Changes in the Pattern of Protein Synthesis during Zoospore Germination in Blastocladiella emersonii

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Using two-dimensional gel electrophoresis, we analyzed the pattern of proteins synthesized during Blastocladiella emersonii zoospore germination in an inorganic solution, in both the presence and absence of actinomycin D. During the transition from zoospore to round cells (the first 25 min), essentially no qualitative differences were noticeable, indicating that the earliest stages of germination are entirely preprogrammed with stored RNA. Later in germination (after 25 min), however, changes in the pattern of protein synthesis were found. Some of these proteins (a total of 6 polypeptides) correspond possibly to a selective translation of stored messages, whereas the majority of the changed proteins (22 polypeptides) corresponds to newly synthesized mRNA. Thus, multiple levels of protein synthesis regulation seem to occur during zoospore germination, involving both transcriptional and translational controls. We also analyzed the pattern of protein synthesis during germination in a nutrient medium; synthesis of specific polypeptides occurred during late germination. During early germination posttranslational control was also observed, several labeled proteins from zoospores being specifically degraded or charge modified.

A fundamental question in developmental biology is the nature of the mechanisms that control the activation and expression of genes that are necessary for the orderly progression of development. Blastocladiella emersonii is a versatile organism well suited for the study of gene regulation during development. Two distinct phases of cell differentiation, germination and sporulation, occur during the life cycle of this fungus. In an attempt to isolate and characterize developmentally regulated genes, we initiated a study of the control of protein synthesis during sporulation and germination in B. emersonii. We have found that dramatic changes occur in the spectrum of proteins synthesized during sporulation and that the control is mainly at the transcriptional level (27). A similar analysis was undertaken during the germination process and is reported in this paper.

Zoospores of B. emersonii germinate rapidly and synchronously upon exposure to nutrient medium or an inorganic salt solution containing certain monovalent ions (32), cyclic AMP (8), or other inducers (10). The motile zoospores encyst by retraction of the flagellum and formation of a thin cell wall within 10 to 15 min after inoculation. Then, these round cells convert into germ cells, with the formation of a visible germ tube that elongates and begins to branch at approximately 60 min (21). Zoospore germination results in a large stimulation of protein synthesis. The zoospore contains all the components required for protein synthesis during germination, including ribosomes, tRNA, protein synthetic enzymes, and mRNA (9, 11). Experiments performed with actinomycin D suggest that the increase in the rate of protein synthesis during early germination is directed almost exclusively by stored mRNA sequences (19, 28, 33). However, the molecular mechanism which leads to this increase in protein synthesis is not known. An important question is whether all the stored mRNAs are recruited with equal efficiency or whether there is selective mobilization of a specific subset of mRNA sequences. Evidence is available that B. emersonii zoospores have some mRNA which has completed initiation but is blocked at one or more of the elongation steps (13). A number of fungi are dormant with regard to protein synthesis at the spore stage, and the block is at the level of translation since mRNA is present in these spores (7, 20, 29). However, it is unknown whether the block is at the initiation or elongation step or both of protein synthesis. The activation of protein synthesis by using stored mRNA in fungi presents an interesting parallel with animal embryonic systems (6).

Another interesting aspect of B. emersonii germination is that very early events are not dependent on concomitant protein synthesis (31). These events involve radical cell architecture rearrangement, including synthesis of a chitinous cell wall and retraction of the flagellum. Therefore, posttranslational and translational controls should be taking place during Blastocladiella germination.

To investigate the regulation of protein synthesis during germination, we administered consecutive 10-min pulses of [35S]methionine during germination in the presence or absence of actinomycin D. The proteins synthesized were analyzed by two-dimensional isoelectric focusing (IEF) and nonequilibrium pH gradient electrophoresis (NEPHGE). Patterns of in vitro translation products encoded by RNA from different stages of germination were also compared. In addition, to verify posttranslational modification of proteins during the very early stages of germination, we followed the decay of labeled proteins from zoospores with two-dimensional gels.

MATERIALS AND METHODS

Culture and labeling conditions. Cultures of B. emersonii were maintained on plates containing 0.13% peptone, 0.13% yeast extract, 0.3% glucose, and 1% agar. Zoospores (3 × 10^6/ml) were inoculated into DM3 medium (3) followed by growth for 13 h at 19°C. Vegetative cells were then starved by filtering them through a Nitex cloth and rinsing and resuspending them in sporulation solution (1 mM Tris maleate buffer [pH 6.8], 1 mM CaCl_2) at a density of 5 × 10^5 cells per ml. After 4 h at 27°C zoospores were released. Zoospore germination was induced in inorganic germination solution (1 mM Tris maleate buffer [pH 6.8] 1 mM CaCl_2, 10
mM MgCl₂, 50 mM KCl) or AL medium (50 mM KCl, 10 mM MgCl₂, 1 mM CaCl₂, 0.3% glucose, 40 ng of thiamine per ml, 1 mM tris maleate [pH 6.8], 0.6 µg of FeSO₄ per ml, 0.1 µg of CuSO₄ per ml, 0.2 µg of ZnSO₄ per ml, 0.2 µg of MnSO₄ per ml, the common 18 amino acids present at 0.2 mM each, except for methionine at 5 µM) by inoculation of zoospores at a density of 10⁷ cells per ml. Cells were incubated with agitation at 27°C. Samples containing 4 x 10⁷ germinating cells were labeled with [³⁵S]methionine (1 to 2 µCi/ml) at the indicated times. The progress and synchrony of germination were monitored by taking samples and examining cell types under a microscope (30).

Extracted of in vivo-labeled proteins. Labeled cells were pelleted and immediately suspended in cold 10% trichloroacetic acid. After 30 min, cells were centrifuged at 3,000 x g for 10 min and treated as described previously (27), to ensure that no proteolysis occurred during cell lysis. The amount of radioactive methionine incorporated into protein was determined by hot-trichloroacetic acid precipitation on Whatman 3MM paper filters and scintillation counting immediately after rupture of the cells by sonication. The radioactivity incorporated into protein during germination in an inorganic germination solution or AL medium in the presence and absence of actinomycin D is shown in Fig. 1. In inorganic solution a rapid increase in the incorporation was followed by a plateau (Fig. 1A); in AL medium the overall rate of synthesis increased exponentially throughout the first hour of germination, although the total percentage of incorporation was low owing to nonradioactive methionine present in this medium (Fig. 1B). In the presence of 5 µg of actinomycin D per ml the kinetics and synchrony of germination were similar to those of the control, at least up to 50 min. After that germ tube elongation seemed to be inhibited. The early increase in protein synthesis was not affected by this inhibitor; however, after 25 min a significant decrease in methionine incorporation in actinomycin-treated cells was observed.

Gel electrophoresis. Proteins prepared as described above were dissolved in 9.5 M urea–2% Nonidet P-40–10 mM dithiothreitol–2% ampholytes. Two methods of two-dimensional gel electrophoresis were used: IEF and NEPHGE (24, 25). In IEF, the ampholyte combination (3.5 to 10 and 5 to 8 in a ratio of 1:4, respectively) provided a pH range of 5 to 8. In the NEPHGE system, pH 3.5 to 10 ampholytes were used. The second dimension in both methods was performed on sodium dodecyl sulfate (SDS)-10% polyacrylamide gels. First-dimensional gels were equilibrated for 1 h, loaded directly on top of the stacking gel, and sealed in place with the stacking gel. A small piece of a first-dimensional-type gel containing molecular weight markers was run on the acidic side. After electrophoresis, gels were stained with Coomassie blue, destained, fluorographed with Amplify (Amer sham Corp., Arlington Heights, Ill.), and exposed to Kodak X-ray film as described previously (18). Exposure times were inversely proportional to the total counts per minute of ³⁵S in protein applied to each gel as judged by counts in material precipitable with trichloroacetic acid (27).

RNA preparation and in vitro translation. RNA was isolated by the procedure of Chirgwin et al. (4), involving sonication of the cells in the presence of guanidinium isothiocyanate and further cesium chloride centrifugation as described by Maniatis et al. (23). The RNA was examined by ethidium bromide fluorescence after electrophoresis through 1.5% agarose gels containing formaldehyde. In vitro translation was as previously described (27).

RESULTS

Protein synthesis during germination. Cells were labeled with [³⁵S]methionine for 10-min pulses during germination in inorganic solution in the presence or absence of actinomycin D. Total proteins were isolated and resolved by using two systems of two-dimensional electrophoresis: IEF-SDS-polyacrylamide electrophoresis, which resolves only acidic proteins, and NEPHGE, which resolves basic as well as acidic proteins (24, 25). Of approximately 400 polypeptides resolved by the IEF-SDS system, synthesis of about 150 was analyzed, and a total of approximately 20 basic polypeptides were unequivocally identified at the basic end of NEPHGE-SDS fluorograms. Figure 2 shows a comparison of proteins synthesized in early germination. During the first pulse (5 to 15 min), no incorporation of [³⁵S]methionine was detected by quantification (Fig. 1); however, a prolonged exposure of the fluorogram allowed the detection of protein synthesis in this initial pulse (Fig. 2A). Comparison of proteins synthesized between 5 to 15 min and 15 to 25 min in the presence or absence of actinomycin D showed almost identical patterns, indicating that zoospores contain preformed mRNA which is translated commencing in the first minutes upon stimulation of germination. The prominent polypeptides were numbered (1 to 39). Only one polypeptide (N41) identified in the IEF system (Fig. 2C) and one basic polypeptide (N40) identified in the NEPHGE system (Fig. 2E) were not synthesized in the presence of actinomycin D.
FIG. 2. Patterns of protein synthesis during early zoospore germination. Zoospores were induced to germinate in an inorganic germination solution either in the presence (B, D, F) or in the absence (A, C, E) of actinomycin D (ACT), which was added at time zero. At the indicated periods of germination (minutes), cells were labeled for 10 min with [35S]methionine, and whole-cell extracts were submitted to two-dimensional gel electrophoresis and fluorography. Arrowheads indicate the position of Coomassie-stained molecular size markers in descending order (68, 55, 43, and 25 kilodaltons).
FIG. 3. IEF-SDS two-dimensional gel electrophoresis of proteins synthesized during late germination in an inorganic solution. As described in the legend to Fig. 2. ACT, Actinomycin D.
Comparison of the patterns after 25 min showed a significant change in the relative rate of synthesis of several proteins. At least 28 new polypeptides appeared between 25 and 55 min, most of them being inhibited by actinomycin D (Fig. 3 and 4). These proteins whose appearance involves newly synthesized mRNA are designated with an N (for new mRNA). However, some new proteins whose synthesis is independent of transcription of new messages were also detected; for example, Fig. 3 shows five new polypeptides (S44, S45, S47, S50, S51) in the 25- to 35-min pulse and one (S58) in the 35- to 45-min pulse which are synthesized in the presence and absence of actinomycin D (designated as S, for stored mRNA). This fact suggests selective translation of stored mRNA during the germination process.

In parallel to the increased synthesis of several proteins after 25 min of germination, a decreased synthesis of several early polypeptides was also observed (polypeptides 3 to 7, 11, 20 to 22, 27, 30, 33 to 35, 38, 39) (Fig. 3 and 4). In several cases the decrement in synthesis was delayed in the presence of actinomycin D (e.g., polypeptides 5, 20, 21, 33 to 35). Moreover, in the presence of actinomycin D a vigorous increase in the rate of synthesis of some proteins was observed when compared with the control after 35 min of germination (Fig. 3D and F), indicating that the half-life of specific mRNAs is affected by the inhibition of transcription.

To distinguish polypeptides specifically related to the germination process, we investigated whether the pattern of protein synthesis during germination in a nutrient medium was different. Zoospores germinating in AL medium were also pulse-labeled with [35S]methionine for 10 min, and the patterns are shown in Fig. 5. Most polypeptides previously identified in the spectrum of proteins synthesized during early germination in inorganic solution were also detected here. Most of the polypeptides classified as N, whose synthesis depends on newly synthesized mRNA, and others classified as S were also identified in AL medium. Some of these proteins had a maximum of synthesis several minutes earlier (frequently a pulse earlier), indicating that the time course of protein synthesis during germination in AL medium is more rapid than under inorganic conditions. Furthermore, a significant number of new polypeptides (about 20) were synthesized exclusively in AL medium. These proteins are designated with a G (for growth). Several of these growth proteins are in fact found during the analysis of the synthesis of vegetatively growing cells (data not shown).

Total RNAs isolated from cells at different stages and conditions of germination were translated in a reticulocyte cell-free protein-synthesizing system, and the translation products encoded by these RNAs were also separated by two-dimensional gel electrophoresis. Although the efficiency of the in vitro translation system was low with total RNA, we could confirm all the main conclusions taken from the actinomycin experiments. All the products encoded by RNA isolated from cells at 20 min of germination were similar to those encoded by RNA extracted from zoospores; among the translation products encoded by messages from 45-min germinating cells in inorganic solution we could identify several corresponding to newly synthesized mRNAs (Fig. 6). By 45 min of germination several polypeptides corresponding to mRNAs stored in the zoospores had disappeared.

Posttranslational modification during early zoospore germi-
FIG. 5. Patterns of proteins synthesized during germination in nutrient medium. Zoospores were induced to germinate in AL medium. Cells were labeled for 10-min periods with \[^{35}\text{S}]\text{methionine}, and whole-cell extracts were submitted to IEF-SDS two-dimensional gel electrophoresis and fluorography. Arrowheads indicate molecular size markers in descending order (68, 55, 43, and 25 kilodaltons).

FIG. 6. Cell-free translation products of RNA isolated from zoospores (A) and 45-min germinating cells (B) in inorganic germination solution. RNA isolated from each sample was translated in a rabbit reticulocyte lysate system. The \[^{35}\text{S}]\text{methionine}-labeled proteins were analyzed by two-dimensional gel electrophoresis and fluorography. Polypeptides encoded by stored mRNAs which are maintained in 45-min cells are indicated by closed triangles, and those that disappear are indicated by open triangles. Arrows refer to translation products encoded by newly synthesized RNA.
FIG. 7. Modifications of prelabeled zoospore proteins during early germination. Labeled zoospores obtained after synchronous sporulation in the presence of [\(^{35}\)S]methionine were washed to remove radioactive methionine and induced to germinate in an inorganic germination medium containing cold methionine (0.2 mM). At 0 (A), 6 (B), 12 (C), 18 (D), or 25 (E) min after the inoculation of zoospores, samples were taken, and proteins were analyzed by IEF-SDS two-dimensional electrophoresis. Arrows indicate proteins which are degraded during early germination.
Labeled zoospores obtained after synchronous sporulation in the presence of [35S]methionine were washed free of exogenous isotope and germinated in an inorganic germination solution containing an excess of nonradioactive methionine (0.2 mM). Samples were collected every 6 min, and labeled proteins were quantified by trichloroacetic acid precipitation and resolved by two-dimensional gel electrophoresis (IEF and NEPHGE). Figures 7 and 8, respectively, show the electrophoretic profiles obtained at these different times. Comparing the profile of zoospores (Fig. 7A and 8A), which represents the spectrum of proteins synthesized during sporulation and conserved into the zoospores, with the profiles shown in Fig. 2 to 4, we can conclude that the population of proteins accumulated during sporulation and present in the zoospore differs significantly from those proteins synthesized during zoospore germination. Zoospore proteins, which were synthesized during sporulation, were relatively stable during very early germination. However, some proteins were processed or degraded during the first minutes and others later in germination. Among the last ones, there was a group of basic proteins with a molecular weight in the range of 40,000 to 55,000, detected in the NEPHGE-SDS system, which disappeared after 18 min. These proteins were synthesized during zoospore differentiation, which occurs at the end of sporulation (27). Modification in the isoelectric point of several proteins was also detected. Some polypeptides became more basic (b, c, d) and others more acidic (a, e), suggesting that mechanisms of phosphorylation-dephosphorylation or glycosylation or both are occurring during early germination.

FIG. 8. NEPHGE-SDS two-dimensional analysis of the modifications of prelabeled zoospore proteins during early germination. As described in the legend to Fig. 7.

DISCUSSION

Germination of Blastocladiella zoospores results in a large stimulation of protein synthesis. This increase in protein synthesis in the first 25 min is mediated by the mobilization of stored mRNA into polysomes. As previously shown, in the zoospores less than 10% of the ribosomes are found in polysomes, but by 30 min of germination this value has risen to about 80% (9, 28). The comparatively small effect of actinomycin D on protein synthesis (19, 28, 33) (Fig. 1) and polyribosome formation (19, 28) early in germination had suggested that zoospores already contained mRNA. The translation of this mRNA between 15 and 30 min could account for the increase in polysome content in germinating cells. As judged by analysis on two-dimensional gels, the increase in the rate of protein synthesis until 25 min is not accompanied by any significant changes in the total protein pattern. Therefore, the increased rate of protein synthesis in early germination (until 25 min) is due to increased translation of the same species of mRNA, not different species. This increase is due to a gradual activation of translation of stored mRNAs, so that the rate of synthesis of the proteins they code for rises linearly over the first 25 min of germination. During sporulation, zoosporangia appear to have the ability not only to store mRNA in a stable form but also to avoid translating mRNAs that are specific for proteins approximately expressed during the first minutes of germination.

After 25 min of germination numerous changes in the pattern of proteins synthesized were observed. The relative
rate of synthesis of several polypeptides decreased, and two
classes of new polypeptides appeared, one corresponding to
de novo mRNA synthesis and the other corresponding to
stored mRNA, which are selectively translated late in ger-
mination. A selectively timed recruitment of some maternal
mRNA also occurs during the fertilization of eggs (34),
meiotic maturation of oocytes (26), or during the de-
velopmental response to light of Volvox sp. (17).

Therefore, multiple levels of regulation of protein synthe-
seem to occur during zoospore germination involving
both transcriptional and translational controls. Superim-
posed on the activation of translation of preformed mRNA is
a control involved in the selection of these messages. Trans-
lation could be regulated at both the level of mRNA avail-
ability and the activity of components of the translational
machinery. Evidence was presented that some dormant
ribosomes in zoospores are arrested at elongation (13),
and inhibitor particles (12) as well as a low-molecular-weight
inhibitor (1) were found in the zoospores. Both adenylation
and deadenylation of preexisting mRNA occur simulta-
aneously during zoospore germination, and experiments with
actinomycin D demonstrated that transcription is not re-
quired for polyadenylation of zoospore mRNA (11). Thus,
changes in the state of adenylation of the preexisting mRNA
population could be involved in the translational selectivity.
Hybridization analysis with cDNA clones for specific
mRNAs of this population of stored messages would clarify
this point.

Preliminary studies of the proteins synthesized during
zoospore germination in the presence and absence of
actinomycin D were performed by Lovett (22). A total of 132
polypeptides were detected in two-dimensional gels, and
minimal changes in the pattern of protein synthesis were
found. However, this author does not describe the condi-
tions of extract preparation nor the conditions of electropho-
resis to allow a direct comparison with our results. Our
conclusions are apparently consistent with the work of
Johnson and Lovett (15) that, on the basis of hybridization
analysis, showed that cells germinated in an inorganic star-
vation medium present a marked loss of the middle- and
high-abundance classes of zoospore poly(A+) mRNA and a
slight enrichment for the low-abundance class. However, it
is difficult to compare our results directly with the hybrid-
ization studies of the Lovett group since the two-
dimensional gel electrophoresis procedure we used should
only detect proteins synthesized by intermediate and abun-
dant classes of mRNA.

In parallel to the increase in protein synthesis during early
germination we found degradation of some polypeptides
which had been synthesized during sporulation and were still
present in the zoospores. Besides a specific degradation of
some polypeptides, others undergo change in their charge,
suggesting that modifications such as proteolysis, phosphor-
ylation, or glycosylation play an important role in the first
minutes of germination, during encystment (transition of
zoospore to round cell), which involves radical morpholog-
ical rearrangements and occurs even in the presence of
cycloheximide. Studies in progress with 32P labeling can
confirm this hypothesis.

We are using B. emersonii as a model to study the
developmental regulation of protein and mRNA synthesis.
Dramatic changes in the synthesis of these macromolecules
occur during sporulation (27). During early zoospore germi-
nation, protein synthesis occurs with stored mRNA tran-
scribed from genes which were probably activated during
sporulation (most of them during the last stages of sporula-
tion) (14). Late in germination, the majority of the changes in
the pattern of protein synthesis can be attributed to newly
synthesized mRNA. Therefore, we are now able to look for
developmentally regulated genes on the basis of their diffe-
rential expression during the life cycle of B. emersonii.

Similar approaches have been used to isolate conidiation-
specific genes of Aspergillus nidulans (35) and Neurospora
crassa (2) and developmentally regulated genes of
Dictyostelium discoideum involved in spore germination (16)
or cell aggregation (5).

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