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Expression, Purification and Characterization of Recombinant Mouse Translation Initiation factor eIF-4E as a Dihydrofolate Reductase (DHFR) Fusion Protein

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Abstract

One of the earliest steps in translation initiation is recognition of the mRNA cap structure (m⁷GpppX) by the initiation factor eIF4E. Studies of interactions between purified eIF4E and its binding partners provide important information for understanding mechanisms underlying translational control in normal and cancer cells. Numerous impediments of the available methods used for eIF4E purification led us to develop a novel methodology for obtaining fractions of eIF4E free from undesired by-products. Herein we report methods for bacterial expression of eIF4E tagged with mutant dihydrofolate reductase (DHFR) followed by isolation and purification of the DHFR-eIF4E protein by using affinity and anion-exchange chromatography. Fluorescence quenching experiments indicated the cap analogue, 7MeGTP, bound to DHFR-eIF4E and eIF4E with a dissociation constant (K_d) of 6 ± 5 and 10 ± 3 nM, respectively. Recombinant eIF4E and DHFR-eIF4E were both shown to significantly enhance in vitro translation in dose dependent manner by 75% at 0.5 μ M. Nevertheless increased concentrations of eIF4E and DHFR-eIF4E significantly inhibited translation in a dose dependent manner by a maximum at 2 μ M of 60% and 90%, respectively. Thus, we have demonstrated that we have developed an expression system for fully functional recombinant eIF4E. We have also shown that the fusion protein DHFR-eIF4E is functional and thus may be useful for cell based affinity tag studies with fluorescently labeled trimethoprim analogs.

Keywords

Translational initiation factor; eIF4E; Fluorescence quenching; DHFR

Introduction

Recruitment of the small ribosome subunit to the 5' end of mRNA is the major rate-controlling event in initiation of eukaryotic protein synthesis. Translation initiation of the majority of eukaryotic transcripts requires assembly of the translational complex eIF4F and its recruitment to the 7MeGTP (m⁷GpppX) cap structure at the 5' end of mRNA[1–4]. The complex eIF4F

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consists of the cap-binding protein eIF4E, the RNA helicase eIF4A, an, and eIF4G, a docking polypeptide, which associates with the 40S ribosomal subunit via contact with the multimeric complex eIF3 [5]. Under physiological conditions, eIF4E is the least abundant component of the initiation machinery, and is therefore considered to be a rate-limiting factor [6]. The eIF4E-mediated translational apparatus functions as a key regulatory hub in the flow of genetic information from the genome to the proteome [7–9]. Recent findings suggest that aberrant dependence on the eIF4E-dependent translational apparatus is integral to pathobiology of human cancer [10–13] and is an attractive target for rational anticancer therapy [7].

Depending upon the species of origin, the molecular mass of eIF4E ranges from 24 to 35 KD. While N – and C-termini of eIF4E vary in length and sequence, the central region contains a conserved core of ~ 170 amino [14]. X-ray crystallographic studies of the cap-analogue 7-Me-GDP bound to mouse [15], human [16], and yeast [17] eIF4E revealed a highly conserved and deep binding cleft sufficient for cap structure recognition. Several groups have reported the isolation and purification of eIF4E protein from several different sources using a range of cloning strategies, expression vectors and purification schemes [18–23]. In general, these methods have relied on immobilized 7MeGDP - affinity column chromatography of recombinant proteins with subsequent elution of eIF4E with a buffer containing 7MeGTP, 7MeGDP or GTP followed by extensive dialysis and/or anion exchange chromatography. However, extensive dialysis may alter the functional integrity of eIF4E [24,25]. K_d values for eIF4E with the cap analogue 7MeGTP have been reported over a broad range from 10^{-5} to 10^{-8} M^{-1} [22,23,25–30]. Recently, Stolarski and co-workers have shown that even after repeated dialysis or anion exchange chromatography, 60% of the resulting recombinant eIF4E can still be bound to cap-analogues used for elution [25]. Hodel and co-workers described the isolation of GST-eIF4E fusion protein by glutathione-agarose affinity chromatography [27]. Although eliminating the need for cap-affinity chromatography, the dimeric (glutathione-S-transferase) GST fusion protein exhibited reduced binding affinity compared to cap-affinity purified eIF4E. In this report, we describe a methodology for the purification of eIF4E as a DHFR fusion protein. In addition, we have characterized and compared the binding affinity and in vitro translation activity of both recombinant eIF4E and the parental DHFR-eIF4E fusion protein.

Materials and Methods

Materials

The construction of the expression plasmid pPH70D containing dihydrofolate reductase (DHFR) and the FLAG peptide was described previously [31]. Cloning vector pSTBlue-1 was obtained from Novagen. E. Coli Novablue, ultracompetent XL-Blue and Tuner (DE3) BL21 strains were purchased from Stratagene (La Jolla, CA). Restriction enzymes, Taq DNA polymerase, T4 DNA ligase, and Dnase were purchased from Promega, Startagene, or Boehringer Mannheim. Wizard plus Miniprep kit was purchased from Promega (Madison, WI). Superdex G-75 size exclusion column for gel filtration chromatography and DEAE cellulose first flow anion-exchange resin was purchased from GE Healthcare (Piscataway, NJ). Bradford protein assay reagent and low range SDS-PAGE marker were purchased from Bio-Rad (Hercules, CA). Lysozyme A, ampicillin, chloramphenicol, thrombin from human plasma, and procainamide were purchased from Sigma-Aldrich (St. Louis, MO). IPTG and DTT were purchased from Fisher Scientific (Chicago, IL) or Sigma-Aldrich (St. Louis, MO). All other chemicals, reagents or HPLC grade solvents, and supplies were obtained from either Fisher Scientific or Sigma Chemical Company. Electrophoresis experiments, protein quantification with Bradford method [32], and DHFR activity assays of the fusion protein were carried out as described previously [31]. 7-methyl GTP – agarose beads were purchased from Amersham Biosciences. Size exclusion chromatography was performed with a Beckman/Coulter Gold

system equipped with a diode array UV detector (Fullerton, CA). Spectrophotometric measurements were made with a Varian Cary 50 UV-Vis spectrophotometer (Palo Alto, CA). Fluorescence spectral measurements were carried out in Cary Eclipse Fluorimeter from Varian, model # EL02025042 (USA). Fluorescence spectra were recorded in quartz-cuvette (Strana Cells, Inc., CA, USA) and a 1.0 cm sample cell path length was employed for absorption and emission spectral measurement.

Construction of *eIF4E-pPH70D* expression recombinant Plasmid

Based on the *eIF4E* gene (*Mus musculus* eukaryotic translation initiation factor) sequence (GeneBank (NCBI, GI: 6681292)), a pair of primers were designed, in which the forward primer (5' CTTACTCGAGAT GGCGACTGTGGAAC 3') included a *XhoI* (CTCGAG) restriction site, and the reverse primer (5' GGCGGTCTAGATTAAACAACAAACCTATT3') contained a *XbaI* (TCTAGA) restriction site. The plasmid pcDNA3-3HA-m4E, (Dr. N. Sonenberg; McGill University, Montreal, Canada) containing the mouse *eIF4E* gene was used as a DNA template. PCR products were purified with SpinPrep Gel DNA kit (Invitrogen Inc.), sequenced and was ligated into pSTBlue-1 with Perfectly Blunt Cloning Kit (Novagen Inc). Novablue Singles Competent Cells were transformed with the ligation mix (6:1), and the transformation mixture was directly plated on LB agar media containing 50 µg/ml carbenicillin, 12.5 µg/ml tetracycline, 70 µg/ml X-gal and 80 µM IPTG. Insert-containing clones were selected by blue-white screening on X-gal/IPTG indicator plates. The resulting plasmid, pSTBlue-1-*eIF4E*, correctly incorporated a single *eIF4E* gene as verified by automated sequencing (Advanced Genetic Analysis Center, University of Minnesota).

Plasmids pSTBlue-1-*eIF4E* and pPH70D [31] containing FLAG-dihydrofolate reductase (DHFR) L54F were each subjected to double digestion with *XhoI* and *XbaI* to obtain the *eIF4E* insert DNA and the modified pFLAG vector DNA containing DHFR L54F, respectively. The digested *eIF4E* DNA and pPH70D plasmid DNA were visualized by staining with ethidium bromide on 1.2% agarose gels, and subsequently purified from the gels with the use of SpinPrep Gel DNA kit (Invitrogen Inc.) and ligated with T4 DNA ligase at 22°C overnight. The resulting plasmid yielded the *eIF4E-pPH70D* expression vector (Figure 1). Fresh competent XL-Blue cells were prepared by the calcium chloride method and transformed with the ligation mixture. The plasmid-containing clones were plated on LB agar media containing 100 µg/ml ampicillin. Single colony was selected and cultured in LB media containing 100 µg/ml ampicillin and the plasmid was removed from the culture via mini-prep kit (Promega). The presence and proper orientation of the *eIF4E* insert in the resulting vector was confirmed by restriction mapping.

eIF4E-Protein Expression from *eIF4E-pPH70D*

The Plasmid *eIF4E-pPH70D* was transformed into fresh competent Tuner (DE3) pLacI cells, and grown on solid LB/carbenicillin (50 µg/ml)/ chloramphenicol (34 µg/ml) plates at 37°C overnight. A 10 mL starter culture was grown from a single colony over night in LB/carbenicillin with shaking at 37°C. A 1L LB/carbenicillin was inoculated with the starter culture and incubated at 37°C with shaking. IPTG (1 mL of 0.2M IPTG; final concentration 0.2mM) was added when the OD of the culture at 600nm reached 0.4. Once the optical density (OD) reached a plateau after 3.5h, the cells were harvested and collected by centrifuging at 5000x g for 30 min. The cell pellets were frozen on dry ice, transferred to a 250 ml centrifuge bottle, weighed and stored at -80°C.

Purification of mouse *eIF4E* –DHFR fusion protein from *eIF4E-pPH70D* by Methotrexate Affinity Chromatography

The Tuner (DE3) pLacI/pPH70D-*eIF4E* cell pellets were thawed on ice and re-suspended in a 12.5 mL volume of degassed lysis buffer A (50mM Tris pH8.0, 5mM EDTA). Lysozyme (Sigma, 12.5mg/per gram cell pellets), sodium azide (final concentration 50mg/ml), 100X

protease inhibitor solution (100 mM PMSF, 100 mM Benzamidine, 1.4 mM Pepstatin A, 2.0 μ M Leupeptin) were added to the above 12.5 mL volume lysis buffer A and mixed. The mixture was incubated for 10 min on ice and swirled once per minute. Lysis buffer B (1.5M NaCl, 100 mM MgCl₂, 100 mM CaCl₂) was added with 20mg Dnase I and incubated for another 5 min on ice. At the end, DDT was added to a final concentration of 5mM. Cell lysate was layered with argon and soluble fraction was obtained by centrifuge at 25,000xg at 4°C for 30 min. The protein concentration of the soluble fraction was determined by Bradford assay and 20 μ g sample was used for SDS-Page and western blot analysis.

The *E. coli* 4E-DHFR fusion protein was purified with a methotrexate (MTX)-agarose column (2.5-ml). The column was initially washed with 40 column volumes of water followed by 80 column volumes of buffer B, high salt phosphate buffer (PB) pH=6.0 (50 mM KH₂PO₄, 1 mM K₂EDTA, 1 M KCl, 1mM DTT) and equilibrated overnight with 1L of PB buffer. Protein was applied onto the MTX column at a flow-rate of 1 mL/min. The flow-through was collected. The column was then washed with 60 column volumes of buffer A, 50 mM PB pH=6.0 (50 mM KH₂PO₄, 1 mM K₂EDTA, 1mM DTT)(3X with 2L). The protein was eluted with folate elution buffer pH=9.0 (10 mM KH₂PO₄, 0.1 mM K₂EDTA, 1M KCl, 3 mM Folate, 1 mM DTT). Fractions were collected at 1 mL/min at 8 min intervals. Maximum amount of protein was eluted with 3mM Folate elution buffer. The total protein amount was 41.4 mg. A small amount of the fusion protein remained on the MTX column as judged from Bradford assay and was eluted with ~60 mL of trimethoprim (256 μ M in buffer B). Each fraction was assayed for protein concentration with the Bradford dye reagent (Biorad). Fractions containing more than 0.1mg/ml of protein were analyzed by 12% SDS-PAGE, and DHFR activity was determined [33,34]. The standard assay mixture contained 50 μ M DHF, 100 mM NADPH and 1mM dithiothreitol in MTEN buffer (50 mM 2-morpholinoethanesulfonic acid, 25 mM tris (hydroxymethyl) aminomethane, 25 mM ethanolamine, and 100 mM NaCl, pH 7.0), and the enzyme in a final volume of 1.0 ml. The reaction was started by the addition of DHF [34]. Half of the pooled fractions of protein solution were subjected to DEAE-cellulose column and the pure DHFR-eIF4E fusion protein was separated from simple DHFR protein by eluting with 0-0.5 M KCl PE buffer (20 mM Tris, 1mM EDTA; 1mM DTT; pH=7.4) gradient. (Figure S2) As judged from SDS page gel electrophoresis two fractions were pooled (about 18mL) which were devoid of any DHFR protein and concentrated.

The fusion protein was dialysed against thrombin cleavage buffer (3 times of 2L) (50 mM Tris, pH 8.0; 0.1 M NaCl; 2.5 mM CaCl₂). Thrombin (250 units) was added to the protein solution and the cleavage was carried out at 4°C for 20h. After thrombin cleavage, the protein solution was centrifuged and applied to DEAE column, which had been washed with 500 ml of water, followed by 1L of 1M KCl PE buffer and equilibrated with 1L of PE buffer (pH=7.4) prior to loading with the protein solution. After loading, the column was washed with 50mL of PE buffer, and the purified eIF4E and DHFR were separated with a gradient of 0-0.4 M KCl PE buffer [31,35]. The total amount of protein obtained was ~2.5 mg. from a 2 L culture. The 12% SDS-PAGE gel confirmed purity of the 4E protein (*vide infra*).

Determination of K_d by Fluorescence Titration

For recombinant eIF4E – cap analog binding studies, samples were excited at 280 nm, and emission spectra collected at between 300 to 600 nm (slit with 5nm). The HEPES/KOH buffer (pH 7.2) was used for all fluorescence spectral studies (50 mM HEPES; 100m KCl; 1mM DTT and 0.5 mM Na₂EDTA) as previously described [25]. Titration experiments were performed in duplicates or in triplicates with a constant eIF4E protein concentration (500 nM). Aliquots, ~1.5 μ l, of compounds were added to 1.5 mL of eIF4E in HEPES buffer. The cuvette chamber was thermostated at 22°C through a temperature controller. The gap between the two measurements was fixed to 90 seconds. The mixing time of 30 seconds with magnetic stirrer

was allowed after each addition of ligand to the protein solution followed by scanning time (45 sec.) required by the Fluorescence spectrophotometer. The steady state data at 342 nm of fluorescence quenching experiments were collected. Generally, 15 to 20 data points were utilized for non-linear regression analysis and the exact fit was obtained with the JUMPIN software using the mathematical expression (Eq.1) deduced previously for enzyme – ligand complex formation [36], and is shown in Figure 6 (B). Appropriate corrections were made to extract intrinsic fluorescence quenching values by taking into account of the dilution effects and the increased fluorescence emission incurred with all the four cap-analogs during titrations. The observed fluorescence, F , can be written in terms of five constants: F_E , fluorescence intensity of the protein without cap binding; F_{EL} , fluorescence intensity of the cap-bound protein; E_T , total concentration of the enzyme; L_T , total concentration of the ligand; K_D , is the dissociation constant of the eIF4E-cap binding equilibria.

$$F = F_E - \frac{\left\{ (E_T + L_T + K_D) - \left[(E_T + L_T + K_D)^2 - 4(E_T)(L_T) \right]^{1/2} \right\} (F_E - F_{EL})}{2E_T} \quad \text{Eq. 1.}$$

In Vitro Translation Assays

To directly assess the level of translation, we employed the dual-luciferase bicistronic reporter construct pcDNA3-rLuc-POLIRES-fLuc (Poulin 1998), which is designed so that the translation of *Renilla reniformis* luciferase (rLUC) is strictly cap-dependent, whereas the translation of firefly luciferase (fLUC) proceeds via an IRES in a cap-independent manner. The plasmid was linearized, and 5'-capped bicistronic luciferase reporter mRNA was generated by *in vitro* transcription (mMESSAGE mMACHINE kit, Ambion, Austin, TX) using T7 polymerase, according to the manufacturer's instructions. Reporter mRNA (0.02 μ g) was added to the reaction mixture (17 μ L Retic lysate, 1 μ L 10 mg/ml L-methionine, 1.25 μ L high salt translation buffer per reaction), as recommended (Retic Lysate IVT™, Ambion, Austin, TX). Purified eIF4E or DHFR-eIF4E (0–3 μ M) were introduced into the reaction mixture, as well as 7-MeGTP (10 μ M) or nuclease-free water (control). *In vitro* translation was carried out at 30°C for 1h. Reaction was stopped by chilling (5 min on ice) and samples were diluted with 100 μ L of nuclease-free water. *Renilla* and firefly luciferase activity/abundance was quantified by luminometry using the Dual-Luciferase Reporter Assay System (Promega) exactly as described in the technical manual in a Lumat LB 9507 Luminometer (EG&G Berthold). Luminescence was measured in relative light units. Assays were performed in triplicate; the mean $\pm$ SEM were determined for each protein concentration. Reporter translation in the samples was compared to the baseline, set at 100.

Results and Discussion

A potential pathway for regulating protein translation is the prevention of eIF4E binding to the 7MeG-Cap of mRNA. In particular, it has recently been shown that cancer cells are more highly dependent on cap-dependent translation than normal cells [37–40]. Consequently, in order to assist in carrying out biochemical, biophysical and pharmacological studies of eIF4E an ample source of pure fully functional cap-free recombinant eIF4E protein is required.

Expression Construct pPH70D-eIF4E

An expression vector pPH70D-eIF4E, which encodes FLAG-DHFR followed by a thrombin sensitive linker and the mouse eIF4E gene has been constructed in our laboratory. The plasmid was generated by replacing the gene encode NAT2 (hamster polymorphic N-acetyltransferase 2) in pPH70D with the gene for eIF4E. After PCR amplification from the cDNA of mouse-eIF4E, which was obtained from the designed forward and reverse primers incorporating XhoI and XbaI restriction sites, the product was cloned into pSTBlue 1. Double digestion with XhoI

and XbaI restriction enzymes followed by sub-cloning into pPH70D resulted in the plasmid pPH70D-eIF4E. (Figure 1 A & B). The DNA for the DHFR insert and the modified pFLAG vector DNA containing the mouse eIF4E were ligated and the proper orientation of the DHFR insert into the final plasmid, pPH70D-eIF4E, confirmed by restriction enzyme analysis.

It was anticipated that this unique expression system offered several significant advantages over the current protocols for purification of homogeneous eIF4E [41]. First, mutant DHFR fusion protein could be purified on a stable, inexpensive, high-capacity (~80 mg/ml), and highly selective methotrexate (MTX) column [31]. Secondly, due to the pH dependence of MTX binding to the DHFR mutant, the fusion eIF4E protein in the cell lysates could be loaded onto the MTX column at pH 6.0 and washed to remove nonspecifically bound proteins, followed by elution of the fusion protein from the MTX column by raising the pH of the buffer around 9 [42]. However, if there is a concern about the stability of the desired protein at such pH, the protein could also be eluted with a folate solution. Regardless, this purification protocol does not require a 7MeGTP-agarose affinity column, or cap-analogue elution [24,43,44].

Expression and Purification of eIF4E-DHFR by Methotrexate Affinity and DEAE anion Exchange Chromatography

E. Coli BL21(DE3) plac I Turner cells transformed with the eIF4E-pPH70D vector were initially grown at 37°C to OD_{600nm} 0.4. The optimal expression level was achieved when the cell culture was induced with IPTG (0.2mM) and grown until the optical density reached a plateau. SDS-PAGE gel electrophoresis showed a significant band of DHFR-eIF4E fusion protein in the soluble fraction of the IPTG induced cell lysate compared to non-induced cell lysate. In IPTG induced cells a significant amount of one fusion protein was also present as inclusion bodies (Figure 2, Lanes 1, 2 and 3).

The soluble fraction of the degassed cell lysates was applied to an MTX-affinity column pre-incubated with a low salt buffer (see experimental section) at pH 6. Nonspecifically bound proteins were removed from the column with a high salt buffer. Elution of the fusion protein DHFR-eIF4E was carried out with 3 mM folate solution at pH 9. The elution profile of MTX column is shown in Figure S1. Similar to the elution pattern reported for DHFR-Hint1[42], we were able to elute the majority of the bound fusion protein ~ 90% as judged from the remaining bound fusion protein, which was latter recovered from the MTX column by trimethoprim (TMP), a potent DHFR inhibitor ($K_i \sim 10^{-12}$ M) [45]. Since DHFR can be eluted with 1mM Folate, the eIF4E-DHFR fusion proteins exhibited increased binding to the MTX column.

The SDS-PAGE analysis indicate that fractions 4–15 (Figure 3, lane 3–8), contain large amount of the fusion protein DHFR-eIF4E (MW ~ 50 KD) and most of these fractions, particularly, 6 and 7 were contaminated with large amounts of proteolytically released DHFR. The collected fractions were pooled and half of the pooled solution was subjected to a second chromatographic column with a DEAE anion exchange resin. This step efficiently separated the DHFR-eIF4E fusion protein from DHFR. The elution profile of the DEAE-anion exchange Column is shown in Fig. 4. SDS-PAGE revealed fractions (Figure 3, lane 3 and 4) that did not contain free DHFR. These fractions were pooled and concentrated to obtain about 4.2 mg of pure DHFR-eIF4E protein. Although later fractions also showed significant amounts of DHFR-eIF4E (Figure 3, lane 5, 6, and 7) protein; these fractions were contaminated with significant amounts of free DHFR. The purity of the final protein after concentration was demonstrated by SEC (Figure S3 (A)). The retention time of the major peak corresponding to purified DHFR-eIF4E protein is 18.5 min. Based on a molecular weight standard curve the calculated molecular weight of the protein was shown to be 50kDa. As seen from Figure S3 (A), two additional minor peaks could be observed at 21.2 and 23.8 min.

Purification of eIF4E by DEAE Anion – Exchange Chromatography

The other half of the pooled protein solution from the MTX column was dialysed against thrombin cleavage buffer. During dialysis insoluble white solid was observed to accumulate in the dialysis tube. It was not, however, shown to be either eIF4E or DHFR-eIF4E. (Data not shown) Complete cleavage of the fusion protein into eIF4E and Flag-DHFR by human thrombin was observed by SEC after a 20h incubation period. The reaction mixture was loaded onto a DEAE cellulose column and the purified eIF4E isolated. (Figure 4)

SDS-PAGE analysis of the protein fractions collected during purification of the expressed protein cell lysates, as shown in Figure 5, clearly indicated that eIF4E (25.4 kDa) was easily separated from DHFR. After concentration of the eIF4E containing fractions, ~2.5 mg of purified protein could be routinely obtained from 2L of bacterial cell culture. The purity of the final concentrate was shown by size-exclusion chromatography to be greater than >99% (Figure S3 B).

Fluorescence Quenching Cap-Analog Binding Studies

The most convenient way to study eIF4E binding to 7MeGTP analogs is by fluorescence quenching [25]. X-ray crystal structure studies of eIF4E binding to 7MeGDP revealed that the cap binding site possesses a deep narrow hydrophobic pocket, in which the side chain of the two conserved tryptophan residues (Trp -102 and Trp-56) support the recognition of cap [15, 16,25,46]. The measurement of cap-binding affinity can be determined by fluorescence quenching of these residues [44]. The fluorescence quenching characteristics of our recombinant eIF4E and eIF4E-DHFR fusion proteins with 7MeGTP, 7MeGDP and 7MeGMP were examined. Measurements of the observed protein fluorescence quenching of freshly purified eIF4E at various concentrations of cap-analogs were carried out with a typical titration curve for 7MeGTP shown in Fig 8 (A). As can be seen from the Figure 6, 63% quenching was observed which is nearly identical to that previously reported [25]. The dissociation constant (K_d) for 7MeGTP was calculated by fitting the observed single exponential decrease in fluorescence to eq.1 with respect to increasing ligand concentration [36]. From a set of three independent measurements the average K_d was found to be 10 (± 3) nM for 7MeGTP, which is nearly identical to the value (9.1 nM) previously reported for eIF4E purified by cap-affinity chromatography [25]. The K_d value is almost 2- orders of magnitude lower than that reported by others [26,29,30]. The fluorescence quenching measurements were also conducted with 7MeGDP, 7MeGMP and 7BnGMP under similar condition and the corresponding K_d values were found to be 45 (± 10) nM, 10,000(± 500) nM and 800 (± 200) nm, respectively. Similar trends in the K_d values for 7MeGDP (49 nM) and 7MeGMP (1240 nM) were reported by Stolarski's and co-workers [25] Although a K_d value determined by fluorescence titration has not been reported for 7BnGMP, a nearly 9-fold greater K_d value (7,000 nM) was recently determined for 7BnGMP by a novel mass spectroscopic technique [47]. The binding affinity of the cap-analog, 7MeGTP, with pure DHFR-eIF4E fusion protein was also studied by fluorescence quenching experiments. The K_d value was found to be comparable (6 nM) to that for purified eIF4E. The binding affinities with 7MeGDP, 7MeGMP and 7BnGMP were also shown to be comparable to the values for eIF4E, indicating that DHFR, in contrast to GST, does not interfere with the substrate binding to eIF4E. [27].

Translational Initiation Studies

In general the ability of eIF4E and DHFR-eIF4E to bind cap-analogs, and therefore by analogy capped-mRNA, is accepted as proof of functional integrity. However, *in vitro* binding studies do not demonstrate whether the recombinant protein is able to not only bind to mRNA; but also correctly interact with other proteins in the eIF4F complex. Consequently, we carried out studies of the effect of eIF4E and DHFR-eIF4E on *in vitro* translational of renilla luciferase mRNA by rabbit reticulocyte extracts. The addition of purified eIF4E or DHFR-eIF4E

enhanced translation in a dose dependent manner up to a concentration of 0.25 μ M by as much as 70% and 40%, respectively. (Figure 7) The amount of free endogenous eIF4E in the reticulocyte extract, therefore, appears to be at least partially limiting. These observations are consistent with previous studies demonstrating that the majority of eIF4E found in rabbit reticulocytes is either bound to eIF4BP or eIF4G [48]. At higher concentrations of both eIF4E and DHFR-eIF4E exhibited significant dose dependent inhibition of translation was observed. At a concentration of 2 μ M, which is nearly 10- fold higher than the concentration needed for maximal translation, the amount of renilla luciferase translated was 3- and 14- fold lower for eIF4E or DHFR-eIF4E, respectively. The induction of translation by either eIF4E or DHFR-eIF4E was inhibited by the addition of 7-Me-GTP, with the difference in translation observed without inhibitor being maintained. (Figure 7). Previously, it has been observed that highly over-expressed levels of intracellular eIF4E (100- fold) can inhibit the expression of luciferase by yeast, while more physiologically relevant levels of over-expression (3- to 10- fold) have only a minimal effect on luciferase translation [20]. Recently, using transient state kinetics, Rhoades and co-workers demonstrated that the binding of the cap analog, 7-MeG-pppG to eIF4E was characterized by concentration dependent double exponential progress curves when protein concentrations exceeded 1 μ M [49]. They concluded that the monomeric form of eIF4E is in equilibrium with one more non-cap-binding eIF4E oligomeric species. This conclusion is consistent with the decrease in translation efficiency we have observed for concentrations of eIF4E and DHFR-eIF4E greater than 0.25 μ M. A rationale for the greater inhibitory effect of DHFR-eIF4E compared to eIF4E is not clear, but maybe useful for experiments in which a greater degree of translation initiation attenuation is sought when the protein is highly expressed intracellularly by transfected mammalian cells.

Summary

A DHFR-eIF4E fusion protein with a thrombin – sensitive linker was constructed. An efficient protocol for over-expression and purification of Cap-analog free soluble eIF4E_{mouse} protein has been developed that does not require protein refolding. Soluble DHFR-eIF4E fusion protein was isolated from bacterial cell extracts by methotrexate affinity chromatography, followed by anion exchange chromatography. Purified eIF4E was obtained after thrombin digestion with a yield of 2.5 mgs per 2 liter of the bacterial culture. (Table 1) Similar to previous studies with eIF4E, fluorescence quenching studies with cap analogs demonstrated that both eIF4E and DHFR-eIF4E are capable of binding cap-analogs with nanomolar binding affinities. The DHFR affinity tag should, therefore, be useful for the isolation of eIF4E mutants with altered cap binding affinities. In addition, both eIF4E and DHFR-eIF4E were shown to be functional, since they were able to enhance in vitro translation in a dose dependent manner. Consequently, DHFR-eIF4E should be considered as a possible intracellular tag for monitoring the location of eIF4E with trimethoprim fluorophores [50]. Our observation of a dose dependent decrease in translation efficiency at concentrations of eIF4E and DHFR-eIF4E greater than 0.25 μ M is consistent with earlier observations that eIF4E exists in rapid equilibrium in solution between cap binding monomeric and non-cap binding oligomeric states. Intriguingly, this observation demonstrates intriguingly the sensitivity of translation initiation to eIF4E concentration; whether this phenomenon is physiologically relevant remains to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

eIF4E, eukaryotic initiation factor, 4E
eIF4G, eukaryotic initiation factor, 4G

4E-BP1, 4E binding protein
 7-MeGTP, 7-methyl guanosine triphosphate
 7-MeGDP, 7-methyl guanosine diphosphate
 7-MeGMP, 7-methyl guanosine monophosphate
 DEAE, diethylaminoethyl
 DHFR, dihydrofolate reductase
 DTT, dithiothreitol
 EDTA, ethylenediaminetetracetic acid
 HPLC, high-pressure liquid chromatography
 IPTG, isopropyl- β -D-thiogalactopyranoside
 LB, Luria–Bertani medium
 MES, 2-[*N*-morpholino]ethanesulfonic acid
 MTX, metho-trexate
 NAT2, polymorphic *N*-acetyltransferase 2
 PA, procainamide
 PAGE, polyacrylamide gel electrophoresis
 PCR, polymerase chain reaction
 SDS, sodium dodecyl sulfate
 TMP, trimethoprim
 BSA, bovine serum albumine
 GST, Glutathione-s-transferase

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Reference

- Gingras AC, Raught B, Sonenberg N. eIF4E initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Ann. Rev. Biochem* 1999;68:913–963. [PubMed: 10872469]
- Gallie DR. Protein-protein interactions required during translation. *Plant Mol. Biol* 2002;50:949–970. [PubMed: 12516864]
- Pestova TV, Hellen CU. Functions of eukaryotic factors in initiation of translation. *Cold Spring Harb. Symp. Quant. Biol* 2001;66:389–396. [PubMed: 12762041]
- Browning KS. The plant translational apparatus. *Plant Mol. Biol* 1996;32:107–144. [PubMed: 8980477]
- Gallie DR. A tale of two termini: a functional interaction between the termini of an mRNA is a prerequisite for efficient translation. *Gene* 1998;216:1–11. [PubMed: 9714706]
- Gingras AC, Raught B, Sonenberg N. mTOR signaling to translation. *Current. Top. Microbial. Immunol* 2004;279:169–197.
- Polunovsky VA, Bitterman PB. The cap-dependent translation apparatus integrates and amplifies cancer pathways. *RNA Biol* 2006;3:10–17. [PubMed: 17114939]
- Raught B, Gingras AC. eIF4E activity is regulated at multiple levels. *Int. J. Biochem. Cell Biol* 1999;31:43–57. [PubMed: 10216943]
- Sonenberg N, Gingras AC. The mRNA 5' cap-binding protein eIF4E and control of cell growth. *Curr. Opin. Cell Biol* 1998;10:268–275. [PubMed: 9561852]
- Avdulov, S SL, Michalek V, Burrichter D, Peterson M, Perlman DM, Manivel JC, Sonenberg N, Yee D, Bitterman PB, Polunovsky VA. Activation of translation complex eIF4F is essential for the genesis and maintenance of the malignant phenotype in human mammary epithelial cells. *Cancer Cell* 2004;5:553–563. [PubMed: 15193258]

11. Larsson O, Li S-h, Issaenko OA, Avdulov S, Peterson M, Smith K, Bitterman PB, Polunovsky VA. Eukaryotic translation initiation factor 4E-induced progression of primary human mammary cells along the cancer pathway is associated with targeted translational deregulation of oncogenic drivers and inhibitors. *Cancer Res* 2007;67:6814–6823. [PubMed: 17638893]
12. Ruggero, L DM, Ma L, Xu W, Londei P, Cardon-Cardo C, Pandolfi PP. The translation factor eIF-4E promotes tumor formation and cooperates with c-Myc in lymphomagenesis. *Nat. Medicine* 2004;10:484–486.
13. Zimmer SG, De Benedetti A, Graff JR. Translational control of malignancy: the mRNA cap-binding protein, eIF4E, as a central regulator of tumor formation, growth, invasion and metastasis. *Anticancer Res* 2000;20:1343–1351. [PubMed: 10928042]
14. Joshi B, Cameron A, Jagus R. Characterization of mammalian eIF4E-family members. *Eur. J. Biochem* 2004;271:2189–2203. [PubMed: 15153109]
15. Marcotrigiano J, Gingras AC, Sonenberg N, Burley SK. Co-crystal structure of the messenger RNA 5'-cap-binding protein (eIF4E) bound to 7-methyl-GDP. *Cell* 1997;89:951–961. [PubMed: 9200613]
16. Tamoo K, Shen X, Okabe K, Nozoe Y, Fukuhata S, Morino S, Ishida T, Taniguchi H, Terashima A, Sasaki M, Katsuya Y, Kitamura K, Miyoshi H, Ishikawa M, Miura K. Crystal structures of 7-methylguanosine 5'-triphosphate (m(7)GTP)- and P(1)-7-methylguanosine-P(3)-adenosine-5', 5'-triphosphate (m(7)GPPA) – bound human full length eukaryotic initiation factor 4E: biological importance of the C-terminal flexible region. *Biochem J* 2002;362:539–544. [PubMed: 11879179]
17. Matsuo H, Li H, McGuire AM, Fletcher CM, Gingras AC, Sonenberg N, Wagner G. Structure of translational factor eIF4E bound to m7GDP and interaction with 4E-binding protein. *Nat. Struct. Biol* 1997;4:717–724.
18. Edery I, Chau LL, Sonenberg N, Pelletier J. An efficient strategy to isolate full-length cDNA based on an mRNA cap retention procedure (CAPture). *Mol. Cellular Biol* 1995;15:3363–3371.
19. Lamphear BJ, Panniers R. Cap binding protein complex that restores protein synthesis in heat-shocked Ehrlich cell lysates contains highly phosphorylated eIF4E. *J.Biol.Chem* 1990;265:5333–5336. [PubMed: 2318815]
20. Lang V, Zanchin NIT, Lünsdorf H, Tuite M, McCarthy JE. Initiation factor eIF-4E of *Saccharomyces cerevisiae*. *J. Bio. Chem* 1994;269:6117–6123. [PubMed: 8119957]
21. Lee S-J, Stapleton G, Greene JH, Hille MB. Protein kinase C-related kinase 2 phosphorylates the protein synthesis initiation factor eIF4E in starfish oocytes. *Dev. Biol* 2000;228:166–180. [PubMed: 11112322]
22. Morino S, Yasui M, Doi M, Inoue M, Ishida T, Morioka H, Ueda H, Uesugi S. Direct expression of a synthetic gene in *Escherichia coli*: purification and physicochemical properties of human initiation factor 4E. *J.Biochem* 1994;116:687–693. [PubMed: 7852292]
23. Ueda H, Maruyama H, Doi M, Inoue M, Ishida T, Morioka H, Tanaka T, Nishikawa S, Uesugi S. Expression of a synthetic gene for human cap binding protein (human IF-4E) in *Escherichia coli* and fluorescence studies on interaction with mRNA cap structure analogues. *J. Biochem* 1991;109:882–889. [PubMed: 1939010]
24. von der Haar T, Ball PD, McCarthy JE. Stabilization of eukaryotic initiation factor 4E binding to the mRNA 5'-cap by domains of eIF4E. *J. Biol. Chem* 2000;275:30551–30555. [PubMed: 10887196]
25. Niedzwiecka A, Marcotrigiano J, Stepinski J, Jankowska-Anyszka M, Wyslouch-Ceiszynska A, Dadlez M, Gingras AC, Mak P, Darzynkiewicz E, Sonenberg N, Burley SK, Stolarski R. Biophysical studies of eIF4E cap-binding protein: Recognition of mRNA 5'-cap structure and synthetic fragments of eIF4G and 4E-BP1 proteins. *J.Mol.Biol* 2002;319:615–635. [PubMed: 12054859]
26. Hagedorn CH, Spivak-Kroizman T, Friedland DE, Goss DJ, Xie Y. Expression of functional eIF-4Ehuman: Purification, detailed characterization, and its use in isolating eIF-4E binding proteins. *Protein Expr. Purif* 1997;9:53–60. [PubMed: 9116502]
27. Hsu P-C, Hodel MR, Thomas JW, Taylor LJ, Hagedorn CH, Hodel AE. Structural requirements for the specific recognition of an m7GmRNA cap. *Biochemistry* 2000;39:13730–13736. [PubMed: 11076512]

28. Miyoshi H, Youtani T, Ide H, Hori H, Okamoto K, Ishikawa M, Wakiyama M, Nishino T, Ishida T, Miura K. Binding analysis of *Xenopus laevis* translation initiation factor 4E (eIF4E) in initiation complex formation. *J. Biochem* 1999;126:897–904. [PubMed: 10544283]
29. Carbery SE, Rhoads RE, Goss DJ. A spectroscopic study of the binding of m7GTP and m7GpppG to human protein synthesis initiation factor 4E. *Biochemistry* 1989;28:8078–8083. [PubMed: 2605173]
30. Wieczorek Z, Niedzwiecka-Kornas A, Chlebika L, M J, Kiraga K, Stepinski J, Dadlez M, Drabent R, Darzynkiewicz E, Stolarski R. Fluorescence studies on association of human translation initiation factor eIF4E with mRNA cap-analogues. *Z. Naturforsch. [C.]* 1999;54:278–284.
31. Sticha KRK, Sieg CA, Bergstrom CP, Hanna PE, Wagner CR. Overexpression and large-scale purification of recombinant hamster polymorphic arylamine N-acetyltransferase as a dihydrofolate reductase fusion protein. *Protein Expr. Purif* 1997;10:141–153. [PubMed: 9179301]
32. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal. Biochem* 1976;72:248–254. [PubMed: 942051]
33. Stone SR, Morrison JF. Kinetic mechanism of the reaction catalyzed by dihydrofolate reductase from *Escherichia coli*. *Biochemistry* 1982;21:3757–3765. [PubMed: 6753919]
34. Morrison JF, Stone SR. Dihydrofolate reductase from *Escherichia coli*: the kinetic mechanism with NADPH and reduced acetylpyridine adenine dinucleotide phosphate as substrates. *Biochemistry* 1988;27:5499–5506. [PubMed: 3052578]
35. Wang H, Vath GM, Kawamura A, Bates CA, Sim E, Hanna PE, Wagner CR. Over-expression, purification, and characterization of recombinant human arylamine N-acetyltransferase 1. *The Protein Journal* 2005;24:65–77. [PubMed: 16003948]
36. Taira K, Benkovic SJ. Evaluation of the importance of hydrophobic interactions in drug binding to dehydrofolate reductase. *J. Med. Chem* 1988;31:129–137. [PubMed: 3275776]
37. Chen H, Fan Y-H, Natarajan A, Guo Y, Iyasere J, Harbinski F, Luus L, Christ W, Aktas H, Halperin JA. Synthesis and biological evaluation of thiazolidine-2,4-dione and 2,4-thione derivatives as inhibitors of translation initiation. *Bioorg. Med. Chem. Lett* 2004;14:5401–5405. [PubMed: 15454234]
38. Ghosh P, Park C, Peterson MS, Bitterman PB, Polunovsky VA, Wagner CR. Synthesis and evaluation of potential inhibitors of eIF4E cap binding to 7-methyl GTP. *Bioorg. Med. Chem. Lett* 2005;15:2177–2180. [PubMed: 15808492]
39. Ghosh, P.; Avdulov, S.; Bitterman, PB.; Polunovsky, VA.; Benyumov, A.; Wagner, CR. Modulation of cap-dependent translation by nucleoside phosphoramidates. 230th ACS National Meeting; Washington DC. 2005.
40. Pillai RS, Bhaattacharya SN, Artus CG, et al. Inhibition of translational Initiation by Let-7microRNA in human cells. *Science* 2005;309:1573–1576. [PubMed: 16081698]
41. Hakes DJ, Dixon JE. New vector of high-level expression of recombinant proteins in bacteria. *Anal. Biochem* 1992;202:293–298. [PubMed: 1519755]
42. Chou T-F, Bieganski P, Shilinski K, Cheng J, Brenner C, Wagner CR. 31P NMR and genetic analysis establish hint as the only *Escherichia coli* purine nucleotide phosphoramidase and as essential for growth under high salt conditions. *J. Biol. Chem* 2005;280:15356–15361. [PubMed: 15703176]
43. Edery, I.; Lee, KAW.; Sonenberg, N. Translational Regulation of Gene Expression. Illan, I., editor. 1987. p. 335-366.
44. Shibata S, Morino S, Tomoo K, In Y, Ishida T. Effect of mRNA cap structure on eIF-4E phosphorylation and cap binding analyses using Ser209-mutated eIF4Es. *Biochem. Biophys. Res. Commun* 1998;247:213–216. [PubMed: 9642105]
45. Blakley, RL. Dihydrofolate reductase, in “Folates and Pterins”. Blakley, RL.; Benkovic, SJ., editors. 1989. p. 191-253.
46. Vasilescu S, Ptushkina M, Linz B, Muller PP, McCarthy JE. Mutant of eukaryotic initiation factor eIF4E with altered mRNA cap binding specificity reprogram mRNA selection by ribosomes in *Saccharomyces Cerevisiae*. *J.Biol. Chem* 1996;271:7030–7037. [PubMed: 8636134]
47. Brown CJ, McNae I, Fischer PM, Walkinshaw MD. Crystallographic and mass spectrometric characterisation of eIF4E with N7-alkylated cap derivatives. *J. Mol. Biol* 2007;372:7–15. [PubMed: 17631896]

48. Rau M, Ohlmann T, Morley SJ, Pain VM. A reevaluation of the cap-binding protein, eIF4E, as a rate-limiting factor for initiation of translation in reticulocyte lysate. *J. Biol. Chem* 1996;271:8983–8990. [PubMed: 8621544]
49. Slepnev SV, Darzynkiewicz E, Rhoads RE. Stopped-flow kinetic analysis of eIF4E and phosphorylated eIF4E binding to cap analogs and capped oligoribonucleotides - Evidence for a one-step binding mechanism. *J. Biol. Chem* 2006;281:14927–14938. [PubMed: 16540463]
50. Calloway, MNTC, Sanz A, Sheetz MP, Miller LW, Cornish VW. Optimized fluorescent trimethoprim derivatives for in vivo protein labeling. *Chembiochem* 2007;8:767–774. [PubMed: 17378009]

A)

B)

Figure 1.

(A & B). Schematic diagram of plasmid construct pPH70D-eIF4E. (A) Construction of bacterial expression plasmid pPH70D-eIF4E. The pPH70D was digested with XhoI and XbaI to get the open reading frame without NAT2 (hamster polymorphic N-acetyltransferase). The cDNA of mouse-eIF4E, contain similarly digested 5' and 3' end and was sub-cloned to the linearized open frame vector through ligation resulting in expression plasmid pPH70D-eIF4E. (B) DNA and protein sequence of the eIF4E-DHFR fusion protein linker region. The recombinant eIF4E product following thrombin cleavage will contain the Gly-Thr-Leu-Glu peptide residues appended at the amino terminus of the eIF4E sequence.

Figure 2.

Coomassie blue-stained of 12% SDS-PAGE gel of DHFR-eIF4E purification. Lane 1: eIF4E-pPH70D cell lysate (without IPTG induction). Lane 2: eIF4E-pPH70D supernatant (with IPTG induction). Lane 3: eIF4E-pPH70D cell pellet (with IPTG induction). Lane 4: Molecular weight markers.

Figure 3.

Coomassie blue-stained 12% SDS-PAGE gel of fractions collected after DEAE anion exchange column: (lane 1) molecular weight markers, (lane 2) 2 μ g dialyzed protein (before elution), (lane 3–9) 20 μ g of eIF4E-DHFR fusion protein (fraction 52–58).

Figure 4.
DEAE anion exchange elution profile of eIF4E-DHFR after thrombin cleavage.

Figure 5.

Coomassie blue-stained of 12% SDS-PAGE gel of proteins present in different steps of purification of recombinant eIF4E. Lane 1: Marker; Lane 2 Dialyzed eIF4E-DHFR after MTX column (before thrombin cleavage); Lane 3–12: After thrombin cleavage (eIF4E + DHFR) followed by DEAE anion exchange column (fractions 30–33 showed only eIF4E), Fractions 46–51 showed only DHFR.

Figure 6. Plot of fluorescence quenching titration data on the association of recombinant eIF4E with increased concentration of 7Me-GTP at 22°C. (□), Experimental data point; (—), Single exponential fit.

Figure 7.

In vitro translational effects on endogenous addition of eIF4E and DHFR-eIF4E in rabbit reticulocyte cell extract.; ■ eIF4E; ▲ 7MeGTP+eIF4E; ◆ DHFR-eIF4E; ● 7MeGP + DHFR-eIF4E.

Table 1Purification of Fusion DHFR-eIF4E and Recombinant eIF4E^a

Purification Step	Protein (mg)	% Recovery
Cell lysate ^b	65	100
MTX column ^b	21	32.3
After DEAE-column ^b (DHFR-eIF4E)	4.2	6.5
After Thrombin/DEAE Column ^c (eIF4E)	2.5	3.8

^aThese results are obtained from 2L cell culture.^bBased on DHFR activity.^cBased on Bradford analysis.

Table 2Thermodynamic Dissociation Constant (K_d) for various eIF4E-cap Analogues at 22°C.^a

Cap-analogues	$K_d \times 10^9 (M)$		
	eIF4E	DHFR-eIF4E	Reported for eIF4E (Ref.)
7MeGTP	10(± 3)	6(± 5)	9.1 (59)
7MeGDP	45(± 10)	60(± 19)	49 (59), 300 ^b (50)
7MeGMP	7500(± 400)	10000(± 500)	1240 (59)
7BnGMP	800(± 200)	700(± 200)	7000 (47)

^a Measurements were based on fluorescence quenching titration data.^b Measurement was made with GST-eIF4E fusion protein.