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Research paper

Regulation of ribosome biogenesis in maize embryonic axes during germination

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ABSTRACT

Ribosome biogenesis is a pre-requisite for cell growth and proliferation; it is however, a highly regulated process that consumes a great quantity of energy. It requires the coordinated production of rRNA, ribosomal proteins and non-ribosomal factors which participate in the processing and mobilization of the new ribosomes. Ribosome biogenesis has been studied in yeast and animals; however, there is little information about this process in plants. The objective of the present work was to study ribosome biogenesis in maize seeds during germination, a stage characterized for its fast growth, and the effect of insulin in this process. Insulin has been reported to accelerate germination and to induce seedling growth. It was observed that among the first events reactivated just after 3 h of imbibition are the rDNA transcription and the pre-rRNA processing and that insulin stimulates both of them (40–230%). The transcript of nucleolin, a protein which regulates rDNA transcription and pre-rRNA processing, is among the messages stored in quiescent dry seeds and it is mobilized into the polysomal fraction during the first hours of imbibition (6 h). In contrast, *de novo* ribosomal protein synthesis was low during the first hours of imbibition (3 and 6 h) increasing by 60 times in later stages (24 h). Insulin increased this synthesis (75%) at 24 h of imbibition; however, not all ribosomal proteins were similarly regulated. In this regard, an increase in RPS6 and RPL7 protein levels was observed, whereas RPL3 protein levels did not change even though its transcription was induced. Results show that ribosome biogenesis in the first stages of imbibition is carried out with newly synthesized rRNA and ribosomal proteins translated from stored mRNA.

1. Introduction

Growth of an organism can be the result of an increase in cell size and/or cell proliferation. In both cases, proteins constitute the main component of the dry cell mass [1]. The amount of proteins that can be synthesized depends on the number of ribosomes; therefore, ribosome biogenesis is a determinant process for organism growth [2,3]. Ribosome synthesis requires the coordinated production of rRNA and ribosomal proteins (RPs) [4]. Cell growth in eukaryotes is regulated by the PI3K-TOR signaling pathway in response to nutrient levels, environmental stress as well as insulin and insulin-like growth factors (IGF's) [5–7]. In this regard, it has been reported that insulin and IGF's show mytogenic activity in animals [8]. In animals and yeast, this pathway is coupled to nutrient availability that regulates ribosome biogenesis by two simultaneous mechanisms: by controlling the 5S rRNA transcription [9], the transcription and processing of the pre-rRNA (which includes the 25S, 18S and 5.8S rRNAs) and by promoting the mobilization of mRNA encoding for ribosomal proteins to the polysomes for translation [10]. Also, it has been shown that insulin increases the rDNA transcription and the number of ribosomes in primary mouse fibroblasts cultures. This increase was not associated with an increase of the RNA polymerase I, but with an increase in the levels of the UBF and PAF53 transcription factors, as well as by the activation of UBF by phosphorylation. These transcription factors participate in the formation of the initiation complex during rDNA transcription and in the regulation of RNA Pol I activity [11–13]. In addition, it has been observed that insulin increases the

Abbreviations: RP, ribosomal protein; IGF's, insulin-like growth factor; ZmIGF, insulin-like growth factor of *Zea mays*; MS, Murashige and Skoog medium; ETS, external transcribed spacer; ITS1, internal transcribed spacer 1; ITS2, internal transcribed spacer 2.

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synthesis of ribosomal proteins in mouse myoblasts [14] by increasing the number of ribosomes [15] while rapamycin, a TOR-kinase inhibitor, blocks the rRNA precursors processing in HeLa cells [16].

There is still little knowledge about the regulation of ribosome synthesis in plants. It has been reported that there is an increase in pre-rRNA levels whereas rDNA transcription is reactivated sequentially in different tissues, going from the cortex to the exterior [17]. Recently, it has been shown that the TOR kinase domain works as a transcription factor regulating the 45S pre-rRNA synthesis in *Arabidopsis thaliana* [18]. Also, the existence of ribosomal proteins with a dual role, that is proteins that have other functions besides participating in the ribosome structure, has been reported. RPL3 is a protein with dual function which according to previous results participates in ribosome biogenesis [19]. During maize seed germination, there is active translation of stored mRNAs among which some ribosomal proteins are present [20].

The presence of IGF's has been reported in spinach, *Lemma gibba* G3 and *Canavalia ensiformis*; some of them stimulate glucose uptake in animals [21,22]. A 5.7 kDa insulin-like peptide named ZmIGF, which accelerates germination and promotes maize seedlings growth has been isolated from maize [23,24]. Even though insulin has not been found in plants, it has been reported that this hormone and IGFs accelerate germination and activity of enzymes of the glyoxysome of several seed species [25]. In this regard, Oliveira et al. [26] reported that the treatment with insulin significantly increases radicles and coleoptiles length and weight of *C. ensiformis* seeds. In addition, these authors isolated insulin-binding proteins, which are probable components of the signaling pathway (receptor, phosphoserine kinases and other associated proteins). Also, orthologues of TOR and S6K proteins have been identified in maize seeds and there are evidences that support the existence of the PI3K-TOR signaling pathway which regulates cell growth in response to insulin in a similar way to what is reported for mammals [27–30].

In previous studies it was observed that ZmIGF induces ribosomal protein synthesis in germinating maize seeds [24,28]. However, not all ribosomal proteins are regulated in the same way by this factor [31]. This differential effect might be explained by the dual function that some of the RPs have besides participating in the ribosomal structure; for example, S6RP [32,33], the acidic proteins [34] and RACK1, which also regulate translation [35,36]. Another RP with dual function is the RPL3; when this protein was silenced in *Nicotiana tabacum*, the accumulation of pre-rRNA and number of cells decreased, supporting its participation in the ribosome biogenesis [19].

Several proteins that participate in rDNA transcription and pre-rRNA processing have been identified in plants. In this regard, Harscoët et al. [37] characterized a nucleolar protein in Arabidopsis, NOF1, which regulates rRNA expression. On another study, a nuclear phosphoprotein, MA16, which interacts with rRNA and with other proteins that participate in the rRNA metabolism was characterized in embryos from maize seeds [38,39]. Also, an enzymatic complex named NF D formed by almost 30 proteins, which participates in the processing of pre-rRNA, was identified in *A. thaliana* [40]. Nucleolin, a non-ribosomal protein, widely distributed from yeast to plants and mammals, which regulates several aspects of the DNA and RNA metabolism, such as chromatin relaxation, rDNA transcription, pre-rRNA processing, ribosomes assembly and nucleus–cytoplasm transportation of pre-ribosomal particles, is part of the NF D complex [41,42].

Since ribosome biogenesis is a pre-requisite for cells to grow and proliferate and it has been demonstrated that ZmIGF/insulin induce selective translation of ribosomal protein mRNAs and regulates growth during maize germination by similar mechanisms; in the present work, insulin was used as an effector for studying ribosome biogenesis in maize seeds during germination.

2. Materials and methods

2.1. Biological material and treatments

Embryonic axes dissected from maize (*Zea mays* L. cv. Chalqueño) seeds were imbibed in Murashige and Skoog (MS) medium during 3, 6, 12, 18 and 24 h. The last 2 h of imbibition, axes were incubated in MS medium with a pulse of 200 μU ml^{-1} of insulin according to the methodology described by Buentello et al. [43]. Then, axes were rinsed with sterile water, frozen with liquid N_2 and stored at −70 °C until they were used. All experiments were performed in triplicate. Insulin concentration and time of treatments have been previously demonstrated to be effective in inducing maize seedling growth [28,44]. Due to its availability, insulin was used instead of ZmIGF to stimulate the maize embryonic axes.

2.2. Isolation of total RNA and RNA from the polysomal fraction

Embryonic axes were incubated for 3 and 6 h with or without insulin as described in Section 2.1. Total RNA was isolated from 0.1 g of embryonic axes pulverized with liquid N_2 using the Trizol reagent (BRL, Life Technologies, Invitrogen, Carlsbad, CA, USA) with the modifications recommended for plant tissues. Polysomes were isolated from 0.5 g of embryonic axes by differential centrifugation according to the methodology reported by Dinkova et al. [28]; RNA was isolated from the obtained polysomes. Both, total and polysomal RNA were resuspended in 50 μL of RNAse free water. Purity of the RNA was determined by the relation A_{260nm}/A_{280nm} and its concentration was quantified using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE. USA). Integrity of all samples was verified using 2 μg of total RNA or RNA from the polysomal fraction following the methodology of Sambrook et al. [45].

2.3. De novo synthesis of ribosomal RNA

Maize seeds were imbibed for 24 h; during the last 2 h they were treated with or without insulin in the presence of 300 μCi of [^{32}P]-orthophosphate. RNA from the polysomal fraction was obtained as described in Section 2.2 [28]. Twenty micrograms of RNA were separated by electrophoresis under denaturing conditions (MOPS/formaldehyde); afterward, the bands corresponding to 18S and 25S rRNA were excised and the [^{32}P] incorporated was determined in a Beckman scintillation counter using 5 ml of scintillation liquid ACS® (Amersham Biosciences). Results were reported as cpm μg^{-1} of polysomal RNA.

2.4. Northern blot analysis of the rRNA precursors

Total RNA (10 μg from each sample) was separated by electrophoresis under denaturing conditions (MOPS/formaldehyde) and then transferred to a nylon Hybond N^+ membrane in 10× SSC buffer. The membrane was cross-linked and prehybridized with PSE buffer (1 M sodium phosphate, 10% (w/v) SDS, 0.5 M EDTA, pH 7.2) for 10 min. Fragments were amplified from pre-rRNA (including ITS1, 5.8S and ITS2 sequences) or mature 18S and 25S ribosomal RNAs based on the published maize sequences (Accession numbers AF019817, AF168884 and AY097330, respectively). For amplifying the pre-rRNA, the following primers were used: Fwd: 5′-TGACCCTTAAACAAAACAGACC-3′ and Rv: 5′-CACCGAGAACAACAACTTG-3′. For 18S rRNA the following primers were used: Fwd: 5′-GTGGCCTAAACGGCCATAGTCCCTC-3′ and Rv: 5′-GGAAACTTACCAGGTCCAGAGATAG-3′ and for the 25S rRNA: