The Phosphorylation State of the Wheat Translation Initiation Factors eIF4B, eIF4A, and eIF2 Is Differentially Regulated during Seed Development and Germination*

(Received for publication, March 3, 1998, and in revised form, June 1, 1998)

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The translation initiation factors (eIF) 4B and eIF2 are phosphoproteins whose phosphorylation state differs between mature seed and leaves. We examined the isoforms of eIF4B and the α and β subunits of eIF2 during the development and germination of wheat seed to determine whether the differences in their phosphorylation state are because of tissue-specific regulation or occur concomitant with changes in protein synthetic activity during development. eIF2βα underwent phosphorylation through several intermediate isoforms that correlated with the increase and subsequent reduction in protein synthetic activity characteristic of seed development. eIF2β and eIF4B, present as highly phosphorylated isoforms during early seed development, underwent dephosphorylation during late development. eIF4B was rapidly phosphorylated within 20 h of germination, whereas eIF2α did not undergo dephosphorylation until 48–60 h of growth. A third factor, eIF4A, was predominantly nonphosphorylated throughout most of seed development and germination. These observations suggest that the phosphorylation state of eIF2α, eIF2β, and eIF4B is developmentally regulated in a way that correlates with the changes in protein synthetic activity but that some differences were also observed.

Protein synthesis undergoes dramatic changes during plant growth, particularly during the development of the seed and its subsequent germination. From the perspective of protein synthetic activity, the development of wheat seed can be divided into three stages: early development (up to 10–12 days after flowering (DAF)), mid-development (approximately 12–30 DAF), and late development (30–45 DAF). During early development, the endosperm and embryo are surrounded by the nucellus, a maternal tissue that provides nutrients to the rapidly growing endosperm (1, 2). The nucellus is the predominant tissue during early seed development but undergoes a progressively programmed cell death to provide room for the expanding endosperm tissue. Following 12 DAF, the nucellus is insignificant and the mass of the seed is dominated by the endosperm and embryo. The highest level of protein synthetic activity in the seed occurs between 12 and 30 DAF when the bulk of the seed storage protein is synthesized in the endosperm and stored to provide the nutrients required for germination (3–5). This developmental stage is followed by a rapid drop in protein synthesis when the endosperm undergoes its own programmed cell death and the embryo prepares for quiescence, a process that continues up to the mature dry stage of the seed by 45 DAF. Despite the reduced protein synthetic activity after 30 DAF, late embryogenesis abundant (lea) proteins are synthesized, which are thought to be required for the embryo to survive desiccation during late seed development (reviewed in Ref. 6). At maturity, the seed is virtually metabolically quiescent, and translational activity ceases despite the continued presence of the translational machinery in the embryo. Protein synthesis resumes quickly following the initiation of germination; formation of polysomes and protein synthesis are detected shortly following the onset of germination (7–10).

Such extreme differences in protein synthetic activity during development in plants presumably requires considerable control over the activity of the translational machinery itself. Translational regulation in yeast and animal cells often occurs through changes in the phosphorylation state of the eukaryotic initiation factors (eIFs) whose function is to aid 40- and 60-S ribosomal subunit binding to the mRNA, resulting in the formation of the 80-S ribosome at the correct start codon. Our previous studies demonstrated that the phosphorylation state of two initiation factors, eIF2 and eIF4B, differed substantially in seed versus leaves of wheat (11), observations suggesting that the activity of these factors may be under developmental regulation.

Wheat eIF4B, a single polypeptide (59 kDa), is thought to assist eIF4A and eIF4F in the ATP-dependent unwinding of secondary structure that may be present within a 5′-leader of an mRNA (12, 13). eIF4B is hypophosphorylated in mature wheat embryos, but is present predominantly as hyperphosphorylated isoforms in leaves (11). The posttranslational modification of eIF4B is similar in both plant and animal cells in that the isoforms are divided into an acidic cluster of four to six isoforms that are phosphorylated forms of a basic cluster of four isoforms (11, 14). The acidic cluster is substantially reduced in mature wheat embryos, but predominates in leaves, and undergoes rapid dephosphorylation in leaves following a heat shock (11). The alteration in wheat eIF4B phosphorylation correlates with the repression of translation that occurs following a thermal stress (15). Further evidence suggesting a relationship between the phosphorylation state of eIF4B and translational activity comes from studies in animal cells: dephosphorylation of mammalian eIF4B occurred following heat shock (16), serum depletion (17), or mitosis (18). Dephos-
phorylation of mammalian eIF4B correlated with the reduction in translation following these treatments, whereas the phosphorylation of eIF4B that occurs following insulin treatment correlated with an increase in translation (19). Moreover, the addition of phosphorylated mammalian eIF4B was partially able to restore translation in an in vitro translation lysate prepared from heat-shocked HeLa cells in which the eIF4B had undergone dephosphorylation (16, 20).

eIF2 is a three-subunit complex in plants as in other eukaryotes and is responsible for binding the initiator Met-tRNA to the 40-S ribosomal subunit (21–23). The three subunits of wheat eIF2 are α (42 kDa), β (38 kDa), and γ (50 kDa) (24). Phosphorylation of eIF2α occurs following amino acid starvation in yeast (reviewed in Ref. 25) or viral infection (reviewed in Ref. 26), heme deprivation, and heat shock in animal cells (27). Its phosphorylation inhibits the eIF2β-directed exchange of GTP for GDP, preventing eIF2 from participating in more than one round of translation initiation (reviewed in Refs. 28–30). In wheat, eIF2α is also subject to phosphorylation: the factor is present in a hyperphosphorylated state in embryos of mature seed but is present in a hypophosphorylated state in leaves (11). However, in contrast to mammalian eIF2, heat shock, whether of short or long duration, had little detectable impact on the phosphorylation state of the α subunit in wheat leaves (11). Mammalian eIF2β is present as a single major form under normal growth conditions (14), but following either a heat shock or serum starvation, both a new acidic and basic species was observed (14, 17). In contrast, approximately four eIF2β isoforms were observed in wheat leaves, the distribution of which did not change significantly following a heat shock (11).

eIF4A (47 kDa) is an ATP-dependent RNA helicase and an RNA-dependent ATPase in plants, animals, and yeast (31, 32). It can be found associated with eIF4G or eIFiso4G or as an independent factor (33, 34). Only a single, dephosphorylated form of mammalian or yeast eIF4A has been observed; however, in plants and Drosophila, a phosphorylated isoform has been detected (34, 35). Although dephosphorylated eIF4A normally predominates in leaves and mature seed, a maximum of 50% of the total available eIF4A can undergo phosphorylation following hypoxia or heat shock (11, 34).

Our previous observations showing that the phosphorylation state of several initiation factors is different in seed versus leaves of wheat (11) suggested that this was because of either tissue-specific differences or to differences in translational activity between mature seed (translationally quiescent) and young leaves (translationally active). This prompted us to examine whether the posttranslational modification of these factors may be subject to developmental control that would correlate with the regulation of protein synthetic activity known to occur during development. We found that the phosphorylation state of eIF4B, eIF2α, and eIF2β (and to a lesser extent, eIF4A) is developmentally regulated during seed development and germination but that the regulation of each differs temporally. The differences in the regulation of the phosphorylation state of these initiation factors may affect message selection or determine translational efficiency during plant development.

EXPERIMENTAL PROCEDURES

Antibody Preparation—eIF4A and eIF2 (31) were purified from wheat germ (commercially prepared wheat embryos) as described. The purification of recombinant eIF4B and eIF4A will be described elsewhere.3 Polyclonal antibodies to eIF2 and recombinant eIF4B and eIF4A were produced in rabbits and affinity-purified as described (33, 36). Antibodies raised against purified PABP were prepared as described previously (37).

Plant Extract Preparation and Two-dimensional Gel Electrophoresis/Western Blot Analysis—For the seed development studies, wheat plants were grown in a greenhouse to minimize any potential environmental effects and were allowed to self-pollinate. Whole seeds were collected at various stages of seed development and frozen in liquid nitrogen. For the germination studies, wheat seed or seedling tissues were dissected at various stages of germination and frozen. Total soluble protein extracts were prepared by grinding tissue in a mortar with liquid nitrogen and then in aqueous buffer (50 mm HEPES, pH 7.5, 120 mm KCl, 5 mm MgOAc, 1 mm dithiothreitol, 1 mm phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μM EDTA, and 0.5 μM okadaic acid). The cell debris was pelleted by centrifugation and the protein-containing supernatant was collected (38).

Depending on the factor examined, 5–100 μg of protein was loaded on IEF tube gels (3.6% acrylamide, 9% urea, 2% ampholytes, 2% Nonidet P-40) and run at 400 V for 4.5 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 electroblotting. 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 NaH2PO4, and 0.14 mm KH2PO4 followed by incubation with primary antibodies diluted 1:2000 for eIF4A and 1:1000 for eIF4B, eIF2α, and eIF2β in TBBS with 1% milk for 1.5 h. The blots were washed twice with TBBS and incubated with goat-anti-rabbit horseradish 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 was typically between 1 and 15 min using chemiluminescence (Amersham Pharmacia Biotech). The range of ampholytes used is indicated in the legend to each figure. 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 ml of 15 mm NaCl.

RESULTS

eIF4B Undergoes Dephosphorylation Late in Seed Development and Is Phosphorylated Early during Germination—To examine whether the phosphorylation state of eIF4B changes during seed development, wheat seed were collected at developmental stages following pollination. Soluble protein extracts prepared from whole seeds were resolved on 2D gels and analyzed using antibodies raised against recombinant eIF4B following Western blotting. The early development of wheat seed is characterized by a small endosperm and embryo and a large nucellus. In the early developmental time points (4–12 DAF), intermediate acidic isoforms of eIF4B were present with a second set of lower molecular weight isoforms also present (Fig. 1). This apparent degradation of eIF4B correlated with the programmed cell death of nucellar tissue that occurs at this stage in the development of the seed (T. Young and D. Gallie, manuscript in preparation). This observation is supported by the presence of only the full-length acidic isoforms of eIF4B at the next developmental stage (16 DAF) by which point the nucellus had virtually disappeared. Also by 16 DAF, the distribution of eIF4B isoforms had shifted to highly phosphorylated (acidic) species and remained unchanged through 30 DAF which corresponds to the period in which the bulk of protein synthesis takes place (3–5). However, at 30 DAF, the basic cluster of eIF4B isoforms appeared for the first time, representing a redistribution of the isoforms away from phosphorylated to dephosphorylated species, a process that continued through late seed development. By 45 DAF, the phosphorylated species had disappeared altogether, leaving only the basic cluster. The eIF4B isoforms were identical in the endosperm and embryo at this stage (data not shown), despite the fact that the embryo is viable whereas the endosperm has undergone programmed cell death (T. Young and D. Gallie, manuscript in preparation), data suggesting that dephosphorylation of eIF4B during development is not a consequence of cell death.

We next examined whether the distribution of eIF4B isoforms was regulated during germination. Imbibing seed were collected at time points and the embryo dissected. The endosperm was not examined as this is a dead tissue in germi-

3 A. M. Metz and K. S. Browning, unpublished data.
nating seed. Upon emergence of the shoot and roots at later stages of germination, these tissues were also collected and the samples were analyzed as described above. eIF4B was present as predominantly hypophosphorylated isoforms in seed before imbibition and up to 10 h of germination (Fig. 2). By 20 h, a shift to more phosphorylated isoforms was observed that was completed by 48 h of germination when the shoot had first emerged. eIF4B present in roots of 48 h germinated seed was also present as highly phosphorylated isoforms (Fig. 2). The observed shift of eIF4B from hypophosphorylated to hyperphosphorylated species during germination correlates with the resumption of protein synthesis in wheat (7–10).

eIF4A Is Phosphorylated at the Nucellus-containing Stage of the Young Seed but Not during Subsequent Seed Development or during Germination—The same extracts were used to determine whether the phosphorylation state of eIF4A changes during seed development and germination. Dephosphorylated eIF4A largely predominates in wheat leaves or maize roots under normal conditions but undergoes phosphorylation to a maximum 1:1 ratio following a severe heat shock or hypoxic treatment (11, 34). During early seed development when the nucellus predominates and undergoes programmed cell death (4–12 DAF), the two isoforms of eIF4A were present in approximately equimolar amounts (Fig. 3). When this maternal tissue had disappeared by 16 DAF, the dephosphorylated form of eIF4A once again predominated and the distribution of the isoforms remained little altered during the remaining development of the seed. This observation suggests that the phosphorylated form of eIF4A is present in the nucellus and may be targeted for inactivation or degradation during programmed cell death. During germination, only the dephosphorylated isoform was observed in the embryo (data not shown) and up through 2 day-old shoots (Fig. 4). However, the presence of both eIF4A isoforms was observed in 2 day-old roots (Fig. 4).

eIF2α Undergoes Progressive Phosphorylation during Seed Development and Is Dephosphorylated during Germination—In the embryo of mature seed, the α subunit of eIF2 is present in its most phosphorylated state (pI of 5.4–5.6) whereas in leaves it is completely dephosphorylated (pI of 6.2–6.4) (11). This large difference in pI is in striking contrast to that observed in animal cells in which the α subunit is subject to a single phosphorylation event at serine-51 (reviewed in Ref. 39) resulting in a small shift in the pI of the subunit from 5.4 to 5.6 (14, 40). This suggests that the α subunit of eIF2 in plants may be multiply phosphorylated. The number of β subunit isoforms also appears to differ between plants and...
FIG. 4. eIF4A is differentially phosphorylated in shoot and root tissue of wheat seedlings. 5 µg of soluble protein extract from leaves and roots (48 h) was resolved in the first dimension using IEF with 50%, pH 5–8, and 50%, pH 2.5–5, ampholytes and on a 10% SDS-PAGE gel for the second dimension. The eIF4A phosphorylated isoform is indicated with a downward pointing arrow.

animals. Whereas a single major mammalian eIF2β species is observed under normal growth conditions (14), at least four eIF2β isoforms were observed in wheat leaves (11). We examined, therefore, whether multiple isoforms of the α and β subunits were present in wheat seed and whether their distribution changed during seed development. As in leaves, approximately four major species were observed for the β subunit during early seed development (Fig. 5), although the distribution in seed included more acidic isoforms not detected in leaves. The two most basic species present at 4–12 DAF (Fig. 5) correspond to the middle two, and most dominant, species in leaves (11). With further development of the seed, a progressive redistribution toward the basic isoforms was observed such that by 35 DAF, only a single basic isoform predominated. This eIF2β basic isoform serves as a convenient reference point (see arrows, Fig. 5) for the changes in the eIF2α subunit isoforms described below. In contrast, small amounts of some of the phosphorylated eIF2β isoforms observed during mid-development remain present in mature embryos (see 0 h, Fig. 7 and (11)), suggesting tissue-specific (endosperm versus embryo) regulation of its phosphorylation state during late development.

At the earliest stages of seed development examined (4–12 DAF), multiple isoforms of eIF2α were detected (Fig. 6) but mostly were basic to intermediate acidic species and those at 12 DAF corresponded to those dephosphorylated species previously observed in young wheat leaves (11). By 16 DAF, a shift to slightly more acidic species was observed (Fig. 6). The most basic of the two α subunits observed at 16 DAF corresponded to the most acidic of the isoforms present at 12 DAF. The distribution of eIF2α isoforms remained unchanged up to 20 DAF. By 25 DAF, the isoforms that had been present at 16–20 DAF had disappeared and a new set of 3–4 highly acidic isoforms (pI range from 5.4 to 5.9) appeared, the most acidic of which (pI 5.4) corresponded to the fully phosphorylated α subunit isoform that was observed in mature wheat embryos (11). At 30 DAF, there was an increasing shift of the α subunit to the most acidic isoform and by 40 DAF, only the most phosphorylated isoform remained. At 40 DAF, a second, smaller molecular weight form of the α subunit was observed in addition to the full-length form. This was also observed with eIF2 purified from mature wheat embryos (11) and may be because of degradation of the α subunit during late seed development. At 45 DAF, the α subunit was no longer detectable despite the fact that the β subunit was still present, data suggesting that the α subunit is degraded during late endosperm development. In contrast, eIF2α remains present in mature embryos (see 0 h, Fig. 7 and (11)). These data demonstrate that the eIF2α subunit is present in a hypophosphorylated state during early seed development but undergoes a progressive shift to a highly phosphorylated state that begins at 16 DAF, is completed by 35 DAF, and is followed by degradation specifically in the endosperm at 45 DAF but not completely in mature embryos.

Upon imbibition, the α subunit was present solely in its highly phosphorylated form in the embryo (Fig. 7). No change in eIF2α phosphorylation in the embryo was observed between 10 and 20 h of germination, the point at which eIF4B was observed to undergo phosphorylation (see Fig. 3). Between 48 and 60 h of growth (see Figs. 7 and 8, respectively), the basic isoforms of the α subunit are detectable in the emerging shoot.
However, substantial levels of the most phosphorylated isoforms remained without the appearance of the intermediate species, suggesting that the two extreme forms of the α subunit temporally coexist during this stage of germination. This differs substantially from the observations made during seed development where the conversion of the α subunit from the hypophosphorylated to hyperphosphorylated state occurred en masse and the two extreme forms of the α subunit were temporally separate. Only by 4 days of growth had the α subunit been completely converted to the basic isoforms (Fig. 8). Similar results were observed in the roots of 4-day-old seedlings where the α subunit was present solely in its dephosphorylated form. Germination in the light promoted the conversion of eIF2α to its dephosphorylated state as dark-germinated seedlings contained nearly equimolar amounts of phosphorylated and dephosphorylated α subunits (Fig. 9). These data show that eIF2α subunit undergoes a shift from being present in a highly phosphorylated state during early germination to a hypophosphorylated state upon emergence of the shoot that occurs over a period of several days.

DISCUSSION

In this study, we observed that eIF4B was present in a hyperphosphorylated state during the period of seed development in which protein synthesis is most active (12–30 DAF) and underwent gradual dephosphorylation (35–45 DAF) during the late stage of development concomitant with the decline in protein synthetic activity that precedes the metabolically quiescent state of the mature seed. eIF4B remained in a hypophosphorylated state up to 10 h following imbibition but was rapidly converted to its hyperphosphorylated state between 20 and 48 h of germination.

In contrast, phosphorylation of eIF2α occurs earlier during seed development than does the dephosphorylation of eIF4B, and eIF2α undergoes dephosphorylation much later during germination than does eIF4B. Interestingly, conversion of eIF2α to a hyperphosphorylated state during seed development occurred in a concerted manner, suggesting either that its phosphorylation occurs in a distributive fashion or that phosphorylation of each site is under temporal control during development. During germination, however, dephosphorylation of eIF2α occurred without the significant appearance of the intermediate isoforms, even though both the hyperphosphorylated and hypophosphorylated isoforms were present simultaneously. This suggests that either eIF2α dephosphorylation occurs in a processive manner or that its phosphorylation is irreversible, and dephosphorylated eIF2α is generated only through new synthesis.

The presence of multiple eIF2α phosphorylation sites in plants appears to differ from the single site present at serine-51 in mammalian eIF2α (41, 42). As in mammalian cells, eIF2α activity in yeast is also regulated through phosphorylation at Ser-51, however, three additional sites close to the C terminus are constitutively phosphorylated in vitro and in vivo by casein protein kinases.
from one developmental stage to another. Whether the translational requirements of mRNAs expressed only during specific developmental stages differs is unknown. However, changes in the phosphorylation status of the protein synthetic machinery may be a means by which necessary changes in the translational environment are achieved during plant development.

Acknowledgments—We thank the Department of Botany and Plant Sciences, University of California, Riverside for the generous use of their greenhouse facilities.

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