The Phosphorylation State of Poly(A)-binding Protein Specifies Its Binding to Poly(A) RNA and Its Interaction with Eukaryotic Initiation Factor (eIF) 4F, eIFiso4F, and eIF4B*

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The poly(A)-binding protein (PABP) interacts with the eukaryotic initiation factor (eIF) 4G (or eIFiso4G), the large subunit of eIF4F (or eIFiso4F) to promote translation initiation. In plants, PABP also interacts with eIF4B, a factor that assists eIF4F function. PABP is a phosphoprotein, although the function of its phosphorylation has not been previously investigated. In this study, we have purified the phosphorylated and hypophosphorylated isoforms of PABP from wheat to examine whether its phosphorylation state affects its binding to poly(A) RNA and its interaction with eIF4G, eIFiso4G, or eIF4B. Phosphorylated PABP exhibited cooperative binding to poly(A) RNA even under non-stoichiometric binding conditions, whereas multiple molecules of hypophosphorylated PABP bound to poly(A) RNA only after free poly(A) RNA was no longer available. Together, phosphorylated and hypophosphorylated PABP exhibited synergistic binding, eIF4B interacted with PABP in a phosphorylation state-specific manner; native eIF4B increased the RNA binding activity specifically of phosphorylated PABP and was greater than 14-fold more effective than was recombinant eIF4B, whereas eIF4F promoted the cooperative binding of hypophosphorylated PABP. These data suggest that the phosphorylation state of PABP specifies the type of binding to poly(A) RNA and its interaction with its partner proteins.

The process of translation initiation in eukaryotes has undergone a paradigm shift in recent years. Until a few years ago, models of translation initiation focused solely on molecular events occurring at the 5′ end of an mRNA. However, several studies in plants, yeast, and animal cells have suggested that the poly(A)-binding protein (PABP), which binds the poly(A) tail at the 3′ terminus of an mRNA, is a necessary participant during translation initiation (reviewed in Ref. 1). Suppressor mutants that overcome the lethality imposed by a pab1 mutation in yeast were found to contain mutations affecting 60 S ribosomal subunit biogenesis, thereby disrupting the ratio of large and small ribosomal subunits (2, 3). The 5′ cap structure (m7GpppN) and the poly(A) tail, both of which are translational regulatory elements, were found to be functionally co-dependent in vivo (4), indicating that PABP participates in a step during translation that requires the participation of those initiation factors associated with the 5′ cap. Yeast Pab1p is required to mediate the increase in translation conferred by the poly(A) tail and the Pab1p-poly(A) tail complex was shown to promote 40 S ribosomal subunit recruitment to an mRNA (5).

During initiation, the 5′ cap is bound by eIF4E, the small subunit of eIF4F. 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) (6–8). In addition to eIF4F, plants contain an isoform called eIFiso4F composed of eIFiso4E and eIFiso4G (9). A direct interaction between PABP and eIF4G or eIFiso4G has been demonstrated in plants, animals, and yeast (10–14). The prediction that such an interaction would maintain the termini of the mRNA in close physical proximity resulting in a circular conformation (15) was visually confirmed using atomic force microscopy (16). In addition to the interaction between PABP and eIF4G, there are additional interactions between PABP and other initiation factors that may be species-specific. An interaction between PABP and eIF4B, a factor that assists in the function of eIF4F, has been demonstrated in plants (10) that has not yet been reported in yeast or animals. An interaction between PABP and PAIP (i.e. PABP-interacting protein), which shares similarity to the eIF3/eIF4A interaction domain of eIF4G, has been reported recently in mammalian cells (17). Moreover, there appear to be differences in the requirements of the PABP-eIF4G interaction among eukaryotes; binding to poly(A) RNA is a prerequisite for yeast PABP to interact with eIF4G (11), whereas plant or animal PABP exhibit no such requirement (10, 13, 18).

Two functional consequences of the interaction between these initiation factors and PABP that are pertinent to the commitment of the translational machinery to an mRNA have been demonstrated. eIFiso4G and eIF4B not only individually increase the binding affinity of PABP for poly(A) but, together, eIF4F (or eIFiso4F) and eIF4B exert a synergistic effect on PABP binding activity (10), suggesting a functional interaction between all three proteins. A second consequence of their in-
teraction is to increase the binding affinity of eIF4F (or eIFiso4F) for the 5' cap structure, demonstrating that the interaction of eIF4G (or eIFiso4G) with PABP results in the mutual stabilization of eIF4E (or eIFiso4E) and PABP to their respective target regulatory elements. Taken together, these studies provide strong evidence suggesting that the role of PABP during translation initiation involves protein-protein interactions with one or more of these initiation factors that, as a result, maintain the 5' and 3' termini of an mRNA in close physical proximity.

The functional interaction between the cap and the poly(A) tail is repressed following serum starvation in mammalian cells but can be reversed by exposure of the cells to insulin. The addition of either insulin or serum promotes rapid phosphorylation of eIF4E, eIF4G, eIF4B, and eIF4F (20–22), and the eIF4E-binding protein (i.e. 4E-BP or PHAS-I), resulting in its release from eIF4E (23, 24), and promotes the association between eIF4G and PABP (14). In plants, PABP and several of the initiation factors are known to be phosphorylated. As in yeast and sea urchin (25), plant PABP is present as multiple phosphorylated species, which is dephosphorylated by alkaline phosphatase, confirming the nature of the modification (26). Plant eIF4B is also present as multiple phosphorylated species and its phosphorylation state is regulated during development or following heat stress (26). eIF4B undergoes dephosphorylation following phosphatase treatment, and the array of phosphorylation eIF4B species observed in vivo can be recapitulated by phosphorylation of nonphosphorylated eIF4B by casein kinase II (26). eIF4A has been shown to be phosphorylated following environmental stress such as hypoxia or heat shock (26, 27). Other plant initiation factors subject to phosphorylation and their interaction with RNA or with each other. To investigate whether their phosphorylation state influences their binding activities, we examined their interaction with one another and with RNA when present in different phosphorylation states. Phosphorylated PABP exhibited cooperative binding to poly(A) RNA even under non-stoichiometric binding conditions (i.e. excess RNA), hypophosphorylated PABP bound non-cooperatively, and their combination resulted in synergistic binding. The phosphorylation state of PABP also determined the specificity of its interaction with initiation factors; the RNA binding activity of phosphorylated PABP was enhanced by eIF4B, whereas the RNA binding activity of hypophosphorylated PABP was enhanced by eIF4F. eIFiso4F enhanced the RNA binding activity of PABP in either phosphorylation state. Those factors that interacted with phosphorylated PABP increased its affinity for poly(A), whereas those that interacted with hypophosphorylated PABP enhanced its cooperative binding to poly(A) RNA. Phosphorylated eIF4B was more than 14-fold more effective in its functional interaction with PABP than was nonphosphorylated eIF4B. Similarly, native eIFiso4G was substantially more enhanced by eIF4B, and eIFiso4G might determine the extent of interaction between the cap and the poly(A) tail, as repressed following serum starvation in mammalian cells but can be reversed by exposure of the cells to insulin (19). The addition of either insulin or serum promotes rapid phosphorylation of eIF4E, eIF4G, eIF4B, and eIF4F (20–22), and the eIF4E-binding protein (i.e. 4E-BP or PHAS-I), resulting in its release from eIF4E (23, 24), and promotes the association between eIF4F and PABP (14). In plants, PABP and several of the initiation factors are known to be phosphorylated. As in yeast and sea urchin (25), plant PABP is present as multiple phosphorylated species, which is dephosphorylated by alkaline phosphatase, confirming the nature of the modification (26). Plant eIF4B is also present as multiple phosphorylated species and its phosphorylation state is regulated during development or following heat stress (26).

For all gel shift assays, radiolabeled poly(A)25 and poly(A)90 RNA were synthesized in vitro and gel-purified to remove the DNA template, unincorporated nucleotides, and less than full-length RNA. 1 ng of radiolabeled RNA and the indicated amount of protein were used for the binding reactions in a 15-μl volume containing 25 mM HEPES, pH 7.5, 1 mM MgAc, 0.1 mM EDTA, 10% glycerol, 1 mM NaF, 1 mM disodium pyrophosphate, 1 mM sodium vanadate. Following dialysis, the solution was brought to 85 μM and 2% (final concentration) amphoters (pH 3–10) were added and the PABP isoforms resolved using a Bio-Rad preparative isoelectric focusing apparatus. Each fraction was examined using two-dimensional/ Western analysis to identify the isoforms present. Each isoform was then rebind to affinity resin and applied to a 4B affinity column equilibrated in Buffer B. PABP was eluted with Buffer B containing 1 M urea and 2 M LiCl and dialyzed against 25 mM HEPES, pH 7.5, 100 mM KCl, 0.1 mM EDTA, 10% glycerol, 1 mM NaF, 1 mM disodium pyrophosphate, 1 mM sodium vanadate. Wheat germ eIF4F, eIFiso4F, and eIF4B were purified as described. Recombinant eIFiso4G and eIFiso4E were purified as described (34).

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Two-dimensional Gel Electrophoresis-Western Blot Analysis—Total soluble protein extracts were prepared from control and heat-shocked (25 °C) seedlings at 24 h following the treatment of leaves by grinding tissue in a mortar with liquid nitrogen and then in aqueous buffer (50 mM HEPES, pH 7.5, 120 mM KOAc, 5 mM MgOAc, 1 mM DTT, 1 mM PMSF, 1 μM leupeptin, 1 μM pepstatin, 1 mM EDTA, and 0.5 mM okadaic acid). The cell debris was pelleted by centrifugation and the protein concentration determined. Depending on the factor examined, 5–100 μg of protein was loaded on an 10% polyacrylamide gel and run at 400 V for 4 h, followed by 0.5 h at 500 V. The protein was then resolved in the second dimension using standard SDS-PAGE and the protein transferred to 0.22-μm nitrocellulose membrane by electrophotography. Following transfer, the nitrocellulose membranes were blocked in 5% milk, 0.01% thimerosal in TBBS (0.1% Tween 20, 13.7 mM NaCl, 0.27 mM KCl, 1 mM Na2HPO4, 0.14 mM NaH2PO4) followed by incubation with primary antibodies diluted typically 1:1000 to 1:2000 in TBBS with 1% milk for 1.5 h. The blots were then washed twice with TBBS and incubated with goat anti-rabbit peroxidase-conjugated antibodies (Southern Biotechnology Associates, Inc.) diluted to 1:10,000 for 1 h. The blots were washed twice with TBBS, and the signal detected typically between 1 and 15 min using chemiluminescence (Amersham Pharmacia Biotech). The pH range of the IEF gel following isoelectric focusing was determined from the measurements of 5-mm sections of a control gel soaked in 1 M NaCl.

mGTP and Poly(A) Affinity Purification—Leaves of seedlings germinated for 60 h were incubated at room temperature or heat-shocked at 45 °C for 25 min in Gamborg's medium supplemented with 10 mM CaCl2, pH 5.8, and then ground in liquid nitrogen and resuspended by grinding in 50 mM HEPES, pH 7.6, 100 mM KCl, 5 mM EDTA, 10 mM MgCl2, 3 mM DTT, 1 mM PMSF, 10 μM/ml pepstatin, 1 μM/ml okadaic acid, 1% each of Triton X-100, Tween 40, and Nonidet P-40. The extract was centrifuged twice at 9000 rpm for 15 min to remove cell debris. Seven mg of soluble protein extract was added to a 100-μl suspension of mGTP-Sepharose or poly(A)-agarose resin and incubated with rotation at 4 °C for 3 h. The resin was collected by
centrifugation (800 × g for 1 min) and washed twice with 400 μl of the grinding buffer. Protein eluted from the resin was then resolved by SDS-PAGE and subject to Western analysis.

RESULTS

The Phosphorylation State of PABP Specifies Its Affinity for and Multimeric Binding to Poly(A) RNA—PABP is present in multiple isoforms in yeast, sea urchin, and wheat (25, 26), and the nature of its modification in plants was demonstrated to be due to phosphorylation through conversion of purified phosphorylated PABP to its hypophosphorylated state following phosphatase treatment (26). In order to investigate how the phosphorylation state affects the RNA binding activity of PABP, the protein was purified from wheat (Fig. 1, top panel) and the isoelectric point determined using preparative isoelectric focusing. Separation of the most phosphorylated isoforms from the hypophosphorylated isoforms was verified using two-dimensional Western analysis (Fig. 1, middle and bottom panels).

To determine whether the phosphorylation state of PABP specifies its RNA binding activity, binding reactions with radiolabeled poly(A) RNA and PABP in each phosphorylated state alone were carried out and the resulting complexes resolved using gel shift analysis. Additionally, combinatorial binding reactions with poly(A)50 RNA were performed in which the concentration of PABP in one phosphorylated state was held constant while PABP in the other phosphorylated state was added in increasing amounts. The size of the poly(A)-binding site of PABP is 12 residues, but its packing density is 25 residues in yeast (35) or approximately 27 residues in mammalian cells (36); consequently, poly(A)50 RNA should be long enough to accommodate at least two molecules of PABP. A single complex was formed between PABP and poly(A)50 RNA when the protein was either phosphorylated (Fig. 2B, lanes 2–6) or hypophosphorylated (Fig. 2A, lanes 2–6). However, migration of the phosphorylated PABP-poly(A)50 complex was considerably retarded relative to the hypophosphorylated PABP-poly(A)50 complex (Fig. 2, compare lanes 2–6 of panel B and panel A, respectively). The slower migration of the phosphorylated PABP-poly(A)50 RNA complex is consistent with multimeric binding of the protein to the RNA, i.e. 2 or more molecules of PABP bound to poly(A) RNA. Moreover, this multimeric binding occurs under non-stoichiometric binding conditions, i.e. an excess of poly(A)50 RNA remained unbound, suggesting that phosphorylated PABP binds poly(A) RNA cooperatively. Phosphorylated PABP exhibited a lower affinity for poly(A) relative to hypophosphorylated PABP; at least a 10-fold greater concentration of phosphorylated PABP was required to achieve a level of RNA binding similar to that observed for hypophosphorylated PABP (Fig. 2, compare lane 5 in panel B to lane 3 in panel A).

To examine whether the RNA binding activity of PABP present in one phosphorylated state could be affected when PABP of the opposite phosphorylated state was present, different combinations of PABP in each phosphorylated state were tested in the binding assay. The addition of just 0.16–0.48 nM hypophosphorylated PABP to a binding reaction containing 2 nM phosphorylated PABP resulted in a substantial increase in multimeric binding (Fig. 2A, lanes 2–6 to lane 7–10). Identical results were obtained when the reverse experiment was performed; the addition of 1–3 nM phosphorylated PABP to a binding reaction containing 0.32 nM hypophosphorylated PABP resulted in enhanced multimeric binding (Fig. 2B, lanes 2–6 to lane 8–10), data suggesting cooperativity between phosphorylated and hypophosphorylated PABP. Cooperative binding by the combination of phosphorylated and hypophosphorylated PABP was more extensive at the lowest concentration tested than that exhibited by phosphorylated PABP alone at the highest concentration tested (Fig. 2B, compare lane 8 to lane 6, respectively), sug-
suggesting that phosphorylated and hypophosphorylated PABP have a greater affinity for one another than exists between molecules of phosphorylated PABP.

To examine the binding characteristics of each PABP isoform to RNAs of different lengths, binding experiments between poly(A)$_{25}$ or poly(A)$_{50}$ RNA and hypophosphorylated or phosphorylated PABP were performed. The multimeric, phosphorylated PABP-Poly(A)$_{50}$ RNA complex (Fig. 3A, lane 8), the monomeric, hypophosphorylated PABP-Poly(A)$_{25}$ RNA complex (Fig. 3A, lane 2), and the synergistic binding exhibited by their combination (Fig. 3A, lane 10) were observed as they had been in Fig. 2. Binding of hypophosphorylated PABP to poly(A)$_{25}$ RNA resulted in a single complex that migrated to the same position as the hypophosphorylated PABP-Poly(A)$_{25}$ RNA complex (Fig. 3A, compare lane 5 to lane 9). A single, rapidly migrating complex was also observed when phosphorylated PABP bound poly(A)$_{25}$ RNA (Fig. 3A, lanes 2 and 3). The phosphorylated PABP-Poly(A)$_{50}$ complex migrated slightly faster than did the hypophosphorylated PABP-Poly(A)$_{50}$ complex (Fig. 3A, compare lanes 4 and 5 to lanes 2 and 3) as a consequence of its greater negative charge. When both phosphorylated and hypophosphorylated PABP were present in the binding reaction containing Poly(A)$_{50}$ RNA, complexes corresponding to those observed for phosphorylated PABP-Poly(A)$_{25}$ and hypophosphorylated PABP-Poly(A)$_{25}$ were observed as was an additional, slower migrating complex (Fig. 3A, lane 6) that corresponded to the multimeric complex formed by phosphorylated PABP-Poly(A)$_{50}$ RNA (Fig. 3A, lane 8), suggesting an interaction between complexes of phosphorylated and hypophosphorylated PABP bound to Poly(A)$_{50}$ RNA that was not observed at a similar concentration of PABP in either phosphorylation state alone. Whether one or both molecules of PABP are bound to Poly(A)$_{25}$ RNA cannot be determined from this experiment.

To determine the binding characteristics of phosphorylated and hypophosphorylated PABP under stoichiometric conditions, binding assays were performed using a high concentration of PABP in each phosphorylation state and either Poly(A)$_{25}$ or Poly(A)$_{50}$ RNA. At a high concentration of phosphorylated PABP, at least four Poly(A)$_{25}$ complexes were observed (Fig. 3B, lanes 6 and 7), including the rapidly migrating complex observed for phosphorylated PABP-Poly(A)$_{25}$ (Fig. 3A, lanes 2 and 3) and the slowly migrating complex observed for phosphorylated PABP-Poly(A)$_{50}$ RNA (Fig. 3A, lane 5). The observation that cooperative binding of phosphorylated PABP to Poly(A)$_{50}$ RNA occurs under excess RNA conditions (Fig. 3B, lanes 6–10) or following near-stoichiometric binding (Fig. 3B, lanes 3) indicates that multimeric binding of hypophosphorylated PABP is limited to when no free RNA is available and to an RNA sufficiently long to accommodate multiple PABPs. In contrast, phosphorylated PABP exhibits cooperative binding to Poly(A)$_{50}$ RNA under non-stoichiometric conditions (Fig. 3B, lanes 2–6). Moreover, the multiple complexes formed following binding of phosphorylated PABP to Poly(A)$_{50}$ RNA suggests extensive protein-protein interactions between the complexes. These data suggest that phosphorylated PABP exhibits a high degree of protein-protein interaction whether it be binding to the same RNA (e.g. Poly(A)$_{50}$ RNA) or between complexes when the RNA is not long enough to accommodate more than one PABP molecule (e.g. Poly(A)$_{50}$ RNA). The fact that the binding of multiple molecules of hypophosphorylated PABP to Poly(A) was observed only with Poly(A)$_{50}$ RNA and only following stoichiometric binding indicates that the binding of hypophosphorylated PABP to Poly(A) is determined by its concentration and the length of the RNA with little contribution made by protein-protein interaction.

When binding to Poly(A)$_{50}$ RNA was analyzed using purified PABP that is unfractionated (i.e. containing all isoforms), complexes were observed (Fig. 4) that were similar to those resulting from the combination of hypophosphorylated and phosphorylated PABP (Fig. 2, A and B, lanes 8–10). Titration of the extract in the Poly(A)$_{50}$ RNA-binding assay demonstrated that
the complexes were observed at even a low PABP concentration (Fig. 4, lanes 2–5 and 12–15) and that under stoichiometric binding conditions when no free RNA remained, the slowest migrating complex predominated (Fig. 4, lanes 7–10, 16, and 17), suggesting that cooperative binding is observed when all cellular PABP isoforms are present.

**The Phosphorylation State of PABP Specifies Its Interaction with the Cap-associated Initiation Factors**—We had previously demonstrated that eIF4F, eIFiso4F, and eIF4B specifically interact with PABP, and one functional consequence of their interaction was to increase PABP binding affinity for poly(A) (10). eIFiso4G was identified as the subunit of eIFiso4F that was responsible for the interaction with PABP and the increased affinity of PABP for poly(A) RNA (10). eIFiso4E, the small subunit of eIFiso4F, did not interact with PABP and had virtually no effect on PABP poly(A) binding activity (10). To investigate whether these initiation factors interact with PABP in a phosphorylation state-specific manner, each factor was tested in binding reactions containing poly(A)_{30} RNA and PABP in each phosphorylated state. eIF4F was observed to enhance specifically the binding of hypophosphorylated PABP to poly(A) RNA (Fig. 5A, lanes 7–10), whereas it had little effect on the binding activity of phosphorylated PABP (Fig. 5A, lanes 3–6). eIF4F promoted cooperative binding of hypophosphorylated PABP, which was not observed when hypophosphorylated PABP was present alone at the same concentration (Fig. 5A, compare lanes 8–10 to lane 7). No binding of eIF4F to poly(A) was observed at this concentration of the factor (Fig. 5A, lane 2). Like eIF4F, eIFiso4F promoted cooperative binding of hypophosphorylated PABP to poly(A) RNA (Fig. 5B, see lanes 7–10). eIFiso4F also enhanced the binding of phosphorylated PABP to a small extent (Fig. 5B, see lanes 3–6). These results indicate that eIF4F and eIFiso4F are similar in that they promote cooperative binding of hypophosphorylated PABP (Fig. 5, lanes 7–10 in panels A and B, respectively). As established previously, eIF4F and eIFiso4F substantially decrease the dissociation rate of PABP from poly(A) RNA but do not remain associated with the shifted complexes during electrophoresis and, as a result, they do not alter the mobility of PABP-poly(A) RNA complex (10). It is likely, therefore, that these factors increase PABP binding to poly(A) by promoting protein-protein interactions between molecules of PABP which in turn stabilizes the association of PABP with poly(A) RNA.

eIFiso4G was identified previously as the subunit of eIFiso4F that is responsible for promoting PABP binding to poly(A) (10). To investigate whether eIFiso4G is responsible for the phosphorylation state-specific interaction observed between eIFiso4F and PABP, recombinant eIFiso4G and eIFiso4E were tested in poly(A)_{30} binding reactions containing PABP in each phosphorylation state. Recombinant eIFiso4G increased the affinity and cooperativity of hypophosphorylated PABP binding to poly(A)_{30} RNA (Fig. 6A, lanes 7–10), similar to that observed with native eIFiso4F (Fig. 5B, lanes 7–10). In contrast, eIFiso4E had little effect on poly(A) binding activity of PABP in either phosphorylation state (Fig. 6B). The combinatorial effect of recombinant eIFiso4G and eIFiso4E on PABP binding was similar to that observed for eIFiso4G alone (Fig. 6C). The small increase in the poly(A) binding activity of phosphorylated PABP observed when native eIFiso4F was present was not observed to the same extent when recombinant eIFiso4G or recombinant eIFiso4E was used (Fig. 6, lanes 3–6 in panels A and C, respectively). The data demonstrating the interaction between eIFiso4G and hypophosphorylated PABP are in good agreement with our previous observations with recombinant eIFiso4G and unfractionated PABP (10). It should be noted that a significantly higher concentration of recombinant eIFiso4G (with or without recombinant eIFiso4E) was required to achieve a similar level of stimulation in PABP RNA binding activity, suggesting that the phosphorylation state of native eIFiso4G, which differs significantly from recombinant eIFiso4G (26), may be required for optimal interaction with PABP.
In addition to the interaction between PABP and eIFiso4G (or the eIF4G subunit of plant eIF4F), an interaction between PABP and eIF4B has been observed in plants (10). Like PABP, eIF4B is a phosphoprotein that can be dephosphorylated following phosphatase treatment (26, 28). The phosphorylation state of eIF4B undergoes dynamic changes during plant development that correlates with changes in protein synthesis; eIF4B is phosphorylated in young leaves or during early seed development when the rate of protein synthesis is high, whereas it is dephosphorylated during late seed development or following heat stress when protein synthesis is repressed (26, 28). To examine whether eIF4B exhibits specificity in its interaction with PABP, native eIF4B was purified from wheat embryos (composed of six isoforms, five of which represent phosphorylated species (Refs. 26 and 28)) and added to poly(A)$_{50}$ RNA binding reactions containing either phosphorylated or hypophosphorylated PABP isoforms. The resulting complexes were resolved using gel shift analysis. 2 nM phosphorylated PABP and 0.34 nM hypophosphorylated PABP were used in the assays where indicated. No complex other than the two prominent ones present in lanes 8–10 was observed in lighter exposures. PABP, hypophosphorylated PABP, PABP-P, phosphorylated PABP.

The effect of native eIF4B on PABP RNA binding activity was greater than that observed for eIF4F or eIFiso4F, which is consistent with previous observations (10). Although native eIF4B exhibited poly(A) binding activity itself (see Fig. 7A, lane 2) in good agreement with previous studies (10, 37), this activity was considerably less than that observed for phosphorylated PABP (Fig. 7A, lane 3) or for unfractionated PABP (10) and cannot account for the increase in poly(A) binding activity observed for phosphorylated PABP. Moreover, the observation that eIF4B did not increase poly(A) binding activity observed for phosphorylated PABP. The affinity of eIF4B for PABP RNA binding activity was greater than that observed for eIF4F or eIFiso4F, which is consistent with the appearance of a band in lanes 9 and 10 in Fig. 7A can be accounted for by the increasing amount of eIF4B) demonstrates that the native eIF4B-mediated increase in phosphorylated PABP binding is specific and could not be accounted for by its low level of poly(A) binding activity. It is unlikely that PABP increases the affinity of eIF4B for poly(A), as eIF4B is not detected in the PABP-pol(A) RNA complex of the type observed in Fig. 7 (10). Like eIF4F and eIFiso4F, eIF4B decreases the dissociation rate of PABP from poly(A) RNA and, therefore, likely stabilizes PABP binding to poly(A) by promoting protein-protein interactions between molecules of PABP.

In order to determine whether the phosphorylation state of eIF4B itself determines the strength or specificity of its interaction with PABP, recombinant eIF4B, representing the factor in a dephosphorylated state (28), was tested in binding reactions with phosphorylated and hypophosphorylated PABP. Recombinant eIF4B increased the affinity for, and cooperative binding of, hypophosphorylated PABP to poly(A) RNA (Fig. 7B, lane 3).
Regulation of the PABP/eIF4G/eIF4B Interaction

The Phosphorylation State of eIF4B and eIFiso4G Specifies Their Association with Polysomes—The selective interactions between PABP and eIF4G, eIFiso4G, or eIF4B might be expected to determine what isoforms of each protein are associated with polysomes. For example, the observed cooperative binding between phosphorylated and hypophosphorylated PABP (see Figs. 2 and 3) suggest that PABP differing in phosphorylation states would be present in polysomal mRNA. Moreover, because the strength of interaction between native (i.e. phosphorylated) eIF4B and PABP is substantially greater than between recombinant (i.e. nonphosphorylated) eIF4B and PABP, phosphorylated eIF4B might be expected to preferentially associate with polysomal mRNA. In order to identify the phosphorylation state of PABP, eIF4B, and eIFiso4G that is associated with polysomal mRNA, the ribosome/polysomal fraction was isolated from wheat embryos and the phosphorylation state of each determined by two-dimensional Western analysis. Analysis of the ribosome/polysomal fraction (Fig. 9, P100) and the non-polysomal fraction (Fig. 9, S100) revealed that all PABP isoforms were present in each fraction as they were in crude extract (Fig. 9, PABP, Unfractionated), suggesting that PABP of differing phosphorylation states binds poly(A) cooperatively. In contrast, the most phosphorylated isoforms of eIF4B were present exclusively in the ribosome/polysomal fraction (Fig. 9, see eIF4B, P100) despite the fact that they represent only a small portion of the total eIF4B present in wheat embryos at this stage of seed development (Fig. 9, see eIF4B, Unfractionated). These data suggest that phosphorylated eIF4B preferentially associates with polysomes, consistent with the observed functional interaction between phosphorylated eIF4B and PABP. Similar to the observations with eIF4B, native eIFiso4G (as part of native eIFiso4F) is more effective in promoting PABP RNA binding activity relative to recombinant eIFiso4G. Native eIFiso4G is present in crude extract as multiple phosphorylated species (Fig. 9), whereas recombinant eIFiso4G runs as a single, highly basic isoform (26) consistent with its predicted pI of 8.0. The same isoforms of eIFiso4G observed in crude extract were observed associated with polysomal mRNA but the most phosphorylated eIFiso4G isoforms predominated (Fig. 9, see eIFiso4G, P100), consistent with the greater effectiveness of native eIFiso4G (as part of eIFiso4F) to increase PABP binding activity relative to recombinant eIFiso4G (as observed in Figs. 5B and 6C, respectively).

The Poly(A) Binding Activity of PABP and Its Interaction with eIF4G, eIFiso4G, and eIF4B Are Reduced following Heat Shock—The above results suggest that the phosphorylation state of PABP and its partner proteins specify their intermolecular interaction as well as the poly(A) binding activity of PABP. We next examined whether in vivo changes in the phosphorylation state of these factors correlate with alterations in the interaction between these proteins or in the binding of PABP to poly(A) RNA. Although no alteration in the phosphorylation state of PABP during development or following heat stress is observed (Fig. 10 and Refs. 26 and 28), heat shock does result in the rapid and near-complete dephosphorylation of eIF4B in wheat leaves (Fig. 10 and Ref. 26). Moreover, a shift from acidic to basic isoforms was observed for eIF4G and eIFiso4G, indicating that each underwent partial dephosphorylation following a heat shock (Fig. 10). Because recombinant (i.e. nonphosphorylated) eIF4B and recombinant eIFiso4G (Figs. 7B and 6A, respectively) were both considerably less

![Image](https://example.com/image.png)

**Fig. 8.** eIF4B, eIFiso4F, or eIF4B increase the poly(A) binding activity of PABP to poly(A)25 RNA. Purified native eIF4B (lanes 4–6), recombinant eIF4B (lanes 7–9), purified, native eIF4F (lanes 10–12), or purified, native eIFiso4F (lanes 13–15) was added to binding reactions containing poly(A)25 RNA and either phosphorylated or hypophosphorylated PABP isoforms. The resulting complexes were resolved using gel shift analysis. 2 nm phosphorylated PABP and 0.34 nm hypophosphorylated PABP were used. PABP: hypophosphorylated PABP, PABP-P, phosphorylated PABP.
effective in stimulating the poly(A) binding activity of PABP than were their native forms (Figs. 7A and 5B, respectively), we examined whether the dephosphorylation of eIF4B, eIF4G, and eIFiso4G resulting from a heat shock correlated with a reduction in PABP poly(A) binding activity. Extracts from control and heat-stressed leaves used for the two-dimensional Western analysis in Fig. 10 were tested for PABP binding activity to poly(A)$_{25}$ or poly(A)$_{50}$ RNA using gel shift analysis (Fig. 11). The addition of purified, unfractonated PABP (composed mostly of hypophosphorylated PABP) resulted in the same complexes as observed in Fig. 4 with the faster-migrating complex predominating (Fig. 11, lane 2). Extract from control wheat leaves resulted in the formation of the same complexes in which the multimeric complex predominated (Fig. 11, lane 3). Addition of anti-PABP antiserum to the binding assay containing the control leaf extract supershifted the multimeric complex, demonstrating that PABP was present in the complex (Fig. 11, lane 4). Poly(A) binding activity was largely abolished when extract from heat-stressed leaves was tested in the assay (Fig. 11, lane 5) and the addition of anti-PABP antiserum to the binding assay did not result in any detectable supershift (Fig. 11, lane 6), indicating that the residual complex observed did not contain PABP. A similar loss in poly(A) binding activity from heat-stressed leaves was observed when poly(A)$_{25}$ RNA was used instead of poly(A)$_{50}$ RNA (Fig. 11, compare lane 9 to lane 8).

To examine whether the observed changes in poly(A) binding activity in heat-stressed leaves correlated with changes in the interaction between PABP and eIF4G or eIFiso4G, the cap-associated factors, eIF4F and eIFiso4F, were isolated from the control and heat-stressed leaves using m$^7$GTP affinity purification and the presence of PABP relative to eIF4G and eIFiso4G determined using Western analysis. PABP co-purified with eIF4F and eIFiso4F isolated from control leaves (Fig. 12A, lane 2, PABP). The heat treatment did not affect the amount of eIFiso4G isolated (Fig. 12A, lane 3, eIFiso4G) but abolished isolation of eIF4G (Fig. 12A, lane 3, eIF4G). Moreover, heat shock abolished the co-purification of PABP with the eIFiso4F (Fig. 12A, lane 3, PABP), suggesting that its association with eIFiso4G was disrupted as a consequence of the heat treatment. A decrease in the amount of eIF4B co-purified as part of the complex was also observed (Fig. 12A, compare lane 3 to lane 2, eIF4B).

Whether heat shock affected the poly(A) binding activity of PABP was confirmed by the extent of binding of the extracts to poly(A)-Sepharose. As expected, PABP from control leaves was greatly enriched following binding to the poly(A) resin (Fig. 12B, compare lane 2 to lane 1, PABP). In good agreement with the data obtained from the gel shift analysis of Fig. 11, a substantial reduction in the binding of PABP was observed (Fig. 12B, compare lane 3 to lane 2, PABP), although a low level of PABP from the heat-stressed leaves was retained on the poly(A) resin as the amount of extract used exceeded the binding capacity (as determined using control extract) in order to provide the greatest sensitivity of detection of binding activity from heat-stressed leaves. A similar loss in PABP binding was observed when the amount of the extract (as determined using control extract) did not exceed the binding capacity of the resin (data not shown). Correlating with the reduced binding activity of PABP from heat-stressed leaves, a reduction in the co-purification of eIF4G or eIFiso4G was also observed. No alteration in the retention of eIF4B was observed from heat-stressed leaves consistent with its ability to bind directly to poly(A) RNA as characterized previously (see lane 2 of Fig. 7A and Ref. 37). Together, these results suggest that heat stress results in a reduction of the poly(A) binding activity of PABP and a reduction in its interaction with the cap-associated partner proteins. These stress-mediated reductions in protein-protein and protein-RNA binding activities do not involve detectable changes in the distribution of the PABP isoforms but correlate rather with alterations in the isoelectric state of eIF4G, eIFiso4G, and eIF4B that are consistent with their heat-induced dephosphorylation. These data support the notion that the stimulation of the poly(A) binding activity of PABP specified by the phospho-
In this study, we have observed that the phosphorylation state of PABP determines its affinity and type of binding to poly(A): phosphorylated PABP exhibited cooperative binding to poly(A)$_{50}$ or even poly(A)$_{25}$ RNA under non-stoichiometric (i.e. excess RNA) binding conditions; binding of hypophosphorylated PABP to poly(A)$_{25}$ RNA resulted in only a monomeric complex even during stoichiometric binding (i.e. excess hypophosphorylated PABP), whereas the binding of multiple molecules of hypophosphorylated PABP to poly(A)$_{50}$ RNA could be detected but only when the protein was in excess. These observations suggest that there is little protein-protein interaction that occurs between molecules of hypophosphorylated PABP, and consequently, the binding of multiple molecules to poly(A) RNA is observed only under stoichiometric binding conditions and only when the RNA is of sufficient length to accommodate multiple molecules of hypophosphorylated PABP. In contrast, the cooperative binding to poly(A) RNA that was exhibited by phosphorylated PABP even under non-stoichiometric binding conditions suggests that its binding activity involves protein-protein interactions either between protein molecules bound to the same RNA (e.g. when bound to poly(A)$_{50}$ RNA) or between protein molecules bound to separate RNAs (e.g. when bound to poly(A)$_{25}$ RNA). The appearance of at least four independent complexes when a high concentration of phosphorylated PABP was used in binding assays with poly(A)$_{25}$ RNA (see Fig. 2B, lane 7) suggests that the interactions can be extensive. It should be noted that the greatest degree of cooperative binding was observed between phosphorylated and hypophosphorylated PABP (see Fig. 2, A and B, lanes 8–10), indicating that an interaction between PABP of differing phosphorylation states is favored. The strength of interaction between PABP molecules, therefore, can be ranked in an order of decreasing affinity: phosphorylated PABP:hyphosphorylated PABP > phosphorylated PABP:phosphorylated PABP > hypophosphorylated PABP: hypophosphorylated PABP. Because PABP binds as multimers to the average poly(A) tail (36, 38, 39) and because the greatest degree of cooperative binding results from the interaction between PABP of differing phosphorylation states, the multimers of PABP bound to the poly(A) tail of a typical mRNA are likely to be heterogeneous in their phosphorylation state. That PABP is present in all phosphorylated states in plant tissues (see Fig. 1; see also Refs. 26 and 31) and polysome-associated PABP includes all observable isoforms (see Fig. 9) indicates that PABP of differing phosphorylation states is available for this interaction in vivo.

The phosphorylated state of PABP not only influenced its cooperative binding to poly(A) RNA but determined its specificity of interaction with those initiation factors previously identified as capable of interacting with PABP, i.e. eIF4F, eIFiso4F, and eIF4B (10). The reduced effect of the phosphorylation state of PABP itself. The reduced effect of the phosphorylation state of PABP on the binding activity of phosphorylated PABP was in agreement with earlier work (10), it was the eIFiso4G subunit of eIFiso4F that was identified as capable of interacting with PABP, whereas native eIF4B increased the binding activity of phosphorylated PABP. In good agreement with earlier work (10), it was the eIFiso4G subunit of eIFiso4F that was specifically responsible for interacting with PABP. However, the native form of eIFiso4G stimulated PABP binding activity to a greater extent than did the recombinant protein. Similarly, native (i.e. phosphorylated) eIF4B was at least 14-fold more effective than recombinant eIF4B in increasing PABP activity (see Fig. 7), indicating that the phosphorylation state of eIF4B and eIFiso4G is as important in determining the strength and specificity of their functional interaction with PABP as is the phosphorylation state of PABP itself. The reduced effect of the recombinant eIFiso4G or eIF4B was not a result of the reduced activity of the recombinant protein, as both are as active as their native form in eIFiso4G- or eIF4B-dependent in vitro translation systems and in an RNA-dependent ATP-hydrolysis assay (34, 40). The observation that the presence of these initiation factors resulted in the appearance of multiple phosphorylated PABP-poly(A)$_{25}$ RNA complexes (see Fig. 8) suggests that they may also promote protein-protein interactions between molecules of an isoform of PABP that does not otherwise exhibit self-interaction. Precedent for the role of phosphorylation in determining the strength of protein-protein interaction that occurs between molecules of phosphorylated PABP.
Regulation of the PABP/eIF4G/eIF4B Interaction

7–10 activity or protein–protein interactions is not a result of a reduction in the level of these proteins following a heat stress (48). The correlation between the heat-mediated dephosphorylation of the cap-associated initiation factors, their reduced co-purification with PABP, and the reduction in the poly(A) binding activity of PABP is consistent with the results obtained in the in vitro binding studies. Consequently, the strength of the interaction between PABP and its partner proteins may be subject to regulation through changes in the phosphorylation state of the initiation factors rather than through alterations in the distribution of the PABP isoforms. Recent evidence demonstrates that serum stimulates the interaction between PABP and eIF4G in mammalian cells (14) and also stimulates mammalian eIF4G phosphorylation in a pathway modulated by phosphoinositide 3-kinase (PI3K) and FKB12-rapamycin-associated protein/mammalian target of rapamycin kinase (49). These observations provide additional support for the conclusion that the protein interactions between the cap-associated factors and PABP may be subject to control through changes in the phosphorylation state of the initiation factors and may constitute a means by which global translation is modulated by developmental or environmental signals.

REFERENCES

The Phosphorylation State of Poly(A)-binding Protein Specifies Its Binding to Poly(A) RNA and Its Interaction with Eukaryotic Initiation Factor (eIF) 4F, eIFiso4F, and eIF4B
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