF4A is described elsewhere. Preparation of satellite tobacco necrosis virus RNA was as described previously (29). Restriction enzymes, T4 DNA ligase, and

* This work was supported by National Science Foundation Grant MCB 9406601 (to K. S. B.). 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.: 512-471-4562; Fax: 512-471-8686; E-mail: kbrowning@mail.utexas.edu.
1 The abbreviations used are: eIF, eukaryotic initiation factor; PAGE, polyacrylamide gel electrophoresis; AA, amino acid(s).

2 K. Browning, unpublished observation.
oligonucleotide primers were from Life Technologies, Inc. Sequencing reagents were from United States Biochemical Corp. DNA amplification reagents were from P/E Express. SDS-PAGE was performed as described previously (30). Protein concentrations were determined by the method of Bradford (31) with bovine serum albumin as a standard. (

---

**TABLE 1**

Primer pairs used to create p86 truncation mutants

| Mutant | Primer 1* | Primer 2 |
|--------|-----------|----------|
| N-52  | 2345'-CTCCACGAGACGTCCAATGCC-3132 | 2345'-CTCCACGAGACGTCCAATGCC-3132 |
| N-90  | 2345'-GCACGAGACGTCCAATGCC-3132 | 2345'-GCACGAGACGTCCAATGCC-3132 |
| N-136 | 4615'-TGACCAACAGAGACCCGCC-488 | 4615'-TGACCAACAGAGACCCGCC-488 |
| N-186 | 14615'-AGAGACCCGCC-488 | 14615'-AGAGACCCGCC-488 |
| C-462 | 2345'-CGACCCTTCACGACGACCCGCC-488 | 2345'-CGACCCTTCACGACGACCCGCC-488 |
| C-489 | 2345'-CGACCCTTCACGACGACCCGCC-488 | 2345'-CGACCCTTCACGACGACCCGCC-488 |
| C-511 | 2345'-CGACCCTTCACGACGACCCGCC-488 | 2345'-CGACCCTTCACGACGACCCGCC-488 |

* Primer pairs used for PCR mutagenesis of each p86 mutant are indicated. Nucleotide positions on the p86 cDNA template are indicated by numbers flanking primer sequences. Changes in base positions are shown in small caps. **NeoI** sites (CCATGG) and **BamHI** sites (GGATCC) are underlined. Initiation (ATG) and stop (TTA) codon sites are in bold.

---

**RESULTS AND DISCUSSION**

A series of deletion mutants from the N terminus and C terminus of p86 were excised from the pET3d expression vector with **NeoI** and **BamHI** and inserted into pGBT-9/N restricted with **NeoI** and **BamHI** by standard procedures. **Two-hybrid Interaction Assays**—Sets of two plasmids were co-transformed as indicated (see Tables III and IV) into yeast strain SBY-526 as per the manufacturer’s instructions (Clontech). Interaction in Table III was scored by the appearance of blue color using the β-galactosidase liquid assay as per the manufacturer’s instructions (Clontech). Interaction in Table IV was quantitated by the β-galactosidase liquid assay as per the manufacturer’s instructions (Clontech).
Fig. 2. Analysis of p86 mutants by SDS-PAGE and immunoblotting. SDS-PAGE was carried out as described under “Experimental Procedures.” The gel contained 12.5% acrylamide and 0.07% bisacrylamide. Each lane contained 10 μg of the indicated purified protein. The gel was stained with Coomassie Brilliant Blue. Lane 1, native eIF(iso)4F; lane 2, recombinant p86; lane 3, N-52; lane 4, N-90; lane 5, N-136; lane 6, N-186; lane 7, C-462; lane 8, C-489; lane 9, C-511. The markers used were: phosphorylase b (M₉ = 94,000), bovine serum albumin (M₉ = 68,000), catalase (M₉ = 58,000), fumarase (M₉ = 48,000), and aldolase (M₉ = 40,000).

| Protein added | Polypeptide synthesis | ATP hydrolysis | Binding of p28 |
|---------------|-----------------------|----------------|----------------|
|               | % control             | % control     | (Yes/No)       |
| Wild-type     | 100⁵                  | 100⁵           | Yes            |
| N-52          | 141 ± 8               | 110 ± 11      | Yes            |
| N-90          | 7 ± 4                 | 109 ± 2       | No             |
| N-136         | 1 ± 0.1               | 85 ± 22       | No             |
| N-186         | 3 ± 2                 | 98 ± 29       | No             |
| C-462         | 36 ± 2                | 14 ± 14       | Yes            |
| C-489         | 101 ± 6               | 58 ± 33       | Yes            |
| C-511         | 99 ± 8                | 106 ± 43      | Yes            |

The ability of the p86 mutants to bind to p28 was measured by the presence of the p86 mutant in the m⁷GTP eluant on an SDS-PAGE as described under “Experimental Procedures.”

30 pmol of [³⁵S]leucine incorporated. The amount incorporated in the absence of p86 was ~6 pmol. The data are an average of three independent experiments.

350 pmol of [γ-³²P]ATP hydrolyzed. The amount hydrolyzed in the absence of p86 was ~80 pmol. The data are an average of six independent experiments.

A schematic of the N-terminal and C-terminal truncation mutants of the p86 subunit of eIF(iso)4F is shown in Fig. 1B. These mutants were expressed in E. coli and purified. An SDS-PAGE of the mutant forms is shown in Fig. 2. There are some minor degradation products in the protein preparations, particularly of the C-terminal truncation mutants. The presence of these products suggests that certain regions in the C terminus are probably more exposed in the truncation mutants and are more susceptible to protease digestion.

The ability of the mutants to form a complex with p28 was measured by retention of the mutant protein on a m⁷GTP-Sepharose column. As shown in Table II, N-terminal deletions past residue 52 (N-90, N-136, N-186) were unable to form a complex with p28. The C-terminal deletions were all able to form a complex with p28.

The yeast two-hybrid system was used to confirm the p28-p86 interactions. A very intense blue color was obtained when wild-type p86 (binding domain vector) and p28 (activation domain vector), as well as the positive controls (pVA3 and pTD1), were co-transformed into yeast cells (see Table III). The negative controls or the plasmids alone (p86, p28, pLAM5, pTD1, or pVA3) gave no color (see Table III). Combinations of p86 with pTD1 or pVA3 or combinations of p28 with pTD1 or pVA3 were also negative. When the p28 was placed into the binding domain vector and p86 into the activation domain vector, an interaction of similar intensity was observed. These results show that the regions of the two molecules that interact are not impaired by the addition of any vector-specific sequences.

A new binding domain vector (pGGB-9/N) was constructed to facilitate the subcloning of the p86 truncation mutations from pET3d into the binding domain vector of the yeast two-hybrid system. As shown in Table III, results identical to the retention on m⁷GTP-Sepharose were obtained for p28 binding by p86 deletions mutants with the two-hybrid system. Interaction of p28 was no longer possible when mutants truncated past residue 52 were used, whereas all the C-terminal mutants were able to interact with p28. These data indicate that the site of interaction of p86 with p28 resides between residues 52 and 90. This result is consistent with the observation that the sequence from amino acids 62 to 73 in p86 (block 1 in Fig. 1A) contains a motif identified as necessary for the binding of the mammalian eIF4E to mammalian eIF4G (or eIF4E-BP1 (33)).

The purified mutants were assayed for the ability to support polypeptide synthesis in the presence of p28 in an eIF(iso)4F-dependent system from wheat germ (28). As shown in Table II, N-terminal deletions past amino acid 52 lost the ability to support translation in the assay system. The N-52 mutant consistently showed a slightly higher activity in this assay system. The removal of these amino acids may be changing the
conformation of the protein such that certain domains are more exposed and increase the activity of the complex. The mutants that were unable to form a complex with p28 (N-90, N-136, and N-186) were also unable to support translation in vitro. These results suggest that a complex of p86 and p28 is necessary to initiate translation. As shown in Table II, truncations from the C-terminal end of the p86 subunit did not adversely affect the activity of p86 to support translation up to residue 462 (C-462).

The ATP hydrolysis activity of p86 is both RNA-dependent and eIF4A-dependent, but does not require the presence of p28 (data not shown). None of the truncations from the amino terminus affected the ATP hydrolysis activity of p86 (see Table II). However, the C-489 mutant, while still supporting translation as well as wild-type, showed ATP hydrolysis activity at 58% of wild-type. C-terminal truncations to C-462 almost completely abrogated ATP hydrolysis activity (to 14% of wild-type). This suggests that at least a portion of the ATP hydrolysis domain on p86 is located in a central region of the protein, around residues 450–500, and a C-terminal truncation to C-462 completely abrogates ATP hydrolysis activity (to 14% of wild-type).

We have demonstrated that the site of interaction of the cap-binding protein (p28) with its large subunit (p86) resides within a region defined by residues 52–90 that contains a motif shown to be required for binding of mammalian eIF4E to mammalian eIF4G (9, 33). We have shown that this region also appears to be required for interaction with eIF4A. This is a novel observation that the N terminus of p86 may also be involved in the binding of eIF4A. The C-terminal portion of mammalian eIF4G has been shown to interact with mammalian eIF4A (9). The ability of p86 to bind the p28 subunit is absolutely required for in vitro protein synthesis activity. However, the ability of the p86 subunit to bind p28 or eIF4A appears to be uncoupled from its ability to stimulate RNA-dependent ATP hydrolysis in the presence of eIF4A. We have also demonstrated that the use of the yeast two-hybrid system will be a very powerful tool in elucidating the complex interactions of the subunits of eIF(iso)4F with each other and with other initiation factors.

REFERENCES

1. Pain, V. M. (1996) Eur. J. Biochem. 236, 747–771
2. Browning, K. S. (1996) Plant Mol. Biol., in press
3. Merrick, W. C. (1994) Biochimie 76, 822–830
4. Rhoads, R. E., Joshi, B., and Minich, W. B. (1994) Biochimie 76, 831–838
5. Sonenberg, N. (1994) Biochimie 76, 839–846
6. Etchison, D., and Milburn, S. (1987) Mol. Cell. Biol. 7, 15–25
7. Webster, C., Gaut, R. L., Browning, K. S., Ravel, J. M., and Roberts, J. K. M. (1991) J. Biol. Chem. 266, 23341–23346
8. Sonenberg, N. (1990) New Biol. 2, 492–499
9. Lamphear, B. J., Kirchwehr, R., Skern, T., and Rhoads, R. E. (1995) J. Biol. Chem. 270, 21975–21983
10. Ohlmann, T., Rau, M., Pain, V. M., and Morley, S. J. (1996) EMBO J. 15, 1371–1382
11. Morley, S. J., and Pain, V. M. (1995) J. Cell Sci. 108, 1751–1760
12. Bu, X., Haas, D. W., and Hagedorn, C. H. (1995) J. Biol. Chem. 269, 4795–4798
13. Manzella, J. M., Ryhlick, W., Rhoads, R. E., Hershey, J. W. B., and Blackshear, P. J. (1991) J. Biol. Chem. 266, 23893–23899
14. Morley, S. J., and Traugh, J. A. (1990) J. Biol. Chem. 265, 10611–10616
15. Lin, T.-A., Kong, X., Haystead, T. A. J., Pause, A., Belsham, G., Sonenberg, N., and Lawrence, J. C., Jr. (1994) Science 266, 653–656
16. Pause, A., Belsham, G. J., Gingras, A.-C., Donéz, O., Lin, T.-A., Lawrence, J. C., Jr., and Sonenberg, N. (1994) Nature 371, 762–767
17. Haghshigt, A., Mader, S., Pause, A., and Sonenberg, N. (1995) EMBO J. 14, 5779–5789
18. Lin, T.-A., Kong, X., Saltiel, A. R., Blackshear, P. J., and Lawrence, J. C., Jr. (1995) J. Biol. Chem. 270, 18531–18538
19. Browning, K. S., Lax, S. R., and Ravel, J. M. (1987) J. Biol. Chem. 262, 11228–11232
20. Browning, K. S., Webster, C., Roberts, J. K. M., and Ravel, J. M. (1992) J. Biol. Chem. 267, 10096–10100
21. Yan, S., Ryhlick, W., Richardson, D., and Rhoads, R. E. (1992) J. Biol. Chem. 267, 23226–23231
22. Goyer, C., Altmann, M., Lee, H. S., Blanc, A., Deshmukh, M., Woolford, J. L., Jr., Trachsel, H., and Sonenberg, N. (1995) Mol. Cell. Biol. 15, 4860–4874
23. Lax, S., Fritz, W., Browning, K., and Ravel, J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 330–333
24. Lax, S. R., Browning, K. S., Maia, D. M., and Ravel, J. M. (1986) J. Biol. Chem. 261, 15632–15636
25. Abramson, R. D., Browning, K. S., Dever, T. R., Lawson, T. G., Thach, R. E., Merrick, W. C., Jr., Trachsel, H., and Sonenberg, N. (1994) Methods Enzymol. 118, 109–128
26. Timmer, R. T., Lax, S. R., Hughes, D. L., Merrick, W. C., Ravel, J. M., and Browning, K. S. (1990) J. Biol. Chem. 265, 24963–24967
27. van Heerden, A., and Browning, K. S. (1994) J. Biol. Chem. 269, 17454–17457
28. van Heerden, A., and Browning, K. S. (1994) J. Biol. Chem. 269, 17454–17457
29. Timmer, R. T., Benkowski, L. A., Schodin, D., Lax, S. R., Meta, A. M., Ravel, J. M., and Browning, K. S. (1993) J. Biol. Chem. 268, 9504–9510
30. Browning, K. S., Humphreys, J., Hobbs, W., Smith, G. B., and Ravel, J. M. (1990) J. Biol. Chem. 265, 17967–17973
31. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
32. Kao, P. C., Huphah, J. D., Kim, H. H., Hanseworth, V. R., Van Heerden, A., Browning, K. S., and Morejohn, L. C. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7120–7124
33. Mader, S., Lee, H., Pause, A., and Sonenberg, N. (1995) Mol. Cell. Biol. 15, 4990–4997
34. Sha, M., Wang, Y. H., Xiang, T., Van Heerden, A., Browning, K. S., and Goss, D. J. (1995) J. Biol. Chem. 270, 28954–28960
35. Schuler, G. D., Altschul, S. F., and Lipman, D. J. (1991) Proteins 9, 180–190
36. Allen, M. L., Metz, A. M., Timmer, R. T., Rhoads, R. E., and Browning, K. S. (1992) J. Biol. Chem. 267, 23223–23226