Identification of a Nucleic Acid Binding Domain in Eukaryotic Initiation Factor eIFiso4G from Wheat*

(Received for publication, September 2, 1998, and in revised form, December 18, 1998)

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Higher plants have two complexes that bind the mG-cap structure of mRNA and mediate interactions between mRNA and ribosomal subunits, designated eIF4F and eIFiso4F. Both complexes contain a small subunit that binds the 5’-cap structure of mRNA, and a large subunit, eIF4G or eIFiso4G, that binds other translation factors and RNA. Sequence-specific proteases were used to cleave native cap-binding complexes into structural domains, which were purified by affinity chromatography. We show here that eIFiso4G contains a central protease-resistant domain that binds specifically to nucleic acids. This domain spans Gln170 to Gln443 and includes four of the six homology blocks shared by eIFiso4G and eIF4G. A slightly shorter overlapping sequence, from Gly202 to Lys445, had no nucleic acid binding activity, indicating that the N-terminal end of the nucleic acid binding site lies within Gln170 to Arg201. The binding of the central domain and native eIFiso4F to RNA homopolymers and double- and single-stranded DNAs was studied. Both molecules had highest affinity for poly(G) and recognized single- and double-stranded sequences.

The group 4 translation initiation factors, eIF4A, B, E, and G, bind and recruit mRNA to 43 S pre-initiation complexes for translation (reviewed in Refs. 1 and 2). Association of eIF4A and eIF4B confers ATPase, RNA helicase, and RNA annealing activities on the intact complex (1–3) and is thought to be necessary for melting of RNA secondary structure and ribosome scanning to the AUG start codon. eIF4E binds to the 5’-cap structure of mRNAs and associates tightly with eIF4G. These two factors together form the core of the mRNA-binding complex, designated eIF4F. Higher plants have two such complexes: eIF4F, which is homologous to the mammalian complex, and eIFiso4F, which is ~10-fold more abundant (4, 5). eIF4G and eIFiso4G differ ~2-fold in molecular mass and show only 25–30% sequence similarity overall, with ~50% similarity in six small homologous regions (6). Yet, despite this substantial divergence, these two factors have very similar functions (1). Either factor alone is sufficient for cap-dependent initiation in vitro (7, 8). eIFiso4G and eIFiso4E have been cloned and sequenced from wheat and have deduced molecular masses of 86.5 and 23.5 kDa, respectively (6).

The eIF4G and eIFiso4G subunits of the mRNA-binding complexes act as a central scaffold for binding of other translation factors, proteins, co-factors, and RNAs. These include binding sites within mammalian eIF4G for eIF4E (aa 409–457) (9), eIF3 (aa 480–886) (10), and two sites for eIF4A (aa 887–1402 and 478–883) (10, 11). Plant eIFiso4G also binds eIFiso4E near its N terminus (aa 52–90) (12) in a small region with sequence homology to the mammalian factor. Binding of eIF4G and eIFiso4G to RNAs, distinct from the cap recognition function of the intact complex, has been demonstrated by several laboratories and appears to play a critical role in the cap-independent translation of viral RNAs. Sha et al. (13) calculated the equilibrium binding constants of recombinant wheat eIFiso4G for both capped and uncapped mRNAs. They found that the binding constants for uncapped message were comparable to those of eIFiso4E and eIFiso4F for capped mRNA, clearly indicating that the large subunit also binds mRNA efficiently and that it does not discriminate between capped and uncapped messages. These findings are consistent with the role of eIF4G in the cap-independent translation of viral mRNAs, following cleavage and loss of the eIF4E binding domain by viral proteases (10, 14). In functional assays for initiation complex formation, Pestova et al. (15) have shown that only eIF4A and the central third of human eIF4G (aa 457–932) are required for binding of encephalomyocarditis virus RNA to 43 S ribosomal complexes, and that this central eIF4G peptide binds, and cross-links to, the viral internal ribosome entry site (IRES). Wheat eIF4E and eIFiso4E were also reported to bind poly(A)-mRNA (16), which implies that the cap-binding complex interacts with both the 5’ and 3’ termini of intact mRNAs, perhaps ensuring that only intact messages are translated. The binding of eIFiso4G to other proteins, in addition to translation factors, has also been reported. The N-terminal region of eIFiso4G appears to bind poly(A)-binding protein (17), thereby increasing its activity. Additionally, recombinant wheat eIFiso4G binds to, and promotes the bundling of microtubules in vitro, and co-localizes with microtubules in vivo, suggesting a role for eIFiso4G in localizing the translational machinery to the cytoskeleton (18). The specific sites and structural motifs in eIFiso4G or eIF4G that mediate most of these RNA and protein interactions have not yet been identified.
In this study, we have used limited proteolysis and affinity chromatography to isolate functional domains of the mRNA-binding complexes of wheat. Protease-resistant domains were fractionated by affinity chromatography on phosphocellulose (phosphate interactions), oligo(dT)-cellulose (phosphodiester or base interactions), and m7GTP-Sepharose (cap recognition). Peptides eluted from the different affinity columns were purified by reverse phase HPLC, sequenced to determine their N terminus, sized by mass spectrometry, and compared with published sequences of cloned proteins to characterize each peptide within the primary amino acid sequence. We report here the identification of a central protease-resistant domain in eIFiso4G, which binds both nucleic acids and phosphocellulose. The specific affinities of both intact eIFiso4F and the central domain of eIFiso4G were determined for RNA homopolymers and ss- and dsDNAs.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Sequencing grade trypsin and endoproteinase glu-C were from Boehringer Mannheim. Oligo(dT)-cellulose and m7GTP-Sepharose 4B were from Amersham Pharmacia Biotech. Phosphocellulose was from Whatman. avidin-conjugated polyA, poly(C), poly(G), poly(U), ssDNA, and dsDNA were from Sigma. Oligonucleotides were from Sigma. All other chemicals were purchased from Sigma, U. S. Biochemicals, Bio-Rad, or Fisher Scientific.

**Protein Purification**—mRNA cap-binding complexes were purified from wheat germ by a modification of the method of Lax et al. (Ref. 19; note that eIFiso4G is designated eIF-4B in this reference). Wheat germ (150 g) was ground with 10% each fine sand and polyvinylpolypyrrolidone (20), homogenized in four volumes of冰冷 fluid (0.25 mM sucrose, 20 mM Tris/Hepes, pH 8.6, 400 mM potassium acetate, 50 mM magnesium acetate, 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.07 mg/ml soybean trypsin inhibitor), and centrifuged 10 min at 10,000 x g. The supernatant was decanted through 70 μm nylon mesh, centrifuged 2 h at 368,000 x g, precipitated with 70% ammonium sulfate, and dialyzed against buffer B120 (buffer B contains 20 mM Hepes/KOH, pH 7.6, 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerin, and the concentration of KCl, in mM, indicated by the suffix). Material from five or six preparations was adsorbed to a 50-ml m7GTP-Sepharose 4B column, and mRNA cap-binding complexes were eluted with 100 mM m7GTP in B120. Approximately 20 mg of protein was recovered from 100 ml of packed resin in microcentrifuge filter cups (MSI, Westboro, MA; 0.45-μm acetate filters). Solutions (300-μl aliquots) were spun through the resin at 500–1,000 rpm for 2–5 min; at least three washes (nine column volumes) were used at each step. Columns were equilibrated in B50 and eluted sequentially with 0.1, 1, or 4 mM NTPs or dNTPs, 200 μM oligo(dT), or oligo(dT)12-20, or with increased KCl, as indicated. All fractions were analyzed as above, but only selected fractions are shown. eIFiso4F, endoproteinase glu-C digests of eIFiso4F, and purified E31 were chromatographed on all affinity resins at least twice.

**Binding studies with ribonucleotide homopolymer and ss and dsDNAs** were carried out in spin columns using 20–100 μg of protein and 100 μl of packed resin in microcentrifuge filter cups (MSI, Westboro, MA; 0.45-μm acetate filters). Solutions (300-μl aliquots) were spun through the resin at 500–1,000 rpm for 2–5 min; at least three washes (nine column volumes) were used at each step. Columns were equilibrated in B50 and eluted sequentially with 0.1, 1, or 4 mM NTPs or dNTPs, 200 μM oligo(dT), or oligo(dT)12-20, or with increased KCl, as indicated. All fractions were analyzed as above, but only selected fractions are shown. eIFiso4F, endoproteinase glu-C digests of eIFiso4F, and purified E31 were chromatographed on all affinity resins at least twice.

**Proteolytic digestion** of cap-binding complexes was performed with trypsin (center panel) or endoproteinase glu-C (right panel) for the hours indicated. Products were analyzed by SDS-PAGE on 14% gels with silver staining. Molecular masses of standard proteins in kDa are indicated to the left.

**RESULTS**

**Affinity Fractionation of Protein Digests**—For the results reported in Fig. 2, 300–500 μg of digested cap-binding complexes were chromatographed on 2-ml phosphocellulose, oligo(dT)-cellulose, or m7GTP-Sepharose columns, and eluted with a 12-ml linear salt gradient (B100 to B1000). All fractions were analyzed for protein composition by SDS-PAGE on 14% gels with silver staining, and by fluorescence spectroscopy on excitation and emission wavelengths of 280 and 350 nm, respectively. Only selected fractions are shown.

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Binding of peptides from cap-binding complexes. Trypsin (left) and endoproteinase glu-C (right) digests were chromatographed on either phosphocellulose (top, PC) or oligo(dT)-cellulose (bottom, dT). Elution profiles were monitored by fluorescence. Insets, SDS-PAGE of selected fractions, run on 14% gels and silver-stained. E4F, elFiso4F standard. T, trypsin digest of cap-binding complexes. E, endoproteinase glu-C digest of cap-binding complex. Numbered lanes correspond to labeled fractions in the elution profiles. The molecular masses of standard proteins in kDa are indicated to the left of each gel.

In order to identify and purify peptides with specific binding affinities, digests of cap-binding complexes were chromatographed in parallel on phosphocellulose, oligo(dT)-cellulose, and m7GTP-Sepharose. When trypsin digests were applied to phosphocellulose only T28, the largest stable fragment, bound and was eluted from the column in ~150 mM KCl (Fig. 2, top left, lane 4). In contrast, none of the peptides generated by trypsin digestion bound to oligo(dT)-cellulose (Fig. 2, bottom left). The second major band at ~26 kDa was primarily undigested elFiso4E, which was resistant to trypsin digestion for up to ~16 h. It bound to m7GTP-Sepharose (data not shown) and had no affinity for either phosphocellulose or oligo(dT)-cellulose (Fig. 2). This binding pattern was the same as for native elFiso4E (data not shown).

Cap-binding proteins digested with endoproteinase glu-C were chromatographed as above (Fig. 2 right). Major bands with apparent masses of 31, 23, and 22 kDa were retained on phosphocellulose, and eluted in 150–200 mM KCl (Fig. 2, top right, lanes 4 and 5). The two smaller bands were also retained on m7GTP-Sepharose (data not shown), while the 31-kDa band bound to oligo(dT)-cellulose (Fig. 2, bottom) and eluted at ~150 mM KCl. Further fractionation of the 31-kDa band by RP-HPLC revealed two peptides with very similar size and hydrophobicity, running as a barely resolved doublet on SDS-PAGE, and eluting from a C18 column at only a 1% difference in acetonitrile concentration (Fig. 3). Only the larger of these two peptides, corresponding to the second RP-HPLC peak, was present in digests of purified elFiso4F, and was designated E31.

E31 binding to phosphocellulose and oligo(dT)-cellulose was due to its direct interaction with the resin, rather than to a protein:protein interaction with the second peptide, or with one of the other peptides retained on the columns. Purified E31, isolated from digests of purified elFiso4F by phosphocellulose chromatography and DEAE-HPLC, had the same affinity and elution profile on phosphocellulose and oligo(dT)-cellulose as did E31 in digests (data not shown).

**Peptide Assignments**—Amino acid sequencing and mass spectrometry were used to identify those peptides that bound to affinity resins. Seven amino acid residues were sequenced from the N terminus of each fragment; each was sequenced twice from separate purifications, and E31 was sequenced following both phosphocellulose and oligo(dT)-cellulose purification. The C-terminal residues were calculated from the total mass and amino acid composition. T28 and E31 were found to be overlapping peptides from the central portion of elFiso4G with 242 amino acids, from Gly202 to Glu443, in common (Table I). The additional 32 amino acids at the N terminus of E31, from Gln170 to Arg201 (Fig. 4A), are required for oligo(dT)-cellulose binding activity. This sequence thus appears to form part of a nucleic acid recognition site.
The second 31-kDa peptide, present in endoproteinase glu-C digests of cap-binding complexes, originated from eIF4G. Its N-terminal sequence was KKYVVGK, which is located 20 amino acids N-terminal to the second region of homology shared by eIFiso4G and mammalian eIF4G (12), beginning at Lys215 in eIFiso4G (Fig. 4A, Region 2). When the sequences of wheat eIF4G and eIFiso4G were compared in this region, the start site of the eIF4G peptide was seen to lie between those of E31 and T28, and to include only seven of the 32 additional residues present in E31 (Fig. 4A). There is limited sequence conservation between factors and species in this region, but several short conserved sequences and amino acids are present in almost all of the factors sequenced to date (Fig. 4B). Most prominent are two small charge clusters, corresponding to KAD194 and EKDR209 in eIFiso4G, which flank conserved Trp, Ser, and Arg residues present at the N terminus of the eIF4G peptide. These elements are also present in yeast eIF4G, but are much more widely spaced (Fig. 4B).

Digestion with endoproteinase glu-C produced two peptides of ~22–23 kDa, which were retained on phosphocellulose (Fig. 2, top right, lanes 4 and 5). These bands were identified as eIFiso4E peptides by their affinity for mGTP-Sepharose (data not shown), their co-migration with the two main fragments generated by digestion of purified eIFiso4E (data not shown), their intense fluorescence (eIFiso4E has an unusually high tryptophan content), and by sequencing of the smaller peptide, which was designated E21. The N-terminal sequence of E21 was AKGPHKL and its mass was 20,852 Da, corresponding to Ala29–Val209 of eIFiso4E (6). Truncation of the N-terminal 28 amino acids did not alter the affinity of E21 for the cap structure, as determined by fluorescence quenching analysis (data not shown). This finding agrees with results reported by Chavan et al. (30), who identified a trypsin-resistant core domain in human eIF4E (aa 47–182), which also retained its affinity for mGTP. Native eIFiso4E does not bind either phosphocellulose or oligo(dT)-cellulose (data not shown), but is retained on both columns when bound to eIFiso4G. Retention of the eIFiso4E peptides on phosphocellulose could thus have resulted from their interaction with E31. This proved not to be the case, however, as purified E21 alone bound to phosphocellulose (data not shown). Apparently the loss of seven Asp and Glu residues from the N terminus leaves several unpaired positive charges, which can interact with phosphate groups.

Purified eIFiso4F and E31 Bind Specifically to Nucleic Acids—Intact eIFiso4F could not be eluted from oligo(dT)-cellulose by free dNTPs, NTPs, or NMPs, even at concentrations as high as 4 mM (data not shown), but were readily eluted by 200 μM oligo(dT)12 (Fig. 5, top). As the concentration of phosphate groups in 4 mM NTP is 5-fold higher than that of the oligo solution, this interaction cannot be due simply to charge. In addition, oligo(dT)6 failed to elute eIFiso4F (Fig. 5, bottom), indicating that the minimum length of the target sequence required for eIFiso4F binding was between 7 and 12 nucleotides.

The binding specificities of intact eIFiso4F and E31 were determined for RNA homopolymers and random sequence ss- and dsDNAs (Table II and Fig. 6). The binding specificity of purified E31 was the same as for E31 in digests, and in each case where binding occurred, free nucleoside mono or triphosphates failed to elute eIFiso4F or E31 from the columns (Fig. 6 and data not shown). eIFiso4F and E31 bound to poly(G) with the highest affinity, and showed no interaction with poly(C). Unlike eIFiso4F, E31 showed no affinity for poly(U) and weak affinity for poly(A), being retarded on poly(A)-agarose but eluting in the wash fractions (data not shown). T28, which did not bind oligo(dT) (Fig. 2), also did not bind poly(G) (data not shown). These results indicate that the central domain of eIFiso4G mediates binding of the eIFiso4F complex to poly(G), oligo(dT), and phosphocellulose, and that this domain recognizes both single- and double-stranded sequences. The lack of interaction of E31 with poly(A) and poly(U) suggests that these lower affinity binding sites lie outside of the central domain. However, it is possible that these nucleic acids are also bound.

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**Table I**

| Fragment | T28 | E31 |
|----------|-----|-----|
| Protease | Trypsin | Endoproteinase glu-C |
| N-terminal sequence | GNLSEKD | QFRNQDQ |
| Mass (Da) | 28,060 | 31,238 |
| No. of amino acids | 244 | 274 |
| Location in sequence | Gly702–Lys445 | Gln170–Glu443 |

**Fig. 4.** Peptide start sites and amino acid sequence comparison of eIF4G isoforms from various species. A, localization of the start sites for the phosphocellulose (T28) and oligo(dT)-cellulose (E31) binding domains of wheat eIFiso4G (wheat 4G), and for the homologous domain from wheat eIF4G (4G<sub>top</sub>, wheat 4G). The eIF4G sequence is shown from Gln170 through Lys215, with the corresponding eIF4G sequence below (see Footnote 2). Residues obtained from N-terminal sequencing are double underlined. The second block of homology shared by mammalian and plant factors begins at Lys215 in eIFiso4G (Region 2). B, conserved sequences around the start site of the oligo(dT) binding domain are shown in bold. Cress, thale cress (Arabidopsis thaliana); i4G, eIFiso4G; 4G, eIF4G; 4G2, eIF4G2. Where known, amino acid residues are numbered at the right. A consensus sequence is given, with spacing between the conserved residues calculated as the average number of intervening amino acids from all species except yeast.
by the central domain, but that additional sequences located elsewhere in the molecule are required to stabilize their interactions.

**DISCUSSION**

We have shown that wheat eIFiso4G contains an endopeptidase glu-C-resistant structural core, which binds nucleic acids with base specificity and poly(G) with highest affinity. This domain is centrally located in eIFiso4G, and spans four of the six small regions of homology it shares with mammalian eIF4G (Fig. 7) (1, 6). A corresponding domain from eIF4G was also purified, and our results suggest that it has many of the same properties; it was resistant to endoproteinase glu-C digestion, bound poly(dT)-cellulose and phosphocellulose, migrated closely with E31 on SDS gels, and had very similar hydrophobicity. The N-terminal end of the nucleic acid recognition site in eIFiso4G lies within Gin^{170}–Arg^{201}, because the shorter tryptic peptide (T28) lacks these 32 residues and fails to bind nucleic acids. Only the later third of this sequence is conserved among eIF4A factors, and only the last seven amino acids, corresponding to Val^{195}–Arg^{201} of eIFiso4G, are also present in the eIF4G peptide (Fig. 4). This suggests that the N-terminal end of the nucleic acid binding site lies within these seven residues, which form part of an additional short block of homology centered at the N terminus of the tryptic peptide (Figs. 4B and 7). This sequence, from Lys^{192} to Arg^{209}, is ~50% similar in eIF4G isoforms from all species, a comparable level of homology to the other six conserved regions (1). Further studies of purified eIF4G are required to define structural and functional properties of its central domain.

The central third of eIF4G is known to have several important functions. First, Pestova et al. (15) have shown that eIF4A and the central third of eIF4G are alone sufficient for cap-independent initiation of encephalomyocarditis virus RNA, and that the eIF4G fragment cross-links to the viral IRES. The wheat eIF4G and eIFiso4G core domains identified here would be included in this larger 476-amino acid fragment, and could be involved in IRES recognition or binding.

Second, it has been known for some time that chromatography of cap-binding proteins on phosphocellulose depletes the complex of eIF4A. The extent to which this occurs varies between laboratories. We have found that, for the plant factors, eIF4F is substantially depleted of eIF4A, while eIFiso4F is completely stripped of eIF4A, both on phosphocellulose (Fig. 1) and poly(dT)-cellulose (data not shown). Why the binding of eIFiso4G to eIF4A and phosphocellulose should be mutually exclusive was not clear, given the lack of overlap in their recognition sites (Fig. 7); the eIF4A binding site on wheat eIFiso4G, as mapped to the N terminus of the molecule by Metz and Browning (12), using eIFiso4G deletion constructs expressed in a yeast two-hybrid system. In contrast, two eIF4A binding domains have now been identified in mammalian eIF4G, one in the C-terminal third of the molecule (10), and a second in the central third (11). Only the eIF4A binding site in the central third of eIF4G is conserved in eIFiso4G, and mutations in this sequence of the mammalian factor abolished eIF4A binding activity (11). This site corresponds to the second
block of homology shared by eIF4G and eIFiso4G (Fig. 4, Region 2; Fig. 7). As RNA recognition motifs are typically 50–100 amino acids long (31), the eIF4A and nucleic acid binding sites may well overlap, with the loss of eIF4A during chromatography resulting from direct competition. This has interesting implications for the mode of action of the mRNA cap-binding complexes, which perhaps deliver eIF4A to the message, and then bind mRNA directly through the same site to stabilize the preinitiation complex.

Third, Lamphear et al. (10) have shown that a ~407-amino acid peptide from the central third of eIF4G binds to eIF3 (Fig. 7). Further proteolysis, resulting in the removal of ~50 amino acids from the C terminus of the peptide, abolished this activity. Thus, at least part of the eIF3 recognition sequence in the mammalian factor lies carboxyl to and outside of the core domain defined here, while the nucleic acid binding site in both plant factors begins at the N-terminal boundary of this structural core. Based on these observations, it seems unlikely that these two sites overlap.

Finally, one can speculate on why a translation factor involved in the binding and recruitment of mRNA to ribosomes should also bind ss- and dsDNA. Such dual specificity in RNA-binding proteins is not unusual, and may have biological significance. However, several examples of RNA-binding proteins that recognize specific DNA sequences have now come to light (32–34). The cap-binding translation factors may also have multiple functions. eIF4E has been found localized to the nucleus in both mammalian (35) and yeast cells (36), and it appears to regulate the expression of cyclin D1 at both the transcriptional and post-transcriptional levels (37). eIF4E has also been shown to cooperate with two oncogenes, v-myc and E1A, in the transformation of primary fibroblast cultures (38). Additional functions for eIFiso4G have also been reported. eIFiso4G was shown to bind, bundle and co-localize with microtubules in maize cells (18), and to promote their growth by end-to-end annealing in vitro (24), suggesting possible roles for eIFiso4F in the localization of translational components to the cytoskeleton (18) or in mRNA transport (1). These relatively ancient RNA-binding proteins may therefore have other functions in both the nucleus and cytoplasm, beyond formation of the 43S preinitiation complex in translation.

Acknowledgments—We thank Karen S. Browning for sharing unpublished sequence information for wheat eIF4G; Rod Nakayama for the preparation of figures; and Fred Kim, Greg Kim, Rick Kim, Dimitri Nikes, David Park, Kenny Park, Tung Nguyen, and John Wingo for factor preparation and technical assistance.

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