Phosphorylation States of Translational Initiation Factors Affect mRNA Cap Binding in Wheat†

Mateen A. Khan and Dixie J. Goss*

Department of Chemistry, Hunter College and the Graduate Center of the City University of New York, New York 10021

Received February 26, 2004; Revised Manuscript Received May 6, 2004

ABSTRACT: Phosphorylation of eukaryotic translational initiation factors (eIFs) has been shown to be an important means of regulating protein synthesis. Plant initiation factors undergo phosphorylation/dephosphorylation under a variety of stress and growth conditions. We have shown that recombinant wheat cap-binding protein, eIF(iso)4E, produced from *E. coli* can be phosphorylated in vitro. Phosphorylation of eIF(iso)4E has effects on m^7G cap-binding affinity similar to those of phosphorylation of mammalian eIF4E even though eIF(iso)4E lacks an amino acid that can be phosphorylated at the residue corresponding to Ser-209, the phosphorylation site in mammalian eIF4E. The cap-binding affinity was reduced 1.2−2.6-fold when eIF(iso)4E was phosphorylated. The in vitro phosphorylation site for wheat eIF(iso)4E was identified as Ser-207. Addition of eIF(iso)4G and eIF4B that had also been phosphorylated in vitro further reduced cap-binding affinity. Temperature-dependent studies showed that $\Delta H°$ was favorable for cap binding regardless of the phosphorylation state of the initiation factors. The entropy, however, was unfavorable (negative) except when eIF(iso)4E was phosphorylated and interacting with eIF(iso)4G. Phosphorylation may modulate not only cap-binding activity, but other functions of eukaryotic initiation factors as well.

Eukaryotic mRNAs have a common cap structure [$m^7G(5')ppp(5')N$, where N is any nucleotide] at the 5′-terminus which plays an important role in stabilizing the mRNA structure and facilitating mRNA binding to the ribosome during initiation (*1*). To allow the efficient translation of mRNA, an interaction is required between the cap structure and eukaryotic initiation factor (eIF)4F,[1] consisting of eIF4G, eIF4E, the cap-binding protein, (*2*) and sometimes eIF4A depending on the preparation. In plants, a second cap-binding protein, eIF(iso)4F, is also present. eIF(iso)4F consists of eIF(iso)4E, a 28 kDa cap-binding protein, and eIF(iso)4G, an 86 kDa polypeptide (*3*). In mammalian cells, it has been indicated that cap-dependent translation is regulated through the interaction of eIF4E with endogenous 4E-binding proteins (4EBP1, 4EBP2, and 4EBP3) and the interrelated phosphorylation of eIF4E and 4EBPs. Thus far, eIF4E-binding proteins have not been identified in plants. eIF4G, the large subunit of eIF4F, serves as a platform for the interaction and assembly of multiple initiation factors, including eIF4E, eIF4A (whose RNA helicase and ATPase activities are required for unwinding RNA secondary structure), and eIF3 (responsible for 40 S ribosomal subunit recruitment) (*4*−*6*).

In eukaryotic protein biosynthesis, the binding of mRNA to the small (40 S) ribosomal subunit is the rate-limiting step and is a key target for regulation (*7*−*11*). The presence of cap structure on eukaryotic mRNA facilitates the recruitment of translational initiation factors to allow ribosome binding and initiation at the correct start site (*2*, *12*−*15*). eIF4E interacts directly with the cap via its concave surface (*16*, *17*) and forms a complex with the scaffold protein, eIF4G (*18*), on its convex surface. eIF4G in turn recruits other initiation factors, such as eIF3, eIF4A, and poly(A)-binding protein (PABP) to the 5′ end of the mRNA, to generate the cap-binding complex. This is believed to promote the efficient unwinding of secondary structure in the 5′-untranslated region (*19*) and the functional circularization of mRNA believed to be necessary to promote efficient translation (*2*, *14*, *15*, *20*, *21*).

The functional interaction between the cap and poly(A) tail is repressed following serum starvation in mammalian cells but can be reversed by exposure of the cells to insulin (*22*). The addition of either insulin or serum promotes rapid phosphorylation of eIF4E, eIF4G, eIF4B (*23*−*25*), and the eIF4E-binding protein (i.e., 4E-BP or PHAS-I). This phosphorylation of 4E-BP or PHAS-I results in its release from eIF4E (*26*, *27*), and promotes the association between eIF4G and PABP (*28*). In plants, PABP and several of the initiation factors are known to be phosphorylated. As in yeast and sea urchin (*29*), plant PABP is present as multiple phosphorylated species, which are dephosphorylated by alkaline phosphatase, confirming the nature of the modification (*30*). Plant eIF4B

† This work was supported in part by National Science Foundation Grant MCB 0076344 (to D.J.G.) and a Professional Staff Congress City University of New York faculty award (to D.J.G.). Center in Minority Institution Award RR-03037 from the National Center for Research Resources of the National Institutes of Health supports the infrastructure at Hunter College.

* To whom correspondence should be addressed. Phone: (212) 772-5383. Fax: (212) 772-5332. E-mail: dgoss@hunter.cuny.edu.

[1] Abbreviations: eIF, eukaryotic initiation factor; kDa, kilodalton(s); 4E-BP, eIF4E-binding protein; PABP, poly(A)-binding protein; TFE, trifluoroethanol; Ant-m^7GTP, anthraniloyl 7-methylguanosine triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

10.1021/bi049602b CCC: $27.50
Published on Web 06/19/2004

is also present as multiple phosphorylated species, and its phosphorylation state is regulated during development or following heat stress (*30*). eIF4B undergoes dephosphorylation following phosphatase treatment, and the array of phosphorylated eIF4B species observed in vitro can be recapitulated by phosphorylation of nonphosphorylated eIF4B by casein kinase II (*30*).

eIF4A has been shown to be phosphorylated following environmental stress such as hypoxia or heat shock (*30*, *31*). Other plant initiation factors subject to phosphorylation include eIF2α, eIF2β, eIF4E, and eIF(iso)4G (*30*, *32*). These observations raise the possibility that the phosphorylation state of these proteins mediates the functional interaction between the cap and the poly(A) tail; i.e., eIF(iso)4E, eIF(iso)4G, and eIF4B might determine the extent of their interaction with m^{7}GTP or with each other. To investigate whether the phosphorylation state of these proteins influences binding activities with one another and with m^{7}GTP, direct fluorescence measurements were used.

EXPERIMENTAL PROCEDURES

Materials. m^7-GTP and trifluoroethanol (TFE) were purchased from Sigma (St. Louis, MO). Sequencing grade modified trypsin was from Promega. Ant-m^{7}GTP was synthesized and purified as described previously (*33*). The concentration of Ant-m^{7}GTP was determined spectrophotometrically using an absorption coefficient of $\epsilon_{332} = 4600\ M^{-1}\ cm^{-1}$.

Purification of Proteins. eIF(iso)4E, eIF(iso)4G, and eIF4B were expressed in *Escherichia coli* containing the constructed pET3d vector in BL21(DE3)pLys as described elsewhere (*34*). A HiTrap Mono-Q ion exchange column and an m^7-GTP-Sepharose 4B affinity column (Pharmacia Biotech. Inc) were used for the purification of eIF(iso)4E. The bound protein was eluted with 10 mM GTP in buffer B-50. A HiTrap SP column from Amersham Pharmacia Biotech was used to purify eIF(iso)4G and eIF4B by the following procedure. *E. coli* cells were disrupted by alumina, suspended in buffer B (20 mM HEPES/KOH, pH 7.6, 0.1 mM EDTA, 1.0 mM DTT, and 10% glycerol) containing 600 mM KCl (B-600), and centrifuged at 45000 rpm for 2 h. The supernatant was diluted with B-0 to a final concentration of 50 mM KCl, loaded onto a 2 $\times$ 5 mL HiTrap SP column, and washed by B-50 buffer until the optical density returned to the baseline. A 50−400 mM KCl linear gradient (total volume 100 mL) was used to elute the proteins, and 1.0 mL fractions were collected. The proteins appeared in the 200−300 mM KCl fractions. After purification by gel filtration, the purity was confirmed by 10% SDS−gel electrophoresis. All steps were carried out in a cold box at approximately 5 °C. The pH of buffer B used for eIF(iso)4E and eIF(iso)4G purification was 7.0; for eIF4B it was 7.5. All samples were dialyzed against buffer B (20 mM HEPES/KOH, 100 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM dithiothreitol, 1.0 mM EDTA, pH 7.6) and passed through a 0.22 μM filter (Millipore) before the spectroscopy measurements were performed. The fractions were concentrated with a Centricon 10 (Amicon Co.) as necessary. Protein concentrations were estimated by the method of Bradford (*35*) using a Bio-Rad protein assay reagent (Bio-Rad Laboratories, California).

Phosphorylation of eIFs. The recombinant eIF(iso)4E, eIF(iso)4G, and eIF4B were phosphorylated by casein kinase II (*31*) in vitro in a reaction mixture containing 50 mM Tris−HCl, pH 7.0, 100 mM NaCl, 10 mM $MgCl_2$, 200 μM ATP, 1.0 mM EDTA, 1.0 mM DTT, and 50 U of casein kinase II. The reaction was incubated for 3 h at 30 °C with stirring. The samples were assayed by nondenaturing gel electrophoresis.

Protease Digestion. The protein was digested in a solution of 50% TFE and 30 mM ammonium bicarbonate buffer, pH $\approx$ 8, in the presence of 10 mM DTT. Trypsin solution was prepared as directed by the supplier and added to the protein mixture in a molar ratio of 1:30 (w/w) protease:protein. The reaction was incubated overnight in a water bath at 37 °C. The digest mixture was sampled and analyzed by mass spectrometry.

Mass Spectrometry. Mass spectrometric analysis was performed with an HPLC−ESI/MS ion trap mass spectrometer (Agilent model 1100 LC/MSD). The protein digestions were separated on a 300 SB-C8 column (2.1 $\times$ 150 mm) at a flow rate of 400 μL/min, eluting with a linear gradient of 0−100% solvent B over 45 min (solvent A, 0.1% formic acid in water; solvent B, 0.1% formic acid in acetonitrile). Peptide separation was performed at room temperature with a fast-rising methanol gradient. The elutant was transferred through a 50 mm inside diameter fused silica capillary from the column to the ion source of the mass spectrometer and electrosprayed at 4.5−5.0 kV. Data were analyzed by a computer running operating system HP'S G1710 AA Chemstation software. Use of this software was accomplished using a utility provided by CSS Analytical Co., Inc.

Fluorescence Titration Measurements. Fluorescence measurements were performed using a Spex Fluorolog τ2 spectrofluorimeter equipped with excitation and emission polarizers. The excitation and emission slits were set at 4 and 5 nm, respectively. The excitation wavelength for Ant-m^{7}GTP was 332 nm, and emission was monitored at 420 nm. The excitation slits were chosen to avoid photobleaching, and the absorbance of the sample at the excitation wavelength was less than 0.02 to minimize the inner-filter effect. Emission spectra were corrected for the wavelength-dependent lamp intensity and monochromator sensitivities. Steady-state fluorescence anisotropy was measured using an L-format detection configuration. All samples were incubated at least 10 min before data were collected.

The samples were thermostated at different temperatures, i.e., 4, 10, 15, 22, and 31 $\pm$ 0.3 °C, controlled with a thermocouple inside the cuvette. Titrations were performed in 20 mM HEPES/KOH, pH 7.6, 100 mM KCl, 1.0 mM $MgCl_2$, and 1.0 mM DTT. Fluorescence intensities, when necessary, were corrected for dilution and for the inner-filter effect. In a mixture of eIFs and Ant-m^{7}GTP, the average anisotropy is related to the fraction of the total fluorophore that is bound as described previously (*36*, *37*), and the association equilibrium constants were obtained by fitting the titration data to the following equation:

$$Y = ((K_{eq}[cap^*]_T + K_{eq}[eIF]_T + 1) - ((K_{eq}[cap^*]_T + K_{eq}[eIF]_T + 1)^2 - (4K_{eq}{}^2[cap^*]_T[eIF]_T))^{0.5})/2K_{eq}[cap^*]_T \quad (1)$$

where $[cap^*]_T$, $[eIF]_T$, and K_{eq} are the total concentrations of Ant-m^{7}GTP and protein and the association equilibrium

```
                1                                                   *   *
Human 4E  MATVEPETTP TPNPPT - - - T EEEKTESNQE VANPEHYIKH PLQNRWALWF
Mouse 4E  MATVEPETTP TTNPPP - - - A EEEKTESNQE VANPEHYIKH PLQNRWALWF
Wheat 4E  MAEDTETRPA SAGAEEREEG EIADDGDGSS AAAAGRITAH PLENAWTFWF
Wheat iso4E MAEVEAALPV AATETP - - - - EVAAEGDAGA AEAKG---PH KLQRQWTFWY
Yeast 4E  MQTEQPPKES QTENTVS - -  E PQEKALRTVF DDKINFNLKH PLARPWTLWF

               51              *                   *
Human 4E  FKNDKSK - - - TWQANLRLIS KFDTVEDFWA LYNHIQLSSN LMPGCDYSLF
Mouse 4E  FKNDKSK - - - TWQANLRLIS KFDTVEDFWA LYNHIQLSSN LMPGCDYSLF
Wheat 4E  DNPQGKSRQVAWGSTIHPIH  TFSTVEDFWG LYNNIHNPSK LNVGADFHCF
Wheat iso4E DIQTKPKPGA  AWGTSLKKGY TFDTVEEFWC LYDQIFRPSK LVGSADFHLF
Yeast 4E  LMPPTPG - - L  EWNELQKNII  TFNSVEEFWG IHNNINPASS L P I KSDYSFF

              101         *                 *                   *
Human 4E  KDGIEPMWED EKNKRGGRWL  ITLNKQQRRS DLNRFWLETL LCLIGESFDD
Mouse 4E  KDGIEPMWED EKNKRGGRWL  ITLNKQQRRS DLDRFWLETL LCLIGESFDD
Wheat 4E  KNKIEPKWED  PICANGGKWT  ISCGR - - - - G KSDTFWLHTL LAMIGEQFDF
Wheat iso4E  KAGVEPKWED PECANGGKWT VISSRK - - - T NLDTMWLETC MALIGEQFDE
Yeast 4E  REGVRPEWED VHNKTGGKWA FQNKGRGG-NALDEMWLTTV LAAIGETLDP

             151                *
Human 4E   YSDDVCGAVV NVRAKGDKIA IWTTECENRE AVTHIGRVYK ERLGLPPKIV
Mouse 4E   YSDDVCGAVV NVRAKGDKIA IWTTECENRD AVTHIGRVYK ERLGLPPKIV
Wheat 4E   - GDEICGAVV SVRQKQERVA IWTKNAANEA AQISIGKQWK  EFLDYKDSIG
Wheat iso4E  -SQEICGVVA SVRQRQDKLS  LWTKTASNEA VQVDIGKKWKEVIDYNDKMV
Yeast 4E   TGQEVMGVVI NMRKGFYRLA VWTKSCNNRE VLMEIGTRFKQVLNLPRSET

              201
Human 4E  IGYQSHADTA TKSGSTTKNR FVV
Mouse 4E  IGYQSHADTA TKSGSTTKNR FVV
Wheat 4E  FIVHEDAKRS DKG - - - PKNR YTV
Wheat iso4E  YSFHDDSRSQ KPS - - - RGGR YTV
Yeast 4E  IEFSAHEDSS  KSGSTRAKTR MSV
```

FIGURE 1: Sequence comparison of the amino acid sequences for cap-binding proteins from human eIF4E (accession number AAH12611), mouse eIF4E (accession number AAH10759), wheat eIF4E (accession number CAA78262), wheat eIF(iso)4E (accession number Q03389), and yeast eIF4E (accession number T43287). Amino acid sequences were aligned using vector NTI. The conserved tryptophan residues are marked with an asterisk. The position of the phosphorylated Ser-207 in wheat eIF(iso)4E is bold.

constant, respectively. Nonlinear least-squares fitting of the data was performed using KaleidaGraph software (version 2.1.3, Abelbeck Software).

Thermodynamic Parameters for eIF Binding to Ant-m^{7}GTP. The temperature dependence of K_{eq} for Ant-m^{7}GTP was analyzed according to the van't Hoff isobaric equation, assuming the entropy change, $\Delta S°$, and the enthalpy change, $\Delta H°$, as constants over the range of temperatures studied:

$$-RT \ln K_{eq} = \Delta H° - T\Delta S° \quad (2)$$

RESULTS

Mass spectrometry was used to determine the phosphorylation site of wheat eIF(iso)4E. Analysis of the intact eIF(iso)4E by mass spectrometry showed an increase in mass corresponding to the addition of one phosphate residue. EST database inspection revealed that wheat, human, mouse, and yeast eIF4E showed the presence of eight tryptophan residues in the same relative positions (Figure 1). Tryptic peptides were prepared from a highly purified preparation of wheat eIF(iso)4E. To identify the location of the phosphoserine residue in wheat eIF(iso)4E, the protein was subjected to trypsin digestion followed by HPLC separation. The masses of the peptides were obtained by mass spectrometry. The peptide sequences were compared with those of the GeneBank database to find potential matches. Two separate experiments were performed, and in both cases, one peak of higher molecular mass (79.9 amu) corresponding to the peptide ^{199}MVYSFHDDSR^{208} was observed (Figure 2). The molecular mass expected for the fragment on the basis of the amino acid sequence is 1255.5 Da, and the addition

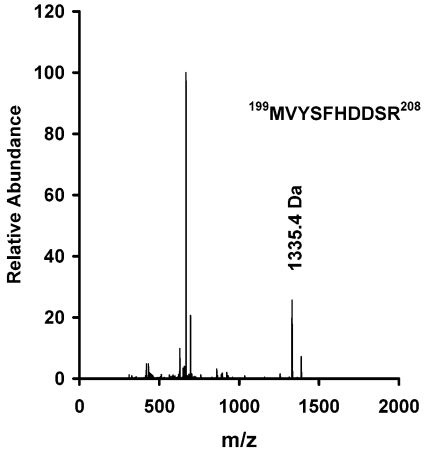

FIGURE 2: HPLC−ESI/MS spectrum of the tryptic digest peptides of wheat eIF(iso)4E. Protein was phosphorylated by casein kinase II and digested by trypsin. The digest was separated by reversed-phase HPLC coupled to the mass spectrometer. The spectrum was recorded by selecting the parent ion and scanning from 300 to 2500 mass units.

of one phosphate increases the expected mass to 1335.4 Da as was observed. Ser-207 represents a casein kinase II consensus site (*38*) and is the in vitro phosphorylation site for eIF(iso)4E.

To investigate the effects of the phosphorylation state on eIF function, we examined whether in vitro changes in the phosphorylation states of these factors alter the interactions between these proteins or the binding of eIF(iso)4E to the fluorescent cap analogue, Ant-m^{7}GTP. When untreated eIF(iso)4E was compared with in vitro phosphorylated eIF(iso)4E, the phosphorylated factor showed about 2.6-fold lower binding affinity for Ant-m^{7}GTP (K_d = 27.4 nM) than the native eIF(iso)4E (K_d = 10.5 nM). The results of titrations are shown in Tables 1 and 2. These affinity differences were temperature dependent. The van't Hoff plots (Figures 3 and 4) showed that phosphorylation of eIF(iso)4E leads to a less favorable enthalpy and more favorable entropy. These results suggest that the electrostatic effects of the phosphate group may be important for binding. Addition of eIF(iso)4G to the eIF(iso)4E increased the binding affinity with Ant-m^{7}GTP for both phosphorylated and nonphosphorylated eIF(iso)4E. In the eIF(iso)4E−eIF(iso)4G complex, phosphorylation of eIF(iso)4G had nearly the same effect as nonphosphorylated eIF(iso)4G; that is, the binding was increased slightly in a temperature-dependent manner. When both eIF(iso)4E and eIF(iso)4G were phosphorylated, the dissociation (K_d = 28.3 nM) increased about 3-fold as compared to that of both nonphosphorylated factors (K_d = 9.2 nM). Further, the interaction of phosphorylated eIF(iso)4G with phosphorylated eIF(iso)4E reduced binding rather than enhanced binding.

Wheat germ eIF4B has been shown to have different phosphorylation states under varying growth conditions. It has been shown that casein kinase II will produce the same electrophoretic species of eIF4B as found in wheat leaves (*30*). To determine the functional effects of eIF4B phosphorylation, cap binding in the presence of phosphorylated and nonphosphorylated eIF4B was determined. Similar to eIF(iso)4G, eIF4B enhanced the binding of eIF(iso)4E to Ant-m^{7}GTP when eIF(iso)4G was present. Addition of eIF(iso)4G/eIF4B to eIF(iso)4E increases the binding affinity

Table 1: Equilibrium Dissociation Constants[a] for the Interaction of Phosphorylated and Nonphosphorylated Initiation Factor Complexes with Ant-m^{7}GTP, Determined by Fluorescence Titration

complex[b]	K_d (nM) at 4 °C	K_d (nM) at 10 °C	K_d (nM) at 15 °C	K_d (nM) at 22 °C	K_d (nM) at 31 °C	$\Delta H°$ (kJ/ mol)	$\Delta S°$ (J/mol)
eIFiso4E	10.5 ± 0.29	20.95 ± 0.66	33.7 ± 0.46	56.69 ± 0.60	112.2 ± 1.08	−64.66 ± 0.27	−80.92 ± 0.94
eIFiso4E*	27.4 ± 0.75	39.2 ± 0.91	50.6 ± 0.98	85.2 ± 0.15	130.0 ± 0.16	−44.71 ± 0.24	−16.18 ± 0.82
eIFiso4E− eIFiso4G	9.2 ± 0.22	16.34 ± 0.35	23.02 ± 0.29	36.9 ± 0.49	76.6 ± 0.92	−53.99 ± 0.19	−41.05 ± 0.66
eIFiso4E− eIFiso4G*	9.5 ± 0.37	16.7 ± 0.47	26.18 ± 0.57	37.01 ± 0.77	80.5 ± 0.46	−55.36 ± 0.18	−46.37 ± 0.63
eIFiso4E*− eIFiso4G	22.7 ± 0.38	31.19 ± 0.35	39.6 ± 0.43	63.4 ± 0.72	95.5 ± 1.40	−38.04 ± 0.11	9.17 ± 0.37
eIFiso4E*− eIFiso4G*	28.3 ± 0.41	37.48 ± 0.44	47.64 ± 0.63	69.3 ± 0.23	98.3 ± 0.4	−33.06 ± 0.10	25.31 ± 0.36

[a] All dissociation constants were obtained by fitting the fluorescence titration data as described in the text. [b] An asterisk indicates the phosphorylated form of the initiation factors.

Table 2: Equilibrium Dissociation Constants[a] for the Interaction of Phosphorylated and Nonphosphorylated Initiation Factor Complexes with Ant-m^{7}GTP, Determined by Fluorescence Titration

complex[b]	K_d (nM) at 4 °C	K_d (nM) at 10 °C	K_d (nM) at 15 °C	K_d (nM) at 22 °C	K_d (nM) at 31 °C	$\Delta H°$ (kJ/mol)	$\Delta S°$ (J/mol)
eIFiso4E− eIFiso4G−eIF4B	6.64 ± 0.28	11.39 ± 0.24	18.04 ± 0.38	29.03 ± 0.55	61.5 ± 0.34	−57.27 ± 0.15	−50.19 ± 0.51
eIFiso4E− eIFiso4G−eIF4B*	8.0 ± 0.43	13.6 ± 0.23	22.67 ± 0.26	35.03 ± 0.89	75.02 ± 0.69	−57.44 ± 0.23	−52.39 ± 0.79
eIFiso4E− eIFiso4G*−eIF4B*	9.2 ± 0.44	16.2 ± 0.29	26.70 ± 0.27	45.40 ± 0.31	86.70 ± 0.72	−58.20 ± 0.20	−56.49 ± 0.69
eIFiso4E*− eIFiso4G*−eIF4B*	30.70 ± 0.99	39.51 ± 0.47	49.6 ± 0.71	63.7 ± 0.93	93.5 ± 1.1	−28.87 ± 0.08	39.72 ± 0.27

[a] All dissociation constants were obtained by fitting the fluorescence titration data as described in the text. [b] An asterisk indicates the phosphorylated form of the initiation factors.

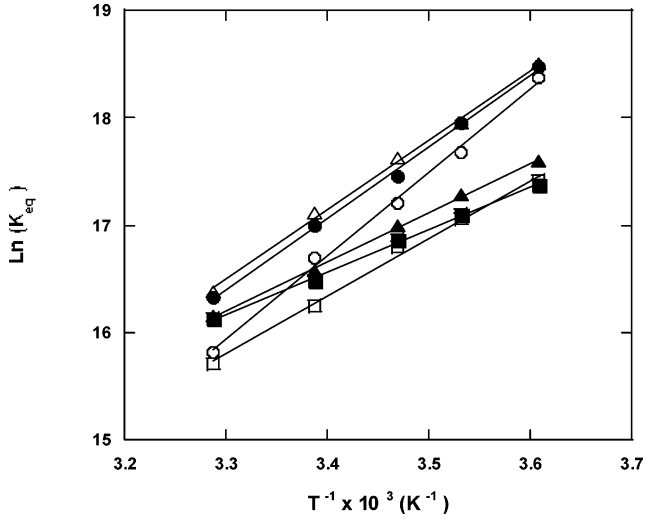

FIGURE 3: van't Hoff plots for the interaction of Ant-m^{7}GTP with (○) eIFiso4E, (□) eIFiso4E*, (△) eIFiso4E + eIFiso4G, (●) eIFiso4E + eIFiso4G*, (▲) eIFiso4E* + eIFiso4G, and (■) eIFiso4E* + eIFiso4G*. All the experimental conditions were the same as described in the text. An asterisk indicates the phosphorylated form of the initiation factors.

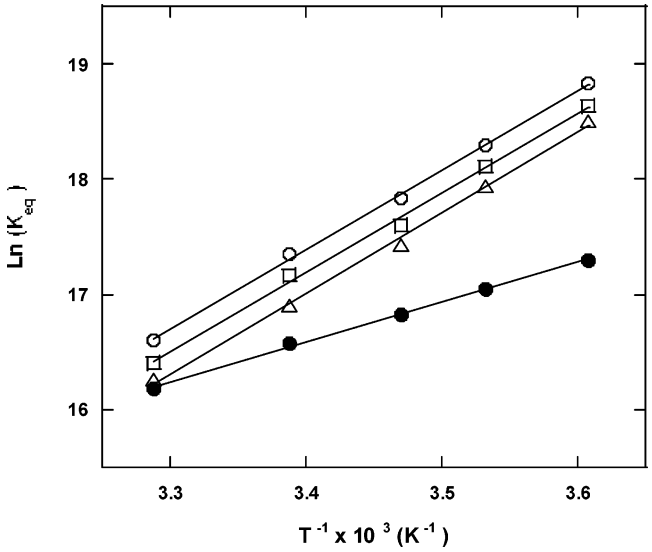

FIGURE 4: van't Hoff plots for the interaction of Ant-m^{7}GTP with (○) eIFiso4E + eIFiso4G + eIFiso4B, (□) eIFiso4E + eIFiso4G + eIF4B*, (△) eIFiso4E + eIFiso4G* + eIFiso4B*, and (●) eIFiso4E* + eIFiso4G* + eIF4B*. All the experimental conditions were the same as described in the text. An asterisk indicates the phosphorylated form of the initiation factors.

up to 1.5 times for Ant-m^{7}GTP as compared to eIF(iso)4E alone. Phosphorylation of eIF4B reduced the effect, but did not completely abolish it. However, when eIF(iso)4G was also phosphorylated, the effect of eIF4B was negligible. The thermodynamic parameters for cap binding to the respective complexes were determined from van't Hoff plots and are shown in Tables 1 and 2. The $\Delta H°$ value is obtained by least-squares fitting from the slope of the plot of ln K_{eq} vs $1/T$, which gave a straight line (correlation coefficient of >0.99) for each of the complexes. The strong specific interaction for eIF(iso)4E (nonphosphorylated and phosphorylated) with Ant-m^{7}GTP has a high enthalpy of association, $\Delta H° = -64.65$ kJ/mol for eIF(iso)4E and −44.71 kJ/mol for eIF(iso)4E (phosphorylated). This compares with the enthalpy of association for murine eIF4E of −74.31 kJ/mol (*39*). Examination of the van't Hoff plots for the interactions between protein complexes containing eIF(iso)4E and Ant-m^{7}GTP shows that they are also enthalpy driven in the range of temperatures studied. However, the binding is entropy-opposed with the resultant $\Delta S° = -80.92$ J/mol for eIF(iso)4E (nonphosphorylated) and −16.18 J/mol for phosphorylated eIF(iso)4E. The entropy of association for murine eIF4E was −98.7 J/mol (*39*).

DISCUSSION

Phosphorylation of eukaryotic initiation factors has been shown to be an important means of regulating protein synthesis. Plant initiation factors have been identified that undergo changes in phosphorylation state as the result of

stress, viral infection, and other environmental conditions (*30*). It has not been clear whether both of the plant cap-binding proteins eIF4E and eIF(iso)4E undergo phosphorylation, nor is it clear what the functional consequences of such modifications might be. Because of the difficulty in isolating large quantities of these proteins from wheat sprouts for biophysical studies, we examined the effects of in vitro phosphorylation. Both eIF4E and eIF(iso)4E contain many of the conserved residues shown to be involved in cap binding (*40*, *41*). However, neither protein contains a phosphorylatable amino acid at the position of Ser-209 in mammals. Treatment of eIF(iso)4E with casein kinase II resulted in no observable change in the isoelectric focusing pattern. However, the functional effects of treatment were very similar to those observed for mammalian eIF4E (*42*, *43*). The in vitro phosphorylation site has been identified as Ser-207. Even though phosphorylation of mammalian eIF4E decreases cap binding, it increases protein synthesis (*42*, *43*). Preliminary data[2] indicate that a similar effect occurs in the wheat germ translation system. The decrease in cap binding associated with eIF(iso)4E phosphorylation suggests that this is a general mechanism of regulation used in all eukaryotes. Phosphorylation of eIF(iso)4G showed a greater effect on binding of cap, in this case reducing affinity, when eIF(iso)4E was phosphorylated. This is in contrast to the enhancement of binding observed for nonphosphorylated eIF(iso)4E.

eIF4B has been shown to be phosphorylated in wheat leaves (*30*), and the isoforms could be reproduced by treatment with casein kinase II (*30*). eIF4B, together with eIF4A and eIF4F, is an RNA-dependent RNA helicase that unwinds secondary structure and enhances mRNA translation. It has been shown that the interaction of eIF4B with poly(A)-binding protein depends on the phosphorylation state of the factors. Since poly(A)-binding protein enhances cap binding, this suggested that eIF4B or a phosphorylated form of eIF4B may also enhance cap binding. This proves to be the case when the factors are not phosphorylated, but there is a decrease in binding when eIF(iso)4E and eIF4B are phosphorylated. Further investigations of the helicase activity and effects with poly(A)-binding protein are in progress.

The temperature dependence of these reactions suggests that not only do plants respond to thermal stress by altering the phosphorylation states of the proteins, but the interactions themselves (K_d) are altered by as much as 10-fold over the temperature range studied. These differences may underlie the selection of mRNA under a variety of stress conditions. Plants are unable to avoid environmental changes and therefore must use a variety of methods to minimize deleterious effects.

Cellular and subcellular changes in pH and developmental stages of plants will alter selection of mRNA and cap-binding activity through phosphorylation of eukaryotic initiation factors and conformational changes. The selection mechanism for mRNA is likely to depend not only on cap-binding affinity but also on protein−protein interactions and helicase activity. A reduction in cap-binding affinity is likely to reduce cap dependence of translation, and changes in helicase activity are likely to influence selection of mRNA depending on secondary structure in the noncoding region. Modulation of not only cap-binding activity but also other functions of eukaryotic initiation factors may be an important regulator of mRNA selection and translation.

[2] Goss, D. J. Unpublished observations.

REFERENCES

1. Reddy, R., Singh, R., and Shimba, S. (1992) Methylated cap structures in eukaryotic RNAs: structure, synthesis and function, *Pharmacol. Ther. 54*, 249−267.
2. Gingras, A. C., Raught, B., and Sonenberg, N. (1999) eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation, *Annu. Rev. Biochem. 68*, 913−963.
3. Lax, S., Fritz, W., Browning, K. S., and Ravel, J. (1985) Isolation and characterization of factors from wheat germ that exhibit eukaryotic initiation factor 4B activity and overcome 7-methylguanosine 5′-triphosphate inhibition of polypeptide synthesis, *Proc. Natl. Acad. Sci. U.S.A. 82*, 330−333.
4. Lamphear, B. J., Kirchweger, R., Skern, T., and Rhoads, R. E. (1995) Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. Implications for cap-dependent and cap-independent translational initiation, *J. Biol. Chem. 270*, 21975−21983.
5. Metz, A. M., and Browning, K. S. (1996) Mutational analysis of the functional domains of the large subunit of the isozyme form of wheat initiation factor eIF4F, *J. Biol. Chem. 271*, 31033−31036.
6. Imataka, H., and Sonenberg, N. (1997) Human eukaryotic translation initiation factor 4G (eIF4G) possesses two separate and independent binding sites for eIF4A, *Mol. Cell. Biol. 17*, 6940−6947.
7. Jagus, R., Anderson, W. F., and Safer, B. (1981) The regulation of initiation of mammalian protein synthesis, *Prog. Nucleic Acids Res. Mol. Biol. 25*, 127−185.
8. Hershey, J. W. B. (1991) Translational control in mammalian cells, *Annu. Rev. Biochem. 60*, 717−755.
9. Mathews, M. B., Sonenberg, N., and Hershey, J. W. B. (1996) in *Translational Control* (Hershey, J. W. B., Mathews, M. B., and Sonenberg, N., Eds.) pp 1−29, Cold Spring Harbor Laboratory Press, Plainview, NY.
10. Standart, N., and Jackson, R. J. (1994) Regulation of translation by specific protein/mRNA interactions, *Biochimie 76*, 867−879.
11. Bi, X., and Goss, D. J. (2000) Wheat germ poly(A)-binding protein increases the ATPase and the RNA helicase activity of translation initiation factors eIF4A, eIF4B, and eIF-iso4F, *J. Biol. Chem. 275*, 17740−17746.
12. Rhoads, R. E. (1999) Signal transduction pathways that regulate eukaryotic protein synthesis, *J. Biol. Chem. 274*, 30337−30340.
13. Raught, B., Gingras, A. C., and Sonenberg, N. (2000) Regulation of ribosomal recruitment in eukaryotes, in *Translational control of gene expression* (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., Eds.) pp 245−294, Cold Spring Harbor Laboratory Press, Plainview, NY.
14. Hershey, J. W. B., and Merrick, W. C. (2000) The pathway and mechanism of initiation of protein synthesis, in *Translational control of gene expression* (Sonenberg, N., Hershey, J. W. B., and Mathews, M. B., Eds.) pp 33−88, Cold Spring Harbor Laboratory Press, Plainview, NY.
15. Morley, S. J. (2001) The regulation of eIF4F during cell growth and cell death, in *Signaling pathways for translation* (Rhoads, R. E., Ed.) pp 1−37, Springer-Verlag, Berlin.
16. Marcotrigiano, J., Gingras, A. C., Sonenberg, N., and Burley, S. K. (1997) Cocrystal structure of the messenger RNA 5′ cap-binding protein (eIF4E) bound to 7-methyl-GDP, *Cell 89*, 951−961.
17. Tomoo, K., Shen, X., Okabe, K., Nozoe, Y., Fukuhara, S., Morino, S., et al. (2002) Crystal structures of 7-methylguanosine 5′-triphosphate (m(7)GTP)- and P(1)-7-methylguanosine-P(3)-adenosine-5′,5′-triphosphate (m(7)GpppA)-bound human full-length eukaryotic initiation factor 4E: biological importance of the C-terminal flexible region, *Biochem. J. 362*, 539−544.
18. Morley, S. J., Curtis, P. S., and Pain, V. M. (1997) eIF4G: translation's mystery factor begins to yield its secrets, *RNA 3*, 1085−1104.
19. Merrick, W. C. (1992) Mechanism and regulation of eukaryotic protein synthesis, *Microbiol. Rev. 56*, 291−315.
20. Gallie, D. R. (1998) A tale of two termini: a functional interaction between the termini of an mRNA is a prerequisite for efficient translation initiation, *Gene 17*, 1−11.

21. Wells, S. E., Hillner, P. E., Vale, R. D., and Sachs, A. B. (1998) Circularization of mRNA by eukaryotic translation initiation factors, *Mol. Cell 2*, 135−140.
22. Gallie, D. R., and Traugh, J. A. (1994) Serum and insulin regulate cap function in 3T3-L1 cells, *J. Biol. Chem. 269*, 7174−7179.
23. Frederickson, R. M., Montine, K. S., and Sonenberg, N. (1991) Phosphorylation of eukaryotic translation initiation factor 4E is increased in Src-transformed cell lines, *Mol. Cell. Biol. 11*, 2896−2900.
24. Morley, S. J., and Traugh, J. A. (1993) Stimulation of translation in 3T3-L1 cells in response to insulin and phorbol ester is directly correlated with increased phosphate labelling of initiation factor (eIF-) 4F and ribosomal protein S6, *Biochimie (Paris) 75*, 985−989.
25. Pause, A., Belsham, G. J., Gingras, A. C., Donze, O., Lin, T. A., Lawrence, J., and Sonenberg, N. (1994) Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5′-cap function, *Nature 371*, 762−767.
26. Hu, C., Pang, S., Kong, X., Velleca, M., and Lawrence, J. (1994) Molecular cloning and tissue distribution of PHAS-I, an intracellular target for insulin and growth factors, *Proc. Natl. Acad. Sci. U.S.A. 91*, 3730−3734.
27. Morley, S. J., and Traugh, J. A. (1990) Differential stimulation of phosphorylation of initiation factors eIF-4F, eIF-4B, eIF-3, and ribosomal protein S6 by insulin and phorbol esters, *J. Biol. Chem. 265*, 10611−10616.
28. Fraser, C. S., Pain, V. W., and Morley, S. J. (1999) The association of initiation factor 4F with poly(A)-binding protein is enhanced in serum-stimulated Xenopus kidney cells, *J. Biol. Chem. 274*, 196−204.
29. Drawbridge, J., Grainger, J. L., and Winkler, M. W. (1990) Identification and characterization of the poly(A)-binding proteins from the sea urchin: a quantitative analysis, *Mol. Cell. Biol. 10*, 3994−4006.
30. Gallie, D. R., Le, H., Caldwell, C., Tanguay, R. L., Hoang, N. X., and Browning, K. S. (1997) The phosphorylation state of translation initiation factors is regulated developmentally and following heat shock in wheat, *J. Biol. Chem. 272*, 1046−1053.
31. Webster, C., Gaut, R. L., Browning, K. S., Ravel, J. M., and Roberts, J. K. M. (1991) Hypoxia enhances phosphorylation of eukaryotic initiation factor 4A in maize root tips, *J. Biol. Chem. 266*, 23341−23346.
32. Le, H., Browning, K. S., and Gallie, D. R. (1998) The phosphorylation state of the wheat translation initiation factors eIF4B, eIF4A, and eIF2 is differentially regulated during seed development and germination, *J. Biol. Chem. 273*, 20084−20089.
33. Luo, Y., and Goss, D. J. (2001) Homeostasis in mRNA initiation: wheat germ poly(A)-binding protein lowers the activation energy barrier to initiation complex formation, *J. Biol. Chem. 276*, 43083−43086.
34. van Heerden, A., and Browning, K. S. (1994) Expression in *Escherichia coli* of the two subunits of the isozyme form of wheat germ protein synthesis initiation factor 4F. Purification of the subunits and formation of an enzymatically active complex, *J. Biol. Chem. 269*, 17454−17457.
35. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem. 72*, 248−254.
36. Bi, X., Ren, J., and Goss, D. J. (2000) Wheat germ translation initiation factor eIF4B affects eIF4A and eIFiso4F helicase activity by increasing the ATP binding affinity of eIF4A, *Biochemistry 39*, 5758−5765.
37. Wei, C. C., Balasta, M. L., Ren, J., and Goss, D. J. (1998) Wheat germ poly(A) binding protein enhances the binding affinity of eukaryotic initiation factor 4F and (iso)4F for cap analogues, *Biochemistry 37*, 1910−1916.
38. Pearson, R. B., and Kemp, B. E. (1991) Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations, *Methods Enzymol. 200*, 62−81.
39. Niedzwiecka, A., Marcotrigiano, J., Stepinski, J., Jankowska-Anyszka, M., Wyslouch-Cieszynska, A., et al. (2002) 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. 319*, 615−635.
40. Sonenberg, N. (1988) Cap-binding proteins of eukaryotic messenger RNA: functions in initiation and control of translation, *Prog. Nucleic Acid Res. Mol. Biol. 35*, 173−207.
41. Carberry, S. E., Rhoads, R. E., and Goss, D. J. (1989) A spectroscopic study of the binding of m7GTP and m7GpppG to human protein synthesis initiation factor 4E, *Biochemistry 28*, 8078−8083.
42. Scheper, G. C., van Kollenburg, B., Hu, J., Luo, Y., Goss, D. J., and Proud, C. G. (2002) Phosphorylation of eukaryotic initiation factor 4E markedly reduces its affinity for capped mRNA, *J. Biol. Chem. 277*, 3303−3309.
43. Zuberek, J., Wyslouch-Cieszynska, A., Niedzwiecka, A., Dadlez, M., Stepinski, J., Augustyniak, W., Gingras, A. C., Zhang, Z., Burley, S. K., Sonenberg, N., Stolarski, R., and Darzynkiewicz, E. (2003) Phosphorylation of eIF4E attenuates its interaction with mRNA 5′ cap analogs by electrostatic repulsion: Intein-mediated protein ligation strategy to obtain phosphorylated protein, *RNA 9*, 52−61.

BI049602B