Regulation of Acidic Ribosomal Protein Expression and Phosphorylation in Maize†

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Received January 15, 2002; Revised Manuscript Received June 3, 2002

ABSTRACT: Acidic ribosomal proteins (ARPs) are highly conserved phosphoproteins in eukaryotic organisms. They participate in translation regulation by interacting with eEF-2 elongation factors in the peptide elongation process. During maize germination, protein synthesis is tightly regulated by different mechanisms that are not yet clearly understood. The objective of this research is to characterize the expression patterns of the two maize ARPs (P1 and P2) and their phosphorylated status in germinating maize embryonic axes. Expression of P1 and P2 mRNA transcripts was analyzed by Northern blots with specific cDNA probes. Results indicated that both transcripts are among the mRNA stored pool of the quiescent axes and each displays a distinctive expression pattern during germination. P1 and P2 synthesis initiates very early in germination, as demonstrated by [^{35}S]methionine pulse-labeling experiments. This synthesis was not insulin/IGF-stimulated as the synthesis of the bulk of ribosomal proteins that was responsive to this stimulus. P1 and P2 proteins were purified from ribosomes of maize embryonic axes and their physicochemical characteristics determined. A cytoplasmic pool of dephosphorylated P1 and P2 proteins was found in axes of quiescent and germinated stages that freely assembled into the ribosomes. IEF analysis of ARPs revealed one P1 (P1-1) and two P2 (P2-1 and P2-2) forms in the ribosomes of 24 h germinated axes. Kinetic studies of ARP phosphorylation during germination revealed a specific order of phospho-ARP appearance, suggesting that this process is under regulation within this period. It is concluded that P1 and P2 phosphorylation rather than ARP expression or assembly into ribosomes is the main step that regulates ARP function in axes during maize germination.

Ribosomes are organelles composed by two unequally sized RNP subunits. A universally conserved feature in the ribosome structure, known as the stalk, has been mapped on one side of the large ribosomal subunit (*1*, *2*). In eukaryotes, the stalk is conformed by five phosphoribosomal proteins (P-proteins), P0, two P1, and two P2 acidic proteins (ARPs),[1] corresponding to their nonphosphorylated prokaryote homologues L10 and L7/L12 dimer (*3*, *4*). This structure has been recognized to participate in the peptide elongation process by interacting with the GTPase domain in the ribosome (*5*) and the elongation translation factor eEF-2 (*6*). In studied eukaryotes, P1 and P2 proteins are not ribosome assembled in the nucleolus with the bulk of ribosomal proteins (rps); instead, ARPs are in the cytoplasm, as a free pool, and incorporate into the ribosomes during translation (*7*, *8*). ARP phosphorylation is a distinctive feature of eukaryote ribosomes that, however, does not seem to be necessary for ribosome assembly (*9*) but rather is relevant for ribosome activity (*10*).

In plants, scarce information is available regarding ARPs; however, it is known that their molecular masses range between 12 and 17 kDa, as in most eukaryotes, and their cDNA deduced amino acid sequences indicate a similar structure to their animal and yeast counterparts (*10*−*12*). P1 and P2 proteins have been isolated from maize tissues (*13*) and their cDNA sequences reported (*14*, *15*), including a P3 cDNA expressed in roots during anoxia (*16*).

In quiescent seeds, protein synthesis is arrested (*17*). At this stage, all elements of the translational machinery are present in the axes tissues, and the ribosomes contain only nonphosphorylated ARPs. During germination, ARPs are phosphorylated, and protein synthesis reinitiates (*18*). At this important physiological stage, translation regulation is most relevant in order to ensure successful germination and further seedling establishment (*17*). Different mechanisms of translational control have been demonstrated to regulate protein synthesis during germination (*19*, *20*). Since ARP proteins play a relevant role in translation, a deep understanding of ARP expression, phosphorylation, and assembly into ribosomes was considered important to further define their functional role in maize germination. In this research, we found that ARP phosphorylation, rather than their expression or assembly into ribosomes, is tightly regulated, suggesting that this process might be the main step that regulates ARP function during germination.

EXPERIMENTAL PROCEDURES

Biological Material. Maize (*Zea mays* L) seeds var. Chalqueño were disinfected and germinated in cotton imbibed with water. The embryonic axes were manually dissected at the indicated periods as previously reported (*21*) and used for the following experiments.

† This work was supported by the Dirección General de Apoyo al Personal Académico (DGAPA), UNAM, Grants IN-201398 and IN-202900.

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[1] Abbreviations: ARP, acidic ribosomal protein; IEF, isoelectric focusing; rps, ribosomal proteins; IGF, insulin-like growth factor.

10.1021/bi020037r CCC: $22.00
Published on Web 07/12/2002

P1 cDNA Probe Synthesized by RT-PCR. Total RNA purified from 24 h germinated maize axes was treated with DNase before being used as template for RT-PCR. Three micrograms of total RNA was used to synthesize first-strand cDNA with an oligo(dT) primer and reverse transcriptase (Gibco-BRL) in a 20 μL volume. For the PCR reaction the oligonucleotide primers used were 5′(TGAGTGGAAGCCGCACGAA) and 3′(CTCGTCGTTGCGCTTCTC). Reactions were performed in a final volume of 50 μL. Twenty-five cycles of amplification were performed in a Gibco-BRL thermocycler. Each amplification cycle consisted of 1 min of denaturation at 94 °C, 1 min of annealing at 53 °C, and 2 min of extension at 72 °C. The amplified products were resolved by electrophoresis on 1% agarose gels. The P2 cDNA probe from maize (500 bp) was kindly provided by Dr. Pedro Ballesta (Centro de Biología Molecular "Severo Ochoa", CSIC-UAM, Spain). P1 and P2 cDNA probes were radiolabeled by random primer following the manufacturer's instructions (NEN's Random Primer Extension Kit; NEN, Boston, MA).

Similarly, a P3 cDNA probe was pursued by RT-PCR synthesis using oligonucleotide primers selected from distinctive regions of the P3 cDNA sequence (*16*) and RNA from 6 to 24 h germinated axes. However, negative results were obtained in all cases, indicating that the P3 transcript is not expressed in maize axes within germination.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated from seed embryonic axes germinated for different times (0, 6, 12, and 24 h) under sterile conditions by the guanidinium thiocyanate method (*22*). RNA (30 μg) was size-fractionated by formaldehyde-denaturing 1.2% agarose gel electrophoresis. RNA bands were blotted onto a Hybond N^+ paper, and the filters were UV-cross-linked and prehybridized in the hybridization buffer (7% SDS, 0.25 M Na_2HPO_4, pH 7.4, 1 mM EDTA, pH 8, 1% BSA) for 2 h in a hybridization incubator at 65 °C. The denatured radiolabeled probe was diluted into the buffer and hybridized for 20 h. The filters were washed once for 20 min at 65 °C in 2× SSC and 0.1% SDS and then for another 20 min at 65 °C in 1× SSC and 0.1% SDS; finally, the filters were washed for 15 min at room temperature in 0.5× SSC and 0.1% SDS.

Ribosome Isolation and ARP−Protein Extraction. Ribosomes were isolated from 24 h germinated axes according to the method described in ref *23*, with modifications as follows: the axes were homogenized to a fine powder in liquid N_2 and resuspended in 8 mL of extraction buffer A1 (20 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 20 mM KCl, 1 mM NaF, and 0.5% β-glycerophosphate). The homogenate was centrifuged at 27000*g* for 30 min and then at 250000*g* through a sucrose cushion (0.5 M sucrose and 0.5 M KCl in buffer A1) for 3.5 h. The ribosomal pellet was resuspended in buffer containing 10 mM Tris-HCl, pH 7.4, 12 mM $MgCl_2$, 80 mM KCl, and 5 mM β-mercaptoethanol plus 1 volume of ethanol−ammonium buffer (1.5 M NH_4Cl, 20 mM $MgCl_2$, 3 mM β-mercaptoethanol, and 20 mM Tris-HCl, pH 7.4) according to ref *24*. The pellet was discarded, and P-proteins were precipitated from the supernatant with 2.5 volumes of acetone at −20 °C overnight. Proteins were measured by the Bradford method (*25*).

Immunoprecipitation of Cytoplasmic ARP Proteins. The postribosomal supernatant from the 250000*g* axes homogenate was precipitated with 5 volumes of acetone at −20 °C, allowed to stand overnight, and centrifuged at 5000*g*. After acetone evaporation, the pellet was ethanol−ammonium extracted, as indicated in the procedure for P-protein isolation. P1 and P2 were resuspended in buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 0.05% Nonidet P-40) and immunoprecipitated with rat P-protein antibody (1:1000) as previously reported (*26*), except that the Sepharose A−IgG P-protein complex was eluted with 9.5 M urea.

Polyacrylamide Gel IEF of Ribosomal Proteins. P1 and P2 proteins were isolated from KCl−sucrose-purified ribosomes and resolved by IEF on 5% polyacrylamide and 8 M urea gels and pH 2.5−5.0 ampholytes (Sigma) for 18 h in a vertical slab-gel unit from Höefer (*8*). Protein bands were visualized by the silver stain method. Slab gels were dried in a gel dryer (Höefer Scientific Instruments, San Francisco, CA).

ARP Protein Synthesis and Their Regulation. Maize seeds (100 seeds) were germinated for 6, 12, 18, and 24 h at 24 °C. The axes were manually obtained and pulse labeled with 100 μCi of [^{35}S]methionine (specific activity 1155 Ci mmol^{-1}; NEN Dupont) and incubated for 2 h in MS medium (*27*) in a final volume of 1.5 mL. The axes were then rinsed three times with deionized water and frozen in liquid N_2 until used. Axes were homogenized and centrifuged, and ARPs were obtained from supernatant proteins (see below). Duplicate aliquots of ARPs from each period were counted in a scintillation counter (Minaxi β from Packard instrument Co.). In another set of experiments, 24 h germinated maize embryonic axes were incubated with [^{35}S]methionine as indicated above, and either 200 microunits of insulin and 0.1 μg of worthmannin or none (control) were added to the system. ARPs were isolated as indicated above, and bulk of the ribosomal proteins was extracted from ribosomes according to Beltrán-Peña et al. (*28*). Both, ARPs and the bulk of ribosomal proteins, were dissolved and counted in a scintillation counter.

RESULTS

Expression Patterns for P1 and P2 Proteins. P1 and P2 transcripts were analyzed in embryonic axes at different germination periods. Embryonic axes from quiescent (0 h) and germinated for 6, 12, and 24 h seeds were dissected and used to extract total RNA. Thirty micrograms of RNA from each sample was resolved by 1.2% agarose electrophoresis, transferred, and tested with labeled P1 or P2 cDNA probes by Northern blot. The RNA from quiescent axes showed two hybrid bands, one of 0.6 kb for P1 and the other of 0.7 kb for P2 cDNA probes (Figure 1), indicating that both mRNAs were pretranscribed and stored in the quiescent tissues. Furthermore, the transcript germination patterns performed along the germination period indicated expression differences between both messages. In repeated independent experiments, P1 showed a rather constant level through the 24 h period whereas P2 consistently increased during all the period, reaching approximately 3−4-fold of its initial value (Figure 1).

Regulation of ARP Synthesis. ARP synthesis was assessed by applying a 2 h pulse of [^{35}S]methionine to maize axes germinated for different periods. ARPs were inmunoprecipitated from the ethanol−ammonium-extracted cyto-

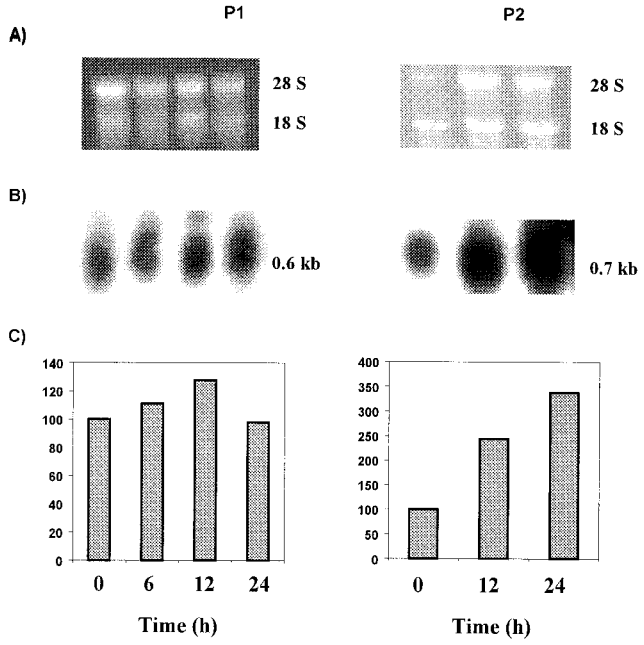

FIGURE 1: Northern blot analysis of ARP transcripts during germination. Total RNA (30 μg) of maize axes at each stated period (0, 6, 12, 24 h) was resolved by formaldehyde−agarose gels, transferred to nitrocellulose membranes, and hybridized with either P1- or P2-labeled cDNA probes. (A) Ribosomal RNA revealed by ethidium bromide was used as load control. (B) Autoradiography of the P1 and P2 cDNA hybrids at 0.6 and 0.7 kb, respectively. (C). Densitrometric analysis of data shown in (B) after correction by RNA loading. Plots are expressed in relative values using 0 h as 100%. The above data are representative of at least three independent experiments.

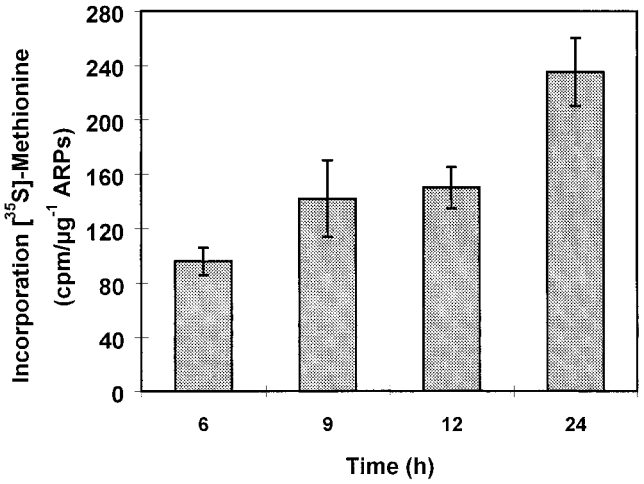

FIGURE 2: ARP synthesis during germination. Embryonic axes of 6, 9, 12, or 24 h germinated seeds were incubated for the last 2 h with [^{35}S]methionine (100 μCi, specific activity 1155 Ci mol^{-1}). ARPs were ethanol-extracted from the acetone-precipitated cytoplasmic proteins, resuspended, and immunoprecipitated with rat ARP antibodies (dilution 1:1000). Aliquots were measured in a scintillation counter, and the values were corrected by total [^{35}S]methionine uptake and plotted per milligram of protein.

plasmic fraction, and the [^{35}S]methionine incorporated was measured. Results showed early incorporation (6 h) of the labeled amino acid into these proteins that progressively increased up to 24 h (Figure 2).

It has been demonstrated that, in both animal (*29*) and plant (*30*) organisms, the synthesis of bulk ribosomal proteins is regulated by an insulin/IGF-induced signal transduction pathway that selectively recruits their mRNAs into polysomes for translation (*31*). To investigate whether ARP synthesis is regulated by this mechanism, axes from 24 h germinated seeds were pulse labeled as above for 2 h with [^{35}S]methionine in the presence of either insulin, insulin plus worthmannin (inhibitor of the insulin-stimulated signal transduction pathway), or water (control). At the end of the period, ARP proteins were isolated from both cytoplasmic and ribosomal fractions, and the incorporated [^{35}S]methionine was measured in each group of axes as before. Results from independent repeated experiments demonstrated a slight but reproducible decrease of [^{35}S]methionine incorporation into ARPs in both the cytoplasmic and the ribosomal fractions of the insulin-stimulated axes as compared with the control (100%) (Figure 3A). Worthmannin reversed the insulin-induced inhibition up to values slightly higher than the control (Figure 3A). On the other hand, the same measurements made on the bulk of the ribosomal proteins showed increased incorporation of [^{35}S]methionine in these proteins that was inhibited by the worthmannin treatment (Figure 3B). These data indicate that neither the synthesis of ARPs nor their assembly into ribosomes is regulated by the insulin/

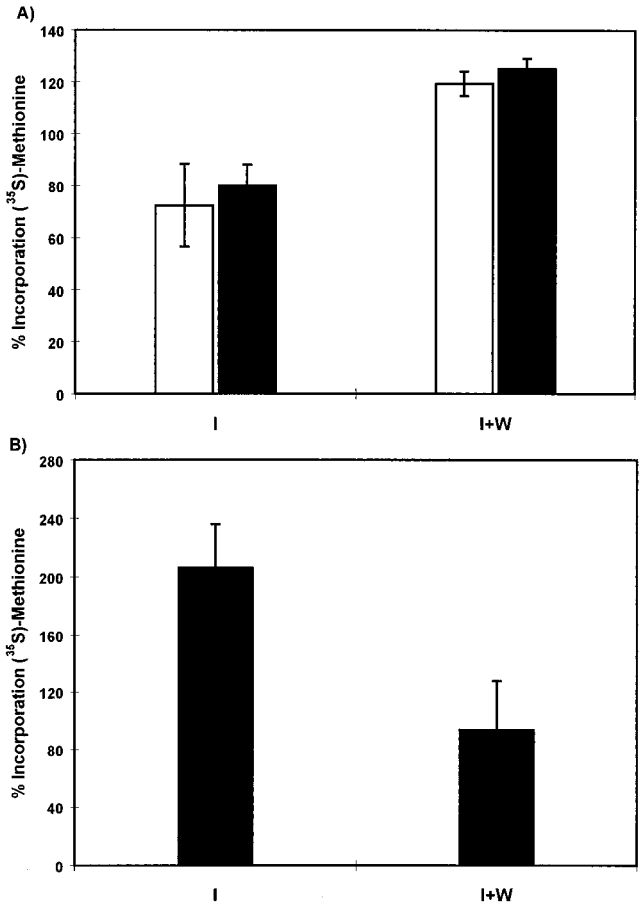

FIGURE 3: Effect of insulin on ARP synthesis. Maize embryonic axes of 24 h germinated seeds were incubated for the last 2 h with [^{35}S]methionine (as in Figure 2) and 200 microunits of insulin (I) [alone or with 0.1 μM worthmannin (W)] or none (control = 100%). After the incubation period, ARPs were isolated from (A) the cytoplasm by acetone precipitation, ethanol−ammonium extraction, and immunoprecipitation (□) or from purified ribosomes (■). (B) The bulk of ribosomal basic proteins was also obtained. The samples were counted as indicated above. Values represent the average ± SD of at least three independent experiments expressed as percentage of incorporation. Control values were taken as 100%.

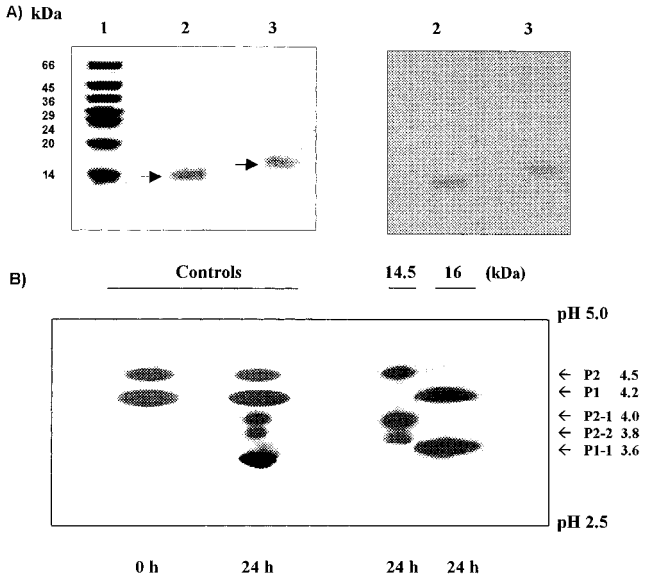

FIGURE 4: Characterization of purified P1 and P2 proteins from maize axes. (A) SDS−PAGE and Western blot. Maize axes from 24 h germinated seeds were used to purify ribosomes through sucrose−KCl buffer by centrifugation. ARPs were ethanol−ammonium extracted, isolated from these ribosomes, and resolved by SDS−PAGE. Each band (P1 and P2) was cut, electroeluted, and analyzed again by SDS−PAGE and Coomasie blue stained (left) (well 1, MM standards) and transferred to nitrocellulose for Western blot (antibody dilution 1:1000) (right). Two bands of 14.5 and 16 kDa were recognized by each assay (wells 2 and 3). (B) Isoelectric focusing of purified P1 and P2 proteins. Twenty to thirty micrograms of each purified 14.5 or 16 kDa ARP obtained by electroelution from the SDS−PAGE was analyzed by IEF from pH 5.0 to 2.5 (wells 2 and 3, respectively). Isolated proteins from 0 and 24 h germinated axes were used as controls. Arrows point to nonphosphorylated P2 and P1 peptides and their more acid forms: P2-1, P2-2, and P1-1.

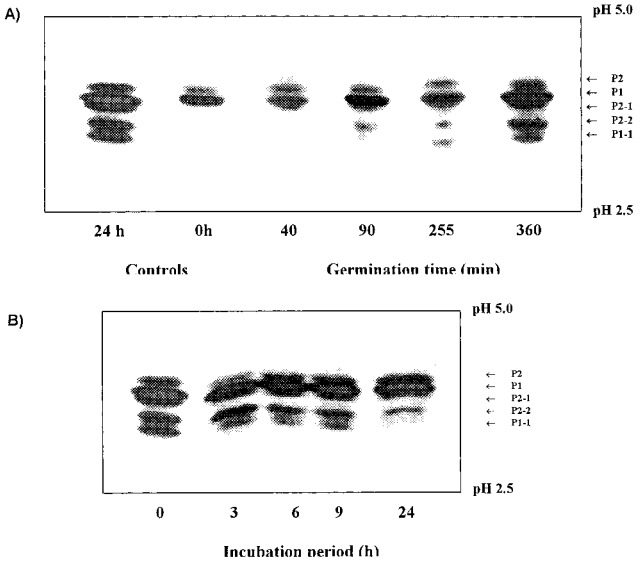

FIGURE 5: Kinetics of ARP phosphorylation/dephosphorylation. (A) In vivo ARP phosphorylation. Maize axes from seeds germinated for 0−6 h were used as a source of ribosomes and their ARPs resolved by IEF (pH 5.0−2.5) and silver nitrate stained. ARPs from 24 h germinated axes were set as control. P2, P1, P2-1, P2-2, and P1-1 proteins are indicated by arrows. (B) In vitro ARP desphosphorylation. Ribosomes obtained from 24 h germinated axes (phosphorylated ARPs) were in vitro incubated with alkaline phosphatase (Alk-P; Sigma) (the 50 μL incubation system contained 1.4 units of Alk-P and 20 mM Hepes−KOH, pH 7.6) for different incubation periods. Their ARPs were resolved by IEF (pH 5.0−2.5). Acidic ribosomal proteins from 24 h germinated axes without alkaline phosphatase were set as control (0 h).

IGF-dependent signal transduction pathway that regulates the bulk of ribosomal protein synthesis (*30*).

Identification of P1 and P2 Proteins and Their Phosphorylated Forms. Previous results from our group (*18*) have shown the presence of phosphorylated P1 and P2 proteins in ribosomes of germinating maize axes and their nonphosphorylated forms in the corresponding cytoplasm. To fully identify and characterize these proteins, purification of each ARP was undertaken. Ribosomes were purified from homogenates of 24 h germinated axes by centrifugation through a high molar KCl−sucrose gradient to eliminate contaminating proteins. This procedure provided ribosomes with OD 260/280 ratios above 1.86. ARPs were ethanol−ammonium extracted from these ribosomes and resolved through SDS−PAGE and Coomasie blue stained. Two main bands of 16 and 14.5 kDa were observed in the gels. Each band was electroeluted, electrophoresed again, and blotted to test its identity by Western blot (Figure 4A). Each ARP was then analyzed by IEF to resolve its phosphorylated forms (more acidic bands). The 14.5 kDa protein showed three bands: a nonphosphorylated band (p*I* 4.5) and two other p*I* 4.0 and 3.8 bands, whereas the 16 kDa protein showed only two forms: the p*I* 4.2 nonphosphorylated form and the more acid p*I* 3.6 (Figure 4B). As control, ARPs from quiescent axes ribosomes (0 h), which contain only the nonphosphorylated P1 and P2 forms, and ARP proteins from 24 h germinated axes, which contain the more acid phosphorylated ARP forms (*18*), were also IEF resolved (Figure 4B). By these means the corresponding phosphorylated and nonphosphorylated bands from each of the two ARPs were identified.

The maize cDNA-deduced amino acid sequence (*16*) of each P-protein indicates that P1 and P2 have a very similar amount of amino acids (112 vs 109 aa) but a higher predicted acidic isoelectric point for P2 than for P1 (p*I*s of 4.36 vs 4.23, respectively), similar to other eukaryote ARPs (*32*). From the above data it was concluded that maize P1 corresponds to the 16 kDa (p*I* 4.2) whereas P2 to the 14.5 kDa (p*I* 4.5). Interestingly, these results indicate that, during germination, maize P2 has two different more acid forms (P2-1 and P2-2) and only one of P1 (P1-1). These patterns also showed that nonphosphorylated P1 protein is more abundant than P2; this is particularly observed in ribosomes from quiescent axes (Figure 4B).

Kinetics of ARP Phosphorylation/Desphosphorylation during Germination. ARP phosphorylation was analyzed in vivo in ribosomes from embryonic axes at different germination periods. Results from IEF analysis showed that, as soon as axes are released from their quiescent stage, ARP phosphorylated forms are detected, first P2-1, then P2-2, and later P1-1 (Figure 5A), indicating a precise phosphorylation sequence for these proteins. To analyze the reverse process, phosphorylated ribosomes from 24 h germinated axes were in vitro incubated with alkaline phosphatase (1.4 units) for different time periods (Figure 5B). The ARP dephosphorylation process was followed by IEF of the corresponding P-protein. Results indicated progressive ARP dephosphorylation that affected first the P1 peptide and then P2, suggesting that the phosphorylated P1 and P2 proteins are not equally

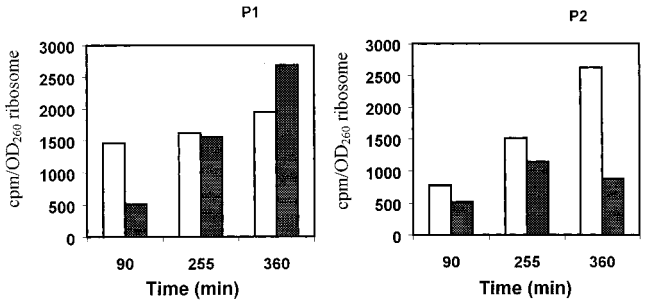

FIGURE 6: ARP assembly into ribosomes. Maize seeds were germinated for different periods, and axes (100 per group) were dissected and incubated for the last 90 min in MS medium with 100 μCi of [^{35}S]methionine (specific activity 1155 Ci mmol^{-1}). Ribosomes were purified from them and ARPs isolated and resolved by IEF (pH 5.0−2.5). The corresponding P1 and P2 bands [(■) phosphorylated form, (□) nonphosporylated form] were cut and counted in a scintillation counter. The average values of two independent experiments after [^{35}S]methionine uptake correction are plotted for P1 and P2.

accessible to the alkaline phosphatase action. It shall be pointed out that the P2-1 band still remained in the IEF profile even after extensive ribosome phosphatase alkaline incubation (48 h incubation with 4.2 units), suggesting that this band is not a P2 phosphorylated form; rather it might correspond to another type of postranslational modified P2. Taken together, the above data indicate that ARP phosphorylation/dephosphorylation is a tightly regulated process in axes ribosomes during germination.

P1 and P2 Assembly into Ribosomes. It was then important to find out whether ARP incorporation/exchange between the nonphosphorylated cytoplasmic ARPs and the P1 and P2 proteins already assembled in the ribosomes occurs simultaneously to the phosphorylation process during germination. To answer this question, pulse-label experiments were performed by incubating maize axes with [^{35}S]methionine at different germination periods, from 90 to 360 min, and the [^{35}S]methionine pulse was applied for the last 90 min. At the end of each period, the ribosomes were isolated, and their assembled P1 and P2 proteins were extracted and resolved by IEF. The amount of labeled P1 and P2 incorporated into the ribosomes was measured as an indication of newly synthesized ARP assembly. [^{35}S]Methionine was found to be incorporated in all ribosome bands, even the nonphosphorylated ones, since the earliest germination period (Figure 6). It shall be pointed out that, at this early stage, phosphorylation were almost negligible in the ribosomes (Figure 5A), strongly suggesting that the ARP cytoplasmic/ribosome assembly process occurs freely during germination and is not dependent on ARP phosphorylation.

DISCUSSION

Regulation of ARP Expression in Germinating Axes. This research describes maize ARP specific features which are shared with other nonplant higher eukaryote ARPs (*11*, *32*, *33*), such as their molecular masses, P1 (16 kDa) and P2 (14.5 kDa) (Figure 4A), and p*I* values for the unphosphorylated native proteins (pH 4.2−4.5) (Figure 4B). This agrees with the notion of ARPs being highly conserved through evolution due to their important role on protein synthesis regulation (*10*).

P1 and P2 transcripts were found among the stored set of mRNAs in quiescent maize axes (Figure 1) similar to other rp mRNAs (*28*). Their transcription patterns during germination, however, differ significantly; i.e., whereas P1 mRNA remains steadily, P2 mRNA dramatically increases during

Organism	Protein	c-Terminal aa sequence	Accession No.
Zea mays	P1	----EKKEEAKEE**S**DDDMGF**S**LFD	U62752
Clamydomonas reinhardtii	P1	-------EKKEP**S**EEEDMGF**S**LFD	X66411
S. cerevisiae	P1α	----KEEEEAKEE**S**DDDMGFGLFD	M26504
S. cerevisiae	P1β	----EKEEEAAEE**S**DDDMGFGLFD	M26507
D. melanogaster	P1	----KKKEEE**S**DQ**S**DDDMGFGLFD	Y00504
Rattus norvegicus	P1	----EAKKEE**S**EE**S**EDDMGFGLFD	X15097
Homo sapiens	P1	----EAKKEE**S**EE**S**DDDMGFGLFD	M17886
Zea mays	P2	-----EKVEEKEE**S**DDDMGF**S**LFD	U29383
Oryza sativa	P2	----EDKVEEKEE**S**-DDDMG**S**LFD	D16065
Arabidopsis thaliana	P2	------KKEEKEE**S**DDDMGF**S**LFE	P51407
Parthenuim argentatum	P2	---PKKEEKSEEE**S**DEELGF**S**LFDDN	P41099
S. cerevisiae	P2α	----EKEEEAAEE**S**DDDMGFGLFD	M26503
S. cerevisiae	P2β	----EKEEEAKEE**S**DDDMGFGLFD	M26505
D. melanogaster	P2	----EEKKEE**S**E**S**EDDDMGFGLFE	X05016
Rattus norvegicus	P2	----DEKKEE**S**EE**S**DDDMGFGLFD	X15098
Homo sapiens	P2	----DEKKEE**S**EE**S**DDDMGFGLFD	M17887

FIGURE 7: P1 and P2 cDNA amino acid deduced sequence alignment. The deduced amino acid C-terminal end of maize P1 and P2 proteins is compared with their homologues from green algae *Chlamydomonas*, plants *Oryza sativa*, *Arabidopsis thaliana*, and *Parthenium argentatum*, and other eukaryotes, *S. cerevisiae* and *D. melanogaster*, and mammals (*Rattus* and *Homo sapiens*). Boxes comprise the amino acid conserved dodecapeptide of the C-terminal end. The conserved putative phosphorylated serine is in bold, and the novel serine present in plant dodecapeptides is also in bold and is indicated by an arrow.

this period (Figure 1) as an indication of different regulation expression mechanims for both transcripts. It, however, remains to be determined whether these ARP transcript patterns are the result of different transcription rates or mRNA stabilities. Indeed, in general terms, there is scarce information regarding P1/P2 expression regulation in higher eukaryotes. In contrast, several reports indicate that the bulk of ribosomal protein (rps) expression is translationally regulated, through an insulin/IGF-stimulated signal transduction pathway (*34*). This mechanism comprises a cascade of reactions leading to S6 rp phosphorylation on the 40S ribosomal subunit and selective mobilization into polysomes of mRNAs with a signal sequence at their 5′UTR known as 5′TOP (track of oligopyrimidines) (*35*). Among this group, rp mRNAs have been identified (*29*, *31*). In maize axes, it has been shown that the bulk of rp synthesis is regulated by a similar pathway stimulated either by insulin (*30*) or insulin-like endogenous maize IGF (ZmIGF) (*36*). Since the 5′UTRs of P1 and P2 cDNAs contain pyrimidine sequences similar to those reported in the 5′TOP mRNAs (*16*), it has been suggested that P1 and P2 synthesis is regulated by the same mechanism (*32*). The experimental data recorded here, however, indicate that it is not the case (Figure 3). Indeed, axes stimulated with insulin did not show an increase in ARP synthesis; on the contrary, slight but reproducible ARP synthesis inhibition was observed (Figure 3). On the other hand, the bulk of rp synthesis showed a strong increment after insulin stimulation, as previously reported (*30*). Further, worthmannin, an specific inhibitor of the insulin-stimulated signal transduction pathway (*31*), inhibited the effect on the bulk of rp synthesis but does not affect ARP synthesis (Figure 3). Considering that the bulk of rps are ribosome-assembled in nucleoli, on a fixed one to one representation basis, whereas P1 and P2 proteins are incorporated into ribosomes from an abundant cytoplasmic free pool, on a variable P1/P2 ratio basis (Figures 3 and 5), it is not surprising that the synthesis of the two types of rps would be regulated by different mechanisms. Following the above reasoning, and considering there is free ribosome assembly of synthesized ARPs (Figures 3 and 6) in germinating maize axes, it can be concluded that P1 and P2 expression regulation does not seem to constitute a limiting step to determine ARP ribosomal content.

ARP Phosphorylation on Maize Axes Ribosomes. ARP phosphorylation has now been recognized not to be necessary for ARP assembly into ribosomes (Figures 5 and 6) (*8*, *18*) but rather to play an important role in protein synthesis regulation (*6*, *37*). The ARP phosphorylation patterns observed in maize axes during germination are consistent with heterogeneity in ribosomal ARP phosphorylated composition. This ribosome heterogeneity would imply functional differences, considering P1/P2 interactions in the pentameric stalk structure (*38*), and that phospho-P2 but not phospho-P1 plays a direct role in the eEF-2-dependent elongation step (*39*) and GTPase activity (*6*) in the translation process. Within this picture, regulation of P2 phosphorylation might represent a translational control mechanism for protein synthesis regulation during specific developmental/differentiation stages.

Finally, an interesting observation is an amino acid change in the maize ARP C-terminal conserved dodecapeptide. The cDNA-deduced amino acid sequence of this C-terminal peptide in all plants reported shows a serine in the place of the last glycine, present in nonplant eukaryotes (Figure 7, arrow). This feature is important considering the strong evolutionary pressure on this ARP dodecapeptide sequence (Figure 7). This difference might also refer to the evolutive divergent period between animal and plant kingdoms.

In summary, the data presented indicate that ARP synthesis is not regulated in maize by the same insulin-sensitive mechanism as the bulk of ribosomal proteins. Further, the tightly regulated ARP phosphorylation process found in maize axes during germination strongly suggests that it constitutes a mechanism of translational regulation.

ACKNOWLEDGMENT

The authors are grateful to Dr. Juan Pedro Ballesta, Centro de Biología Molecular, Universidad Autónoma de Madrid, Spain, for providing maize P2 cDNA and for fruitful discussions on the research subject.

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BI020037R