Protein Science (1997), 6:125–131. Cambridge University Press. Printed in the USA.

Identification of the cap binding domain of human recombinant eukaryotic protein synthesis initiation factor 4E using a photoaffinity analogue

DIANA E. FRIEDLAND,[1] MICHAEL T. SHOEMAKER,[2] YIPING XIE,[3] YUHUAN WANG,[3] CURT H. HAGEDORN,[3] AND DIXIE J. GOSS[1]

[1]Department of Chemistry, Hunter College of the City University of New York, New York, New York 10021
[2]Division of Medicinal Chemistry and Pharmaceutics, College of Pharmacy, University of Kentucky Medical Center, Lexington, Kentucky 40536-0082
[3]Department of Medicine and Genetics Program of the Winship Cancer Center, Emory University School of Medicine, Atlanta, Georgia 30322

(RECEIVED August 20, 1996; ACCEPTED September 27, 1996)

Abstract

Binding of eIF-4E to the 5′ m^7G cap structure of eukaryotic mRNA signals the initiation of protein synthesis. In order to investigate the molecular basis for this recognition, photoaffinity labeling with [γ-^{32}P]8-N_3GTP was used in binding site studies of human recombinant cap binding protein $_r$eIF-4E. Competitive inhibition of this cap analogue by m^7GTP and capped mRNA indicated probe specificity for interaction at the protein binding site. Saturation of the binding site with [γ-^{32}P]8-N_3GTP further demonstrated the selectivity of photoinsertion. Aluminum (III)-chelate chromatography and reverse-phase HPLC were used to isolate the binding site peptide resulting from digestion of photolabeled $_r$eIF-4E with modified trypsin. Amino acid sequencing identified the binding domain as the region containing the sequence Trp 113–Arg 122. Lys 119 was not identified in sequencing analysis nor was it cleaved by trypsin. These results indicate that Lys 119 is the residue directly modified by photoinsertion of [γ-^{32}P]8-N_3GTP. A detailed understanding of eIF-4E·m^7G mRNA cap interactions may lead the way to regulating this essential protein–RNA interaction for specific mRNA in vivo.

Keywords: cap binding site; eukaryotic initiation factor 4E; photoaffinity labeling; protein–RNA interactions; protein synthesis

The initiation phase of eukaryotic protein synthesis is characterized by recognition of the m^7(5′)Gppp(5′)N cap structure at the 5′ terminus of eukaryotic mRNA by initiation factors (reviewed in Rhoads, 1988 and Sonenberg, 1988). Of the eIFs that interact at or near the cap, only eIF-4E (alone or as part of the eIF-4F complex) has been shown to interact directly with the cap (Sonenberg, 1981; Sonenberg et al., 1981; Tahara et al., 1981; Hellmann et al., 1982; Grifo et al., 1983; Webb et al., 1984). This initial recognition of the cap structure represents the first committed step in the initiation phase of protein synthesis.

Reprint requests to: Dixie J. Goss, Department of Chemistry, Hunter College of the City University of New York, New York, New York 10021; e-mail: goss@mvaxgr.hunter.cuny.edu.

Abbreviations: m^7G, 7-methyl guanosine; eIF, eukaryotic initiation factor; $_r$eIF-4E, recombinant eukaryotic initiation factor; DTT, dithiothreitol; TFA, tri-fluoro acetic acid; TCA, tri-chloro acetic acid; PTH, phenylthiohydantoin; 8-N_3GTP, 8-azidoguanosine 5′-triphosphate; FPLC, fast protein liquid chromatography.

Evidence that cap recognition by eIF-4E is a limiting step in protein synthesis is supported by the observation that it is the least abundant of all the initiation factors and thus may serve a regulatory function (Hiremath et al., 1985; Duncan et al., 1987).

In addition, a number of other observations indicate that this protein is a key point for posttranscriptional control of gene expression. They include the rapid stimulation of eIF-4E phosphorylation by epidermal growth factor and other growth regulatory peptides in intact cells (Donaldson et al., 1991; Proud, 1992; Rhoads, 1993; Sonenberg, 1994); evidence in two different cellular systems that hyperphosphorylation of eIF-4E promotes the recruitment of other eIF-4F subunits to the m^7G mRNA cap (Bu et al., 1993; Morley et al., 1993); and a 3–4-fold increase in the affinity of eIF-4E binding to mRNA caps (Minich et al., 1994). The regulation of eIF-4E function was shown to be even more complicated by the identification of eIF-4E binding proteins that are regulated physiologically by insulin (Lin et al., 1994). When bound to eIF-4E, these proteins inhibit initiation of protein synthesis. To understand fully the mechanisms by which these covalent modifications and translational repressor 4E binding proteins exert their effects,

a more detailed understanding of eIF-4E structure and binding site sequence will be required.

The amino acid sequence for the cap binding protein has been reported previously for several species, including yeast (Altmann et al., 1987), wheat (Metz et al., 1992), human (Rychlik et al., 1987),[4] and mouse (Altmann et al., 1989). The numbers and positions of the eight tryptophans are highly conserved, with one additional tryptophan residue in p26 of wheat germ eIF-4F (see Table 1).

To date, no X-ray crystallographic or structural NMR data of the eIF-4E·cap complex is available. Previous photoaffinity labeling of eIF-4E narrowed the active site domain to 63% of the amino acid residues (Chavan et al., 1990). Furthermore, mutational analysis suggested that a glutamic acid residue, which is located three amino acid residues to the carboxy side of tryptophan 5,[5] participates in the binding of the mRNA cap to human eIF-4E via direct hydrogen bond formation with the 2-amino group of the m^7G base (Ueda et al., 1991a, 1991b). Others proposed that a glutamic acid residue two amino acid residues to the carboxy side of tryptophan 7 participates in this hydrogen bond interaction (Koch et al., 1988; Ueda et al., 1988). However, the cap binding subunit of wheat germ eIF-4F lacks any such acidic residue in these regions (Metz et al., 1992). These conflicting results and the lack of structural information indicated the need for a more precise localization of the eIF-4E binding site for the 5′ m^7G cap of mRNAs.

Photoaffinity labeling of nucleotide binding sites has provided a highly selective method for identifying the participating amino acids of many proteins (Salvucci et al., 1992; Shoemaker & Haley, 1993; Jayaram & Haley, 1994). Photoactivation of the azido moiety with UV light results in the generation of a nitrene and the subsequent covalent attachment of the probe to the binding domain through the remaining nitrogen (Potter & Haley, 1983). Immobilized aluminum (III)-chelate chromatography has facilitated the isolation of binding site peptides due to the presence of highly charged phosphates on the photoinserted azidonucleotide that interact with the Al^{3+} (Anderssen, 1991; Salvucci et al., 1992; Shoemaker & Haley, 1993; Jayaram & Haley, 1994).

In the present study, [γ-^{32}P]8-N_3GTP was used to label selectively the m^7GTP cap binding domain of human recombinant eIF-4E ($_r$eIF-4E). The photolabeled peptide resulting from proteolysis was isolated using aluminum (III)-chelate chromatography followed by reverse-phase HPLC. The amino acid sequence of this peptide was determined and is reported here.

Results

Photoaffinity labeling of $_r$eIF-4E

The specificity of [γ^{32}P]8-N_3GTP was demonstrated by saturation effects with the probe and by protection studies with m^7GTP, unmethylated nucleotides, and a capped mRNA. Photoinsertion was saturated at 70 μM with an apparent K_d of approximately 19 μM (Fig. 1A). Increasing concentrations of m^7GTP resulted in decreased photoinsertion by [γ^{32}P]8-N_3GTP, with more than 75% maximal protection observed at 15 μM m^7GTP with an apparent K_d of 7 μM (Fig. 1B). The percent maximal protection of 12% is determined by dividing the [γ^{32}P]8-N_3GTP concentration (40 μM) by the total maximum nucleotide plus probe concentration (340 μM). This result provides evidence that photoinsertion of [γ-^{32}P]8-N_3GTP was indeed occurring at the m^7G cap binding site of eIF-4E.

[4] In this paper the amino acid numbering is according to this reported sequence.

[5] Numbering of tryptophan residues proceeds from the amino terminus to the carboxy terminus.

Table 1. *Amino acid sequence of human eIF-4E*[a]

MATVEPETTP	TPNPPTTEEE	KTESNQEVAN
PEHYIKHPLQ	NR**W**AL**W**FFKN	DKSKT**W**QANL
RLISKFDTVE	DF**W**ALYNHIQ	LSSNLMPGCD
YSLFKDGIEP	M**W**EDEKNNRG	GR**WLITLNKQ**
QRRSDLDRF	WLETLLCLIGE	SFDDYSDDVC
GAVVNVRAKG	DKIAI**W**TTEC	ENREAVTHIG
RVYKERLGFP	PKIVIGYQSH	ADTATKSGST
TKNRFVV		
Sequence: 217 AA;	MW: 25,117	

[a] Bold indicates tryptophan residues and cap binding domain.

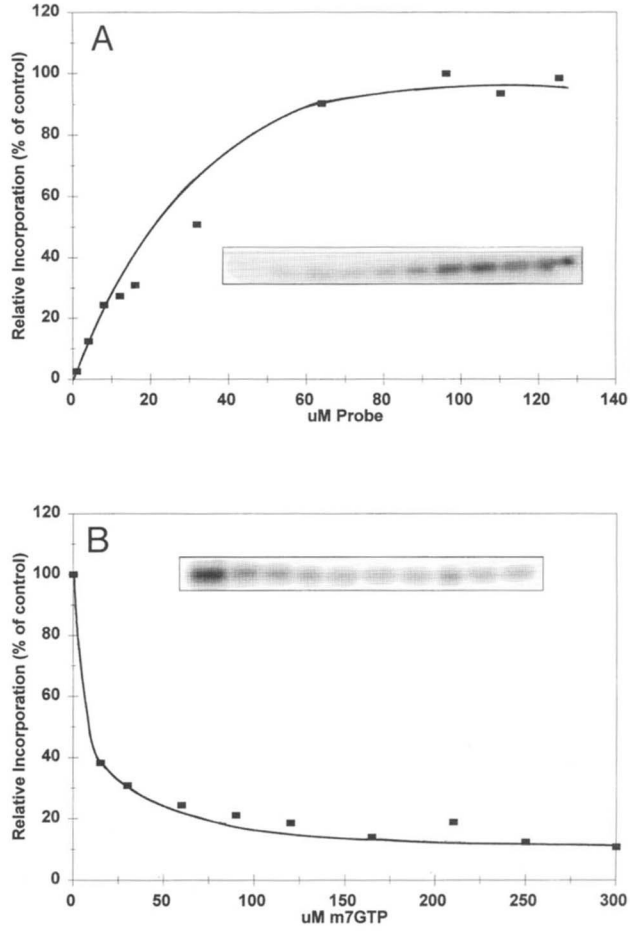

Fig. 1. A: Saturation of [γ-^{32}P]8-N_3GTP photoincorporation into $_r$eIF-4E. $_r$eIF-4E (4 μg) was incubated with the indicated concentrations of [γ-^{32}P]8-N_3GTP in 50 μL of photolysis buffer. The reaction mixture was irradiated with UV light for 90 s and analyzed by SDS-PAGE. ^{32}P incorporation was determined by radioisotopic imaging and quantitation. **B:** Prevention of [γ-^{32}P]8-N_3GTP photoincorporation into $_r$eIF-4E by m^7GTP. $_r$eIF-4E (4 μg) was incubated with 40 μM [γ-^{32}P]8-N_3GTP in the presence of increasing concentrations of m^7GTP under the conditions described for Figure 1A. ^{32}P incorporation was determined by radioisotopic imaging and quantitation.

To further study the specificity of photoinsertion of the probe into the m^7G mRNA cap binding site, the effect of other nucleotides and a capped mRNA was studied (Table 2). Among the unmethylated nucleotides, GTP (21% of control), GDP (26% of control), and ATP (36% of control) proved to be the best inhibitors to photoinsertion. Although other nucleotides were also able to act as inhibitors, they were much less effective. Capped mRNA was able to reduce photoinsertion dramatically (5% of control) compared with equimolar concentrations of m^7GTP (18% of control) and unmethylated nucleotides (maximum of 21% of control).

The observation that nucleotides other than m^7GTP caused some protection lead to a further determination of azido GTP binding to the active site. A fluorescent derivative of m^7GTP, Ant-m^7GTP, has been synthesized recently in our laboratory (Ren & Goss, 1996). This probe binds competitively with m^7GTP for the active site. GTP and other nucleotides do not compete for binding. Because this fluorescent probe binds the active site, 8-azido GTP competition was tested with this probe. Figure 2 shows Lineweaver–Burk plots of the binding. In the case of competitive inhibition, Lineweaver–Burk plots meet at the same y-axis intercept, as is clearly shown in Figure 2.

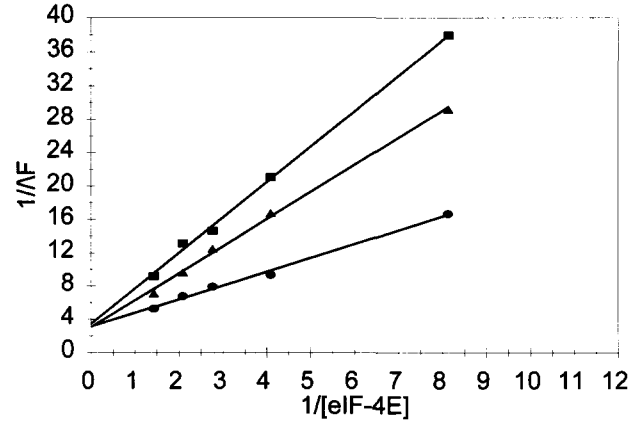

Fig. 2. Lineweaver–Burk plot for the competition of Ant-m^7GTP and 8-N_3GTP in binding to ,eIF-4E. The fluorescence spectrum was measured in a buffer containing 20 mM HEPES, 1 mM DTT, 100 mM KCl, and 2 mM $MgCl_2$. Fluorescence measurements were made at 21 °C with 1.5 μM Ant-m^7GTP and 8-N_3GTP as indicated. Samples were excited at 332 nm and the emission was measured at 420 nm. Data are indicated as follows: circle, 0 μM 8-N_3GTP; triangle, 3.4 μM 8-N_3GTP; square, 6.4 μM 8-N_3GTP.

Isolation and identification of [γ-^{32}P]8-N_3GTP photolabeled peptides

,eIF-4E was photolabeled as described above to determine the amino acid sequence that was modified covalently by [γ-^{32}P]8-N_3GTP within the m^7G cap binding site. The second round of photolysis was performed with nonradioactive 8-N_3GTP to increase the amount of binding domain peptide while reducing nonspecific radiolabeling. TCA was used initially to precipitate photolabeled ,eIF-4E; however, this approach resulted in low yields of peptide for sequencing, probably due to the disruption of the acid sensitive probe–peptide bond. However, acetone precipitation of photolabeled ,eIF-4E resulted in higher yields of labeled peptide.

Photolabeled ,eIF-4E was digested overnight with modified trypsin. The labeled peptide was isolated by affinity chromatography and further purified by reverse-phase HPLC, using the methods described above. To locate the photolabeled peptide in the various buffer solutions, the flow through fractions (fractions 1–5), washes (fractions 6–25), and phosphate elutions (fractions 26–31) (Fig. 3) were pooled separately and subjected to reverse-phase HPLC. The flow through and wash fractions contained no radioactive peaks, as determined by liquid scintillation counting, and thus no labeled peptides (data not shown). However, these fractions did contain many peptide fragments, demonstrating that most of the peptides are unmodified and not retained on the Al^{3+} resin. The HPLC absorbance profile at 220 nm and the corresponding ^{32}P cpm pro-

Table 2. *Percent of photolabeling of ,eIF-4E by 40 μM [γ ^{32}P]8-N_3GTP in the presence of various nucleotides and capped mRNA*

Nucleotide	Nucleotide concentration (μM)	% of Control
None	0	100
GTP	300	21
GDP	300	26
GMP	300	73
CTP	300	43
ATP	300	35
ADP	300	51
AMP	300	89
UTP	300	58
UDP	300	93
UMP	300	93
m^7GTP	300	16
Capped mRNA[a]	300	5

[a]Capped mRNA, 34-base long oligoribonucleotide.

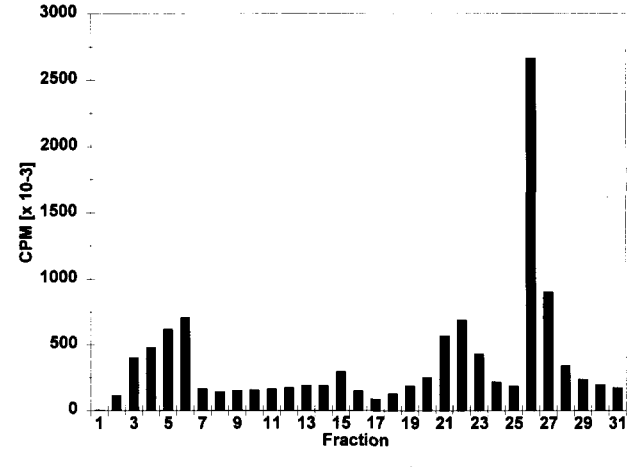

Fig. 3. Radioactivity profile of immobilized Al^{3+} chromatography of tryptic peptides. ,eIF-4E (5 mg) was photolabeled with 50 μM [γ-^{32}P]8-N_3GTP followed by a second photolabeling with 50 μM 8-N_3GTP. Following digestion with modified trypsin, Al^{3+} chelate chromatography proceeded as described in Materials and methods. Fractions 1–5 represent flow through, 6–25 represent various buffer washes, and 26–31 represent fractions eluted with 10 mM K_2HPO_4. ^{32}P levels were determined by liquid scintillation counting.

file for the phosphate elution (fractions 16–31) is shown in Figure 4. Two major radioactive peaks were observed that also gave a UV absorbance. The first peak at 3 min represents the flow through and injection disturbance and contained no peptides, as revealed by sequencing data. The radioactivity detected with this peak was free photolyzed [γ-^{32}P]8-N_3GTP, or probe hydrolyzed during HPLC due to the lability of the N-glycosyl bond to acidic conditions (King et al., 1991). The second major radioactive peak at 23 min was concentrated for amino acid sequence analysis. Photolabeling, peptide isolation, and sequencing were repeated in three separate experiments to ensure that only one binding domain was modified covalently by [γ-^{32}P]8-N_3GTP. In all experiments, the radioactivity coeluted with fraction 23. The sequenced peptide consisted of residues 113–122 of human eIF-4E, which includes tryptophan 6 (see Table 3). In addition, the lysine residue at position 119 remained uncleaved by trypsin. This information, combined with the lack of identification of Lys 119 by sequence analysis, indicates that the residue is the likely site of photoinsertion. No minor peptides were detected in the sequencing data.

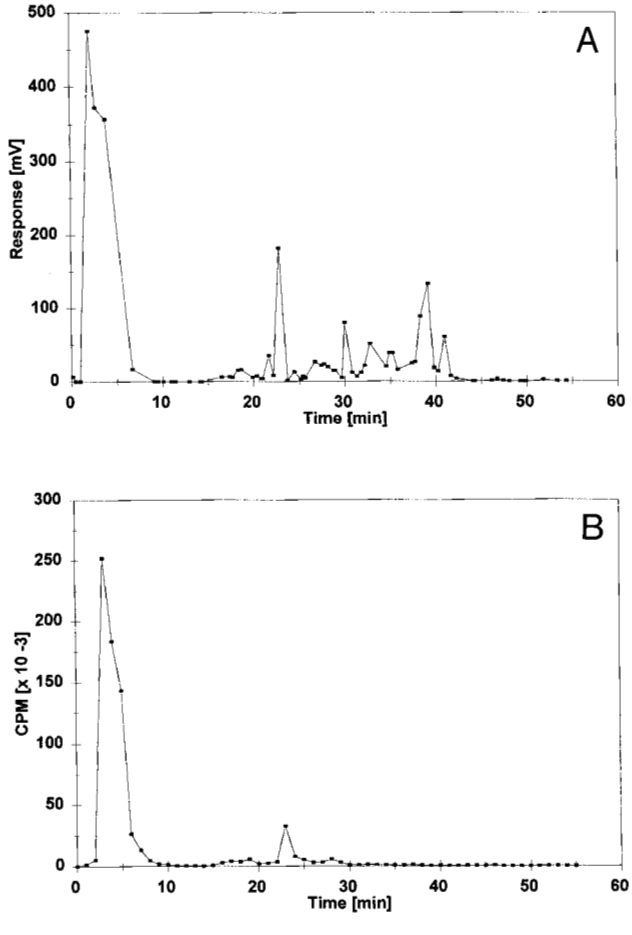

Fig. 4. Radioactivity profile of microbore, C_8 reverse-phase HPLC of tryptic peptide fractions from Al^{3+} chelate chromatography. **A:** Typical UV profile of the radioactive fractions resulting from K_2HPO_4 elution of the Al^{3+} chelate column. **B:** Corresponding ^{32}P cpm profile of the fractions resulting from the HPLC in A. Radioactivity levels were determined by liquid scintillation counting.

Table 3. *Sequence of the [γ-^{32}P]8-N_3GTP photolabeled tryptic peptide from the radioactive peak at 23 min in the HPLC chromatogram*

Cycle number	Identified residue	Picomoles observed
1	W	5.6
2	L	17.5
3	I	14.1
4	T	10.6
5	L	11.7
6	N	7.0
7	K	NF[a]
8	Q	6.4
9	Q	4.9
10	R	2.1

[a]NF, not found.

Discussion

Binding studies of m^7GTP, m^7GpppG, and various capped mRNAs with eIF-4E have provided insight into the nature of the eIF-4E•cap interaction (Carberry et al., 1989, 1992; Goss et al., 1990). Tryptophan stacking and hydrogen bonding with glutamic acid residues have been suggested to be important factors (Adams et al., 1978; Kamiichi et al., 1987; Ishida et al., 1988; Ueda et al., 1991a). Additionally, the involvement of a protonated histidine has been proposed (Carberry et al., 1989). These results have allowed for much speculation regarding the specific amino acid residues that constitute the m^7G cap binding site.

The present study demonstrates the ability of [γ-^{32}P]8-N_3GTP to serve as a substitute for the m^7(5′)Gppp(5′)N cap structure of mRNA in binding to eIF-4E. Selectivity of the probe for the active site was determined in saturation studies with [γ-^{32}P]8-N_3GTP, competitive binding studies with Ant-m^7GTP, and in protection studies utilizing m^7GTP, various nucleotides, and capped mRNA as inhibitors to photoinsertion. Photoinsertion into eIF-4E was saturated with 70 μM [γ-^{32}P]8-N_3GTP and the apparent K_d was 19 μM. The observation that photoinsertion of [γ-^{32}P]8-N_3GTP was inhibited by 90% in the presence of 90 μM m^7GTP demonstrates the specificity of this binding site. The ability of the purine nucleotides GTP, GDP, and ATP to reduce photoinsertion is not surprising because the cap binding site is a dinucleotide site and the probe used in these studies was a mononucleotide. Additional evidence for the site-specific insertion of [γ-^{32}P]8-N_3GTP into the cap binding domain is that of capped mRNA (m^7G(5′)′ppp(5′)G, 34 nucleotides) as the inhibitor that reduced photoinsertion of the probe drastically to 5% of the control (no inhibitor). Competitive binding experiments with Ant-m^7GTP, which has been shown to bind the active site, give further evidence for site specificity.

The peptide resulting from photoinsertion of [γ-^{32}P]8-N_3GTP and subsequent isolation has been identified as the tryptic peptide consisting of residues 113–122 and thus contains tryptophan 6. Detection of the actual site of nitrene insertion is often difficult due to the lability of the N-glycosyl bond to the acidic conditions of HPLC (King et al., 1991). However, these data suggest that Lys 119 is the modified residue for two reasons: the photolabeled binding site peptide is the product of tryptic cleavage at Arg 112 and Arg 122, yet Lys 119 remained uncleaved; and amino acid

sequence analysis was unable to identify Lys 119 despite the continuation of sequencing for three residues beyond this position. Such a gap in sequencing usually indicates reaction of the probe with the missing amino acid (Shoemaker & Haley, 1993). The amino acid is "missing" because it does not elute in the expected position when the probe has been attached to it covalently.

Previous fluorescence quenching studies have indicated the involvement of a tryptophan residue (Carberry et al., 1989). Additionally, the blue-shifted fluorescence emission maximum (330 nm) suggests that the tryptophan resides in a somewhat hydrophobic environment (Carberry et al., 1989). Computer-predicted secondary structure obtained using Chou and Fasman sequence analysis (Chou & Fasman, 1974) indicate that tryptophan 6 comes after a putative turn region and would be exposed only partly (McCubbin et al., 1988). The calculations also suggest that this residue lies in a region of β-sheet structure. CD data from our laboratory reveal a large conformational transition upon binding of mRNA cap analogues by wheat eIF-(iso)4F. These studies suggest a structural β-sheet interaction motif and that this conformational transition may have a regulatory role (Wang et al., 1995).

Fluorescence and CD studies have been performed with Trp → Lys mutants of tryptophan 6 of yeast eIF-4E, which indicate that this residue may be responsible for imparting specificity to cap recognition (McCubbin et al., 1988). It was found that 7-methylated analogues of GDP and G(5′)ppp(5′)G bind strongly to the mutant, but unmethylated analogues also have binding affinity. The presence of the phosphate groups results in a stacking interaction with the indole ring, regardless of the state of methylation. This has been attributed to electrostatic or hydrogen bonding between the tryptophan side chain and the highly charged phosphates (Kamiichi et al., 1987). The substitution of Gly 111 by aspartic acid in yeast eIF-4E reduced cap binding activity as measured by binding of eIF-4E to m^7GDP agarose affinity columns (Altmann & Trachsel, 1989). This glycine is two residues to the N-terminal side of tryptophan 6.

The identification of residues 113–122 as playing a role in m^7GpppG cap recognition by eIF-4E is consistent with previous studies. The eight tryptophans in yeast eIF-4E have been classified into three separate groups on the basis of the mRNA crosslinking activity of Trp → Phe mutants: (1) tryptophans that are strongly required for eIF-4E crosslinking to the mRNA cap, which include tryptophans 1, 2, 5, and 8; (2) tryptophans that reduce the crosslinking ability of eIF-4E, which include tryptophans 3, 6, and 7; and (3) tryptophans that are not required for cap recognition, which includes tryptophan 4. Furthermore, the mutation of tryptophans 1 and 8 completely obliterated cap binding activity (Altmann et al., 1988). The large number of residues affecting binding may indicate that the overall folding of eIF-4E plays a crucial role in cap recognition. Alteration of the tryptophan residues may interfere with the proposed β-sheet structural motif necessary for binding, rather than changing direct base stacking interactions between tryptophan and the m^7G moiety.

Identification of amino acid residues that participate in the m^7G(5′)ppp(5)N binding site of eIF-4E will aid in further mutational studies designed to understand the molecular details of eIF-4E·cap recognition. In addition, further knowledge of the structural motif required for eIF-4E·mRNA cap interactions will aid in our understanding of precisely how translational repressor 4E-binding proteins function (Lin et al., 1994; Mader et al., 1995).

Sequencing of the cap binding site peptides of the wheat germ factors eIF-(iso)4F and eIF-4F is underway currently.

Materials and methods

Materials

Sequencing grade modified trypsin was from Boehringer Mannheim Biochemica (Indianapolis, Indiana). Electrophoresis reagents were from Bio-Rad (Melville, New York). HPLC reagents were from E.M. Science (Gibbstown, New Jersey). All other reagents were obtained from Sigma Chemical Company (St. Louis, Missouri) and were molecular biology grade unless otherwise noted.

Recombinant eIF-4E

Human recombinant eIF-4E was purified from *Escherichia coli* containing a T7 polymerase-driven vector and will be described in detail elsewhere.[6] Recombinant eIF-4E was purified from bacterial lysates by m^7GTP Sepharose affinity chromatography followed by Mono Q FPLC (Pharmacia, Piscataway, New Jersey) (Haas & Hagedorn, 1991; Baker et al., 1992; Bu & Hagedorn, 1992). The FPLC purification step was performed in 50 mM HEPES, pH 8.0, 1 mM $MgCl_2$, 10% glycerol, 1 mM DTT, and used a 0–500 mM gradient of NaCl. Fractions that contained greater than 95% pure recombinant eIF-4E were identified by measuring absorbance at 280 nm and SDS-PAGE analysis. The isoelectric point of the recombinant eIF-4E was identical to that of the dephosphorylated iso-species of eIF-4E isolated from cultured human cells (Bu & Hagedorn, 1992). In addition to its ability to bind the m^7GTP cap structure of mRNA, the recombinant eIF-4E prepared using these methods is phosphorylated in vitro by protein kinase C at the same sites as native eIF-4E (Haas & Hagedorn, 1992).

Synthesis of photoaffinity probe

The radioactive photo probe [γ-^{32}P]8-N_3GTP (specific activity, 32 mCi/μmol) was synthesized and purified as reported previously (Potter & Haley, 1983).

Photoaffinity labeling of ,eIF-4E

To demonstrate saturation effects, samples containing 4 μg of ,eIF-4E in photolysis buffer (10 mM Tris·Cl, no salt, no DTT, pH 7.6) were incubated at 4 °C in 1.5-mL Eppendorf tubes with the appropriate concentration of [γ-^{32}P]8-N_3GTP for 60 s. The reaction mixture was then irradiated for 90 s from a distance of 1 cm with a hand-held 254-nm UVB UV lamp (intensity, 1,400 μW/cm^2). Total reaction volume was 50 μL. The reaction was quenched by the addition of 250 μL of cold acetone. The sample was kept at 4 °C for 3 h and then centrifuged in a Savant HSC10K high-speed centrifuge at 10,000 rpm for 20 min. The supernatant was drawn off and the pellet was resuspended in a protein solubilizing mixture (PSM) consisting of 10% SDS, 3.6 M urea, 2.5% (w/v) DTT, 2% (w/v) pyronin Y (tracking dye), and 20 mM Tris·Cl, pH 8.0. For studies of protection against photoinsertion, 4 μg of ,eIF-4E were incubated in photolysis buffer for 60 s at 4 °C with the required competitor. At 60 s, 40 μM [γ-^{32}P]8-N_3GTP was added and at 60 s, the samples were irradiated, precipitated, and solubilized as described above.

[6] Unpublished experiments.

SDS-PAGE, scintillation, and radioisotopic imaging and quantitation

Following solubilization, photolabeled samples were analyzed by SDS-PAGE according to Laemmli (1970). The gels were fixed in 25% isopropanol/10% acetic acid for 1 h with frequent changes of fixing solution, and dried at 80 °C on a Bio-Rad Model 583 slab gel dryer. ^{32}P incorporation into $_r$eIF-4E was determined with an Ambis 4000 radioisotopic imaging and quantitation system (Scanalytics/CSPI, Billerica, Massachusetts).

Photoaffinity labeling and enzymatic digestion of $_r$eIF-4E for binding domain peptide isolation

$_r$eIF-4E (5 mg) was photolabeled with 50 μM [γ-^{32}P]8-N_3GTP in a 4-mL total volume of photolysis buffer. The reaction mixture was incubated at 4 °C for 5 min in a plastic weighing boat followed by irradiation for 2 min. This was followed by a second incubation and irradiation with nonradioactive 8-N_3GTP under identical conditions. The reaction was quenched and the protein precipitated by the addition of 4 mL of cold acetone followed by incubation at 4 °C for 3 h. The sample was collected by centrifugation for 20 min at 10,000 rpm. The pellet was resuspended in 2 mL of 2 M urea in 75 mM NH_4HCO_3 and the pH adjusted to 8.5–9.0 by the addition of concentrated NH_4OH. $_r$eIF-4E was proteolyzed by the addition of modified trypsin (10% w/v), with shaking overnight at 20 °C.

Immobilized aluminum (III)-chelate chromatography

Iminodiacetic acid-epoxy-activated Sepharose (2 mL) was placed into a 15-mL centrifuge tube and washed as follows: 3 × 10 mL distilled water, 4 × 10 mL 50 mM $AlCl_3$, 3 × 10 mL distilled water, 3 × 10 mL buffer A (50 mM NH_4OAC, pH 6.0), 3 × 10 mL buffer B (100 mM NH_4OAC and 0.5 M NaCl, pH 7.0), 3 × 10 mL buffer A. The resin was then transferred to a 10-mL disposable column (Bio-Rad). The labeled $_r$eIF-4E digestion mixture was brought to a total volume of 3 mL with buffer A, the pH was adjusted to 6.0 with concentrated acetic acid, and the sample was loaded onto the column. To increase the recovery of the labeled peptide, the sample tube was washed with 2 mL buffer A. The column was eluted successively with 2 × 10 mL buffer A, 1 × 10 mL buffer B, 1 × 10 mL buffer A, and the radiolabeled peptide was eluted with 6 mL buffer C (10 mM K_2HPO_4, pH 8.0). Chromatography was conducted at room temperature with a flow rate of 0.5 mL/min. All fractions were assayed for radioactivity on a Wallac Model 1409 Liquid Scintillation Counter (99% counting efficiency for ^{32}P).

Reverse-phase HPLC and amino acid sequencing of photolabeled peptide

The radioactive fractions resulting from buffer C elution from the aluminum (III)-chelate chromatography were pooled and concentrated in a Savant SVC100H speed vac concentrator until a volume of 1.5 mL was reached; 250-μL samples were then analyzed by reverse-phase HPLC using a microbore C_8 column (Brownlee Lab) and an Applied Biosystems 130A separation system. The mobile system consisted of a 25 μM $AlCl_3$/0.1% TFA solution (X) and a 0.1% TFA/70% acetonitrile (Y) solvent system. The gradient was as follows: 100% X at a flow rate of 50 μL/min for 5 min, 0–100% Y at a flow rate of 125 μL/min for 45 min, and 100% Y at a flow rate of 125 μL/min for 5 min. Photolabeled peptides were detected by exact correlation of absorbance at 220 nm and ^{32}P cpm. Fractions containing photolabeled peptides were concentrated to 20 μL and subjected to amino acid sequence analysis at the Hunter College Sequencing and Synthesis Facility using an Applied Biosystems 477A protein sequencer and an Applied Biosystems 120A analyzer with on-line PTH derivative identification.

Competitive binding experiments

The fluorescent m^7GTP cap analogue, anthraniloyl-m^7GTP (ant-m^7GTP) was synthesized as described previously (Ren & Goss, 1996). The competitive substitution reactions were performed at constant Ant-m^7GTP concentration (1.5 μM) and increasing amounts of 8-azido GTP. The buffer used for all fluorescence measurements consisted of 20 mM HEPES·KOH and 1 mM DTT, pH 7.6. All chemicals were molecular biology grade. Fluorescence measurements were made at 25 °C on a SPEX Fluorolog-τ2 spectrofluorometer equipped with a high-intensity (450 W) xenon arc lamp. An excitation wavelength of 332 nm was used to monitor the Ant-m^7GTP fluorescence emission at 420 nm. Excitation and emission slit widths of 2.0 mm were used and a 1.0-cm sample cell pathlength was employed. Lineweaver–Burk plots of $1/\Delta F$ versus 1/[eIF-4E] at varying concentrations of 8-azido GTP will intersect at the same point on the y-axis for competitive inhibition.

Acknowledgments

We thank Dr. Boyd Haley for providing the probes used for this study and for helpful discussion. Also we thank Steve Bobin for amino acid sequence analysis. D.J.G. was supported by grants from the National Science Foundation (GER-9023681 and MCB 9303661), the American Heart Association (AHA-NYC Established Investigator ship), and a P.C.-CUNY Faculty Award. C.H.H. was supported by a grant from the National Institutes of Health (NIH-CA63640) and the AGA Foundation. D.E.F. was supported by grants from the National Science Foundation (pre-doctoral fellowship DGE-9553549), Glaxo-Wellcome Co. (Gertrude Ellion Fellowship), and a Helen Samuels Schectman Scholarship.

References

Adams BL, Morgan M, Muthukrishmam S, Hecht SM, Shatkin AJ. 1978. The effect of "cap" analogs on reovirus mRNA binding to wheat germ ribosomes. *J Biol Chem 253*:2589–2595.

Altmann M, Edery I, Trachsel H, Sonenberg N. 1988. Site-directed mutagenesis of the tryptophan residues in yeast eukaryotic initiation factor 4E. *J Biol Chem 263*:17229–17232.

Altmann M, Handschin C, Trachsel H. 1987. Yeast mRNA cap binding protein gene: Cloning of the gene encoding protein synthesis initiation factor eIF-4E. *Mol Cell Biol 7*:998–1003.

Altmann M, Mueller PP, Pelletier J, Sonenberg N, Trachsel H. 1989. A mammalian transition initiation factor can substitute for its yeast homolog in vivo. *J Biol Chem 264*:12145–12147.

Altmann M, Trachsel H. 1989. Altered cap recognition activity of initiation factor 4E in the yeast cell cycle division mutant CDC33. *Nucleic Acids Res 9*:1643–1656.

Anderssen L. 1991. Recognition of phosphate groups by immobilized aluminum (III) ions. *J Chromatogr 539*:327–334.

Baker BF, Miraglia L, Hagedorn CH. 1992. Modulation of eucaryotic initiation factor-4E binding to 5′ capped oligoribonucleotides by modified anti-sense oligonucleotides. *J Biol Chem 267*:11495–11499.

Bu X, Haas DW, Hagedorn CH. 1993. Novel phosphorylation sites of eukaryotic initiation factor-4F and evidence that phosphorylation stabilizes interactions of the p25 and p220 subunits. *J Biol Chem 268*:4975–4978.

Bu X, Hagedorn CH. 1992. Phosphoroprotein phosphatase 2A dephosphorylates eIF-4E and does not alter binding to the mRNA cap. *FEBS Lett 301*:15–18.

Carberry SE, Friedland DE, Rhoads RE, Goss DJ. 1992. Binding of protein synthesis initiation factor 4E to oligoribonucleotides: Effects of cap accessibility and secondary structure. *Biochemistry 31*:1427–1432.

Carberry SE, Rhoads RE, Goss DJ. 1989. A spectroscopic study of the binding of m7GTP and m7GppG to human protein synthesis initiation factor 4E. *Biochemistry* 2:8078–8083.

Chavan AJ, Rychlik W, Blaas D, Kuechler E, Watt D, Rhoads RE. 1990. Phenyl azide- and benzophenone-substituted phosphonamides of 7-methyl guanosine 5′-triphosphate as photoaffinity probes for protein synthesis initiation factor eIF-4E. *Biochemistry 29*:5521–5529.

Chou PY, Fasman GD. 1974. Prediction of protein confirmation. *Biochemistry 13*:222–245.

Donaldson RW, Hagedorn CH, Cohen S. 1991. Epidermal growth factor or okaidaic acid stimulates phosphorylation of eukaryotic initiation factor 4F. *J Biol Chem 266*:3162–3166.

Duncan RF, Milburn SC, Hershey JWB. 1987. Regulated phosphorylation and low abundance of He La cell initiation factor eIF-4F suggest a role in translational control. *J Biol Chem 262*:380–388.

Goss DJ, Carberry SE, Dever TE, Merrick WC, Rhoads RE. 1990. A fluorescence study of the interaction of protein synthesis initiation factors 4A, 4E, and 4F with mRNA and oligonucleotide analogs. *Biochem Biophys Acta 1050*:163–166.

Grifo HA, Tahara SM, Morgan MA, Shatkin AJ, Merrick WC. 1983. New initiation factor activity required for globin mRNA translation. *J Biol Chem 258*:5804–5810.

Haas DW, Hagedorn CH. 1991. Casein kinase I phosphorylates the 25-kDa mRNA cap-binding protein. *Arch Biochem Biophys 284*:84–89.

Haas DW, Hagedorn CH. 1992. Protein kinase C phosphorylates both serine and threonine residues of the mRNA cap binding protein eIF-4E. *Second Messengers and Phosphoproteins 14*:55–63.

Hellmann GM, Chu LY, Rhoads RE. 1982. A polypeptide which reverses cap analog inhibition of cell-free protein synthesis. *J Biol Chem 257*:4056–4062.

Hiremath LS, Webb NR, Rhoads RE. 1985. Immunological detection of the messenger RNA cap-binding protein. *J Biol Chem 260*:7843–7849.

Ishida T, Doi M, Ueda H, Inoue M, Scheldrick GM. 1988. Specific ring stacking interaction on the tryptophan-7-methylguanine system: Comparative crystallographic studies of indole derivatives-7-methylguanine base, nucleoside, and nucleotide complexes. *J Am Chem Soc 110*:2286–2294.

Jayaram B, Haley BE. 1994. Identification of peptides within the base binding domains of the GTP and ATP-specific binding site of tubulin. *J Biol Chem 269*:3233–3242.

Kamiichi K, Doe M, Nabae M, Ishida T, Inoue M. 1987. Structural studies of the interaction between indole derivative and biologically important aromatic compounds. Part 19. Effect of base methylation on the ring-stacking interaction between tryptophan and guanine derivatives: A nuclear magnetic resonance investigation. *J Chem Soc Perkin Trans II*:1739–1745.

King S, Kim H, Haley B. 1991. Strategies and reagents for photoaffinity labeling of mechanochemical proteins. *Methods Enzymol 196*:449–466.

Koch AE, Cho M, Burrows J, Leibovich SJ, Polverini PJ. 1988. Inhibition of production of macrophage-derived angiogenic activity by the anti-rheumatic agents gold sodium thiomalate and auranofin. *Biochem Biophys Res Commun 154*:205–212.

Laemmli UK. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature 227*:680–685.

Lin TA, Kong X, Haystead TA, Pause A, Belsham G, Sonenberg N, Lawrence C. 1994. PHAS-1 as a link between mitogen-activated protein kinase and translation initiation. *Science 266*:653–656.

Mader S, Lee H, Pause A, Belsham G, Sonenberg N. 1995. The translation initiation factor eIF-4E bind to a common motif shared by the translation factor eIF-4 gamma and the translational repressor 4E-binding proteins. *Mol Cell Biol 15*:4990–4997.

McCubbin WD, Edery I, Altmann M, Sonenberg N, Kay CM. 1988. Circular dichroism and fluorescence studies on protein synthesis initiation factor eIF-4E and two mutant forms from yeast *Saccharomyces cerevisiae*. *J Biol Chem 263*:17663–17671.

Metz AM, Timmer RT, Browning KS. 1992. Isolation and sequence of a cDNA encoding the cap binding protein of wheat eukaryotic protein synthesis initiation factor 4F. *Nucleic Acids Res 20*:4096.

Minich WB, Balasta ML, Goss DJ, Rhoads RE. 1994. Chromatographic resolution of in vivo phosphorylated and nonphosphorylated eukaryotic translation initiation factor eIF-4E: Increased cap affinity of the phosphorylated form. *Proc Natl Acad Sci USA 91*:7668–7672.

Morley SJ, Rau M, Kay JE, Pain VM. 1993. Increased phosphorylation of eukaryotic initiation factor 4 alpha during early activation of T lymphocyte correlates with increased initiation factor 4F complex formation. *Eur J Biochem 218*:39–48.

Potter RL, Haley BE. 1983. Photoaffinity labeling of nucleoside binding sites with 8-azidopurine analogs: Techniques and applications. *Methods Enzymol 91*:613–633.

Proud CG. 1992. Protein phosphorylation in translational control. *Curr Top Cell Regul 32*:243–369.

Ren J, Goss DJ. 1996. Synthesis of a fluorescent 7-methylguanosine analog and fluorescence spectroscopic study and its reaction with cap binding proteins. *Nucleic Acids Res*. Forthcoming.

Rhoads RE. 1988. Cap recognition and the entry of mRNA into the protein synthesis initiation cycle. *Trends Biochem Sci 13*:52–56.

Rhoads RE. 1993. Regulation of eukaryotic protein synthesis by initiation factors. *J Biol Chem 268*:3017–3020.

Rychlik W, Domier L, Gardner PR, Hellman GM, Rhoads RE. 1987. Amino acid sequence of the mRNA cap-binding protein from human tissues. *Proc Natl Acad Sci USA 84*:945–949.

Salvucci ME, Chavan AJ, Haley BE. 1992. Identification of peptides from the adenine binding domains of ATP and AMP in adenylate kinase: Isolation of photoaffinity-labeled peptides by metal chelate chromatography. *Biochemistry 31*:4479–4487.

Shoemaker MT, Haley BE. 1993. Identification of a guanine binding domain peptide of the GTP binding site of glutamate dehydrogenase: Isolation with metal-chelate affinity chromatography. *Biochemistry 32*:1883–1890.

Sonenberg N. 1981. ATP/Mg^{++}-dependent crosslinking of cap binding protein to the 5′ end of eukaryotic mRNA. *Nucleic Acids Res 9*:1643–1656.

Sonenberg N. 1988. Cap binding proteins of eukaryotic messenger RNA (a) Functions in initiation and control of translation. *Prog Nucleic Acid Res Mol Biol 35*:173–207.

Sonenberg N. 1994. Regulation of translation and cell growth by eIF-4E. *Biochemie (Paris) 76*:839–846.

Sonenberg N, Guertin D, Cleveland D, Trachsel H. 1981. Probing the function of the eucaryotic 5′cap structure by using a monoclonal antibody directed against cap-binding proteins. *Cell 27*:563–572.

Tahara SM, Morgan MA, Shatkin AJ. 1981. Two forms of purified m7G-cap binding protein with different effects on capped mRNA translation in extracts of uninfected and poliovirus-infected HeLa cells. *J Biol Chem 256*:7691–694.

Ueda H, Iyo H, Doi M, Inoue M, Ishida T. 1988. A possible recognition mode of mRNA cap terminal structure by peptide: Cooperative stacking and hydrogen-bond pairing interactions between $m^7$6pppA and TRP-LEU-GLU. *Biochem Biophys Res Commun 154*:199–204.

Ueda H, Iyo H, Doi M, Inoue M, Ishida T. 1991a. Cooperative stacking and hydrogen bond pairing interactions of fragment peptide in cap binding protein with mRNA cap structure. *Biochim Biophys Acta 1075*:181–186.

Ueda H, Iyo H, Doi M, Inoue M, Ishida T, Morioka H, Tanaka T, Nishikawa S, Uesugi S. 1991b. Combination of Trp and Glu residues for recognition of mRNA cap structure. Analysis of m^7G base recognition site of human cap binding protein (IF-4E) by site-directed mutagenesis. *FEBS Lett 280*:207–210.

Wang Y, Sha Xiang T, Sha M, Balasta ML, Friedland DE, Goss DJ. 1995. Circular dichroism and fluorescence spectroscopy studies of wheat germ protein synthesis mRNA cap binding protein. In: Merlin JC, Turrell S, Huvenne JP, eds. *Spectroscopy of biological molecules*. Kluwer Academic Publishers.

Webb NR, Chari RVJ, DePillis B, Kozarich JW, Rhoads RE. 1984. Purification of messenger RNA cap-binding protein using a new affinity medium. *Int Biochemistry 23*:177–181.