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Effects of poly(A)-binding protein on the interactions of translation initiation factor eIF4F and eIF4F·4B with internal ribosome entry site (IRES) of tobacco etch virus RNA

Mateen A. Khan [a], Hasan Yumak [a], Daniel R. Gallie [b], Dixie J. Goss [a,*]

[a] Department of Chemistry, Hunter College and the Graduate Center of the City University of New York, New York, NY 10065, USA
[b] Department of Biochemistry, University of California, Riverside, CA 92521-0129, USA

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ABSTRACT

In wheat germ, the interaction between poly(A)-binding protein and eukaryotic initiation factor eIF 4G increases the affinity of eIF4E for the cap by 20–40-fold. Recent findings that wheat germ eIF4G is required for interaction with the IRES, pseudoknot 1 (PK1), of tobacco etch virus to promote cap-independent translation led us to investigate the effects of PABP on the interaction of eIF4F with PK1. The fluorescence anisotropy data showed addition of PABP to eIF4F increased the binding affinity ~2.0-fold for PK1 RNA as compared with eIF4F alone. Addition of both PABP and eIF4B to eIF4F enhance binding affinity to PK1 about 4-fold, showing an additive effect rather than the large increase in affinity shown for cap binding. The van't Hoff analyses showed that PK1 RNA binding to eIF4F, eIF4F·PABP, eIF4F·4B and eIF4F·4B·PABP is enthalpy-driven and entropy-favorable. PABP and eIF4B decreased the entropic contribution 65% for binding of PK1 RNA to eIF4F. The lowering of entropy for the formation of eIF4F·4B·PABP–PK1 complex suggested reduced hydrophobic interactions for complex formation. Overall, these results demonstrate the first direct effect of PABP on the interaction of eIF4F and eIF4F·4B with PK1 RNA.

1. Introduction

Initiation of protein synthesis in eukaryotes is a complex multi-step process, which requires the participation of at least eleven initiation factors. In most eukaryotes, mRNA is required to have both a 5′ cap (m^7GpppX) and a poly(A) tail for efficient translation and message stability [1–6]. These two elements act synergistically to increase translational efficiency, and evidence suggests that they communicate during translation [7–9]. The cap serves as the binding site for initiation factors eIF4F and eIFiso4F, an isozyme form of eIF4F present in higher plants. eIF4F interacts with the poly(A) binding protein (PABP) through the eIF4G subunit, the larger subunit of eIF4F. eIF4G in turn recruits other initiation factors, such as eIF3, eIF4A and eIF4B to the 5′ end of the mRNA, to generate the cap-binding complex. In the wheat germ system, the cap-associated proteins have a very high affinity for PABP in the absence of poly(A) [9] but require poly(A) in yeast [8]. Binding of eIF4F, eIF4B and eIF4A are believed to catalyze the efficient unwinding of secondary structure in the 5′-untranslated region [10]. In combination with PABPs, these factors promote the functional circularization of mRNA believed to be necessary for efficient translation [11–15].

PABP and eIF4G interaction is conserved among plants, animals, and yeast [8,9,16] and serves to stabilize the binding of eIF4F to the 5′-cap [17]. The interaction between PABP and eIF4G or eIF4B increases the poly(A) binding activity of PABP by 2-fold [9,18] and increases the binding affinity of eIF4F to the cap 40-fold [17]. eIF4G and eIF4B not only individually increase the binding affinity of PABP for poly(A) RNA but also together exert a synergistic effect on PABP activity [9], which indicates that the physical interaction between all three proteins serves to stabilize their association with their respective binding sites to increase their function during translation initiation.

eIF4B is essential to stimulate the ATPase and RNA helicase activity of eIF4A in mammalian systems [19–25] and increases the ATP affinity of eIF4A and the processivity of its helicase activity [26,27]. In contrast, wheat eIF4B only moderately stimulates but is not required for the ATP hydrolysis and RNA helicase activities of eIF4A [28–31]. The effects of eIF4B and PABP on formation of a cap-independent internal ribosome entry site initiation factor complex have not been demonstrated.

Tobacco etch virus (TEV) is a member of the picornavirus supergroup of positive strand RNA, which infects plants. The genomic RNA of TEV is polyadenylated and contains an internal ribosomal entry site (IRES) in the 143-nucleotide (nt) 5′-leader sequence. This leader sequence is sufficient to ensure efficient translation of the RNA. The 143-nt leader folds into a complex structure composed of two domains, each of which contains a pseudoknot (PK). PK1 (residues 28–77) is an H-type pseudoknot with two stems, S1 (6 bp) and S2 (5 bp). The PK1 of TEV 5′-leader is sufficient to confer cap-independent translation to the viral RNA [32–34]. It is functionally

Abbreviations: m^7G, 7-methyl-guanosine; eIF, eukaryotic initiation factor; PABP, poly(A)-binding protein; TEV, tobacco etch virus; IRES, internal ribosomal entry site; nt, nucleotide; PK, pseudoknot; PMSF, phenylmethylsulfonyl fluoride; DEAE, diethylaminoethyl

* Corresponding author. Tel.: +1 212 772 5383; fax: +1 212 772 5332.
E-mail address: dgoss@hunter.cuny.edu (D.J. Goss).

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analogous to a cap and interacts with the poly(A) tail to promote efficient translation [34]. eIF4F/eIFiso4F depleted wheat germ lysate [35] has shown that eIF4F is required for IRES-driven translation. The addition of eIF4F into the depleted lysate increased translation of TEV IRES up to 167-fold, whereas addition of eIFiso4F did not increase translation. The translational advantage conferred by the TEV IRES is lost when the concentration of eIF4F is no longer limiting, whereas TEV IRES recruits eIF4F when the factor is present in limiting amounts. These studies confirm that eIF4F alone is sufficient for the translation of TEV IRES. Further quantitative binding studies [36] showed that PK1 preferentially binds with eIF4F rather than eIFiso4F.

PABP has been shown to be necessary for binding of 40 S ribosomal subunits to mRNA and for formation of the 48 S initiation complex [8,37]. Interaction of PABP·eIFiso4F has been shown to enhance the binding of both cap analogs and poly(A) by about 40-fold [17]. Further studies have shown that the cap and poly(A)-tail can be bound by the protein complex simultaneously [17]. It was concluded that at least part of the enhancement of translation could be accounted for by increased binding affinity for the 5′-cap by the protein complex. We have previously investigated the mechanism of eIF4F and eIFiso4F interacting with TEV RNA [36]. eIF4F was shown to be required for efficient cap-independent translation. However, whether PABP has the same dramatic effect on the binding of PK1 RNA with eIF4F as it does for eIFiso4F and cap has not been established. In order to understand the assembly of the initiation complex, we analyzed the binding effects of PABP on the binding of PK1 RNA to eIF4F in the presence and absence of eIF4B.

2. Materials and methods

2.1. Materials

Fluorescein covalently attached to the 5′-end of pseudoknot (^{Fl}PK1) RNA was synthesized by Gene Link, Inc. Hawthorne, New York. The PK1 domain (nucleotide, Fl-CAAAACAAACGAAUCUCAAG-CAAUCAAGCAUUCUACUUCUAUUGCAGCAA) was used for RNA binding studies as described previously [38]. Wheat germ (partially crushed) was purchased from Bob's Red Mill, Natural Foods, Inc. (Milwaukie, Oregon, USA) and was stored at −20 °C until used. DEAE Cellulose was purchased from Whatman International Ltd. Maidstone England. Sephadex G-25 was purchased from Pharmacia Fine Chemicals Inc. Piscataway, N.J. The HiTrap SP, HiTrap Mono Q Column, and PreScission Protease were purchased from Amersham Pharmacia Biotech, Inc. m^{7}GTP-Sepharose-4B was purchased from Amersham Biosciences Co. Ltd. His-bind Ni-resin was purchased from Novagen an affiliate of Merck Co. Ltd. The concentration of ^{Fl}PK1 was determined spectrophotometrically using an absorption coefficient ε_{490} of 79,000 M^{-1} cm^{-1}.

2.2. Purification of eIF4F

Wheat germ eIF4F was isolated from the 0–40% ammonium sulfate fraction of the 120 mM KCl post-ribosomal supernatant fraction as described previously [39]. The active fractions were pooled and dialyzed. To further increase the purity of eIF4F the dialyzed sample was applied to 2 ml m^{7}GTP-sepharose-4B column and the eIF4F was eluted with 100 mM GTP. The purified fractions were pooled and concentrated in a Centricon-10 microconcentrator (Amicon Co.). Purity of protein was confirmed by running 7.5% SDS-polyacrylamide gel electrophoresis with Coomassie brilliant blue staining.

2.3. Expression and purification of PABP and eIF4B

PABP was expressed in *Escherichia coli* containing the constructed pET19b vector in BL21 (DE3) pLysS. Cells were cultured in LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37 °C. At 600 nm, optical density of 0.5, expression was induced for 5 h with 0.1 g/l isopropyl-1-thio-β-D-galactopyranoside, after which cells were harvested by centrifugation. Subsequent steps were performed at 4 °C. Cell pellets were resuspended into binding buffer (20 mM Tris–HCl, pH 7.9, 0.5 M NaCl, and 5 mM imidazole) containing 0.5 ml of aprotinin, 1.0 mM phenylmethylsulfonyl fluoride (PMSF) and 100 μg/ml soybean trypsin inhibitor, disrupted by sonication, and the lysate was centrifuged. Supernatant was applied to a 5 ml His-bind nickel column equilibrated with binding buffer. The column was washed with binding buffer, and the bound protein was eluted with elution buffer (20 mM Tris–HCl, pH 7.9, 0.5 M NaCl, and 200 mM imidazole). The protein was dialyzed against buffer (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, and 5% glycerol). The purity of the PABP was confirmed by 10% SDS-PAGE. eIF4B was expressed in *E. coli* containing the constructed pET3d vector in BL21 (DE3) pLysS as described elsewhere [40]. The purity of eIF4B was confirmed by 10% SDS-PAGE.

All protein samples were dialyzed against Titration Buffer (20 mM Tris–HCl, pH 7.6, 150 mM KCl, 2.0 mM $MgCl_2$, and 1.0 mM DTT) and passed through a 0.22 μM filter (Millipore) before the spectroscopy measurements were performed. The samples were concentrated with a Centricon 10 (Amicon Co.) as necessary. The concentrations of protein were determined by a Bradford assay with bovine serum albumin as standard [41] using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, CA).

2.4. Fluorescence anisotropy measurements

Fluorescence anisotropy measurements were performed using a Spex Fluorolog τ2 spectrofluorimeter equipped with excitation and emission polarizers. Anisotropy was measured using an L-format detection configuration. The protein–RNA (eIFs–PK1) interactions were studied by direct fluorescence anisotropy titration using a 10-mm path-length quartz cuvette. The fluorescein labeled pseudoknot (^{Fl}PK1) of TEV RNA (50 nM) was incubated with varying concentration of eIF4F, eIF4B, PABP, eIF4F·4B, eIF4F·PABP and eIF4F·4B·PABP complex (0–1.1 μM) in Titration Buffer. All samples were incubated at least 10 min before data were collected. The sample temperature was maintained at 25 °C for all experiments unless otherwise stated. Interaction of initiation factors (eIFs) with PK1 RNA was measured by the increase in anisotropy of ^{Fl}PK1 RNA emission. The anisotropy of each sample was measured by excitation with vertically polarized light at wavelength of 490 nm and the emission was measured at 519 nm in the horizontal and vertical directions. The excitation and emission slits were 4 nm and 5 nm, respectively. The anisotropy data was fitted to equation (1) to determine the dissociation equilibrium constant [42,43],

$$r_{obs} = r_{min} + \left\{(r_{max} - r_{min})/\left(2 \times [^{Fl}PK1]\right)\right\}\left\{b - \left(b^2 - 4[^{Fl}PK1][eIFs]\right)^{0.5}\right\} \quad (1)$$

where, $b = K_d + [^{Fl}PK1] + [eIFs]$, r_{obs} is the observed anisotropy for any point in the titration curve, r_{min} is the minimum observed anisotropy in the absence of protein, r_{max} is the maximum anisotropy at saturation and is fit as a parameter. [^{Fl}PK1] and [eIFs] are the PK1 RNA and protein concentrations (eIFs = eIF4F, eIF4B, PABP, eIF4F·4B, eIF4F·PABP and eIF4F·4B·PABP complex). K_d is the equilibrium dissociation constant. For all equilibrium measurements, three independent titration experiments were performed, and the average value was reported. Values for K_d determined from individual duplicate experiments varied by less than 20%.

The concentrations of protein and RNA were measured using an Ultrospec 1100 Pro UV-visible absorption spectrophotometer. Non-linear least squares fitting of the titration data was performed using KaleidaGraph software (version 2.1.3; Abelbeck Software). Uncertainties in K_d values are reported for one sigma as determined from data fitting.