THE EFFECTS OF PULSE CYCLOHEXIMIDE TREATMENTS ON THE LIGHT-INDUCED RECOVERY OF MITOTIC DIVISIONS IN ANTHERIDIAL FILAMENTS OF *CHARA VULGARIS*

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(Received: January 15, 1998. Accepted: February 12, 1999)

ABSTRACT

Within the proliferative period of spermatogenesis in *Chara vulgaris*, the progression throughout successive cell divisions in antheridial filaments is greatly influenced by changes in photoperiodic conditions. The extended (4-day) period of total darkness brings about cell cycle arrest in the early G2 phase.

The recovery of mitosis requires about 20 hours of exposition to light. In the present study, a series of 8 pulse incubations of plants in cycloheximide (Cx; 2.5 mg/l, 2.5 h each pulse) were performed within the period elapsing till the resumption of mitotic divisions. Depending on the time of treatment, the effects induced by Cx vary considerably. Within the first 10 hs of exposition to light, incubations with Cx result in the delays of mitoses; within the period between the 10th and the 17th h, mitotic divisions become blocked, and, following the 17.5 h of light-induced recovery, no influence of Cx is noticed on mitotic activity, as compared with the untreated control plants. The obtained results provide a starting point for the characteristic of proteins synthesized during the G2 phase and a preliminary study on those mechanisms, which become engaged in the regulation of the G1-deficient cell cycle evidenced in antheridial filaments of *Chara*.

KEY WORDS: cycloheximide, cell cycle, darkness, antheridial filaments, *Chara vulgaris*.

INTRODUCTION

The course of the cell cycle and the regulatory processes involved in mitotic divisions still provide a focus of many current experimental studies and a challenging question of modern cell biology. Despite recent advances, particularly in our understanding of some universal factors that govern the expression of genetic information during the cell cycle progression, much further work will be needed to conclusively determine the nature of the controls functioning at various levels of cellular organization. Very much less is known about the cell cycle of the S+G2+M type (without the G1 period), which is widely believed to represent a more primitive form, although on the phylogenetic grounds one might expect to find the underlying physiological mechanisms less complicated. The cell division cycles of this kind has been found typical for plasmodial slime molds *Physarum* (Eon-Gerhardt 1981) and *Dictyostelium* (Michaelis and Weeks 1992), many amoebae (Mills and Bell 1981), chlorophyte unicellular alga *Micrasterias* (Tourte 1975), and during the proliferative developmental pathway in antheridial filaments of mono- and dioecious species of *Chara* (Olszewska and Godlewski 1972; Maszewski 1991; Maszewski and Kołodziejczyk 1991).

A number of qualitative and quantitative analyses have been made previously in order to characterize cell division cycles in antheridial filaments of *Chara*; these included autoradiographic studies using ³H- and ¹⁴C-labeled precursors of nucleic acids and proteins (Olszewska and Godlewski 1972;

Godlewski and Olszewska 1973; Maszewski 1977, 1991; Kwiatkowska and Maszewski 1979c), cytochemical investigations of various histones (Olszewska 1974b; Olszewska and Marciniak 1977) and fractions of proteins containing disulfide bonds (Olszewska and Marciniak 1980), analyses of nuclear and cytoplasm dry mass contents based on interference-microscopy (Kuran 1975; Maszewski and Kwiatkowska 1982), investigations using radioactive substances binding with tubulin (³H-colchicine; Olszewska 1975) and the transcriptionally active DNA fractions (³H-actinomycin D; Olszewska 1974a; Kwiatkowska and Maszewski 1979a; Godlewski 1988). Moreover, in situ ³H-rRNA-DNA hybridization was carried out (Kwiatkowska et al. 1987), the extent of histone acetylation was determined (Marciniak and Olszewska 1979), and DNA methylation during antheridial filament development was analysed using in situ nick-translation driven by methylation-sensitive restriction enzymes (Olszewska et al. 1997). Another series of electron-microscopic studies allowed to link ultrastructural changes with modifications evidenced in the metabolic activities during different stages of the cell cycle (Kwiatkowska and Maszewski 1976, 1978, 1979b, 1980; Kwiatkowska 1981a, b).

Although much of the research work has been already made, including experiments using plant growth substances (Godlewski 1977, 1980, 1982, 1988; Godlewski and Kwiatkowska 1980; Kwiatkowska and Godlewski 1980, 1988; Kwiatkowska et al. 1993; Kwiatkowska 1991, 1995; Kwiatkowska and Malinowski 1995), inhibitors of DNA (Bilecka

1979) and protein synthesis (Olszewska et al. 1982), as well as the studies concerning the regulatory influence of light (Maszewski 1977, 1981; Maszewski and Kwiatkowska 1982; Kwiatkowska and Maszewski 1980; Kwiatkowska et al. 1980; Kwiatkowska 1990; Kwiatkowska et al. 1992, 1995), the problem of cell cycle regulation in antheridial filaments of *Chara* remains still open.

The aim of our present investigations was to get a closer insight into the process of mitotic reactivation of cells blocked by dark-treatment, using cycloheximide, an inhibitor of 80S ribosomes-mediated protein synthesis. Our previous ultrastructural, morphometric, cytophotometric, interferometric and autoradiographic data have shown that the dark-induced cell cycle arrest point is located within the early stages of the G2 phase (Kwiatkowska and Maszewski 1980; Maszewski and Kwiatkowska 1982). Furthermore, it has been evidenced that about 20 h period of light treatment is needed to induce mitotic recovery. In the present study, using pulse incubations with cycloheximide during successive 2.5 h intervals, we have been able to estimate the time period during which proteins essential for photoinduction of mitotic divisions are synthesized. These data may serve as a starting point for our further investigations aimed at characterization of proteins engaged in the mechanisms regulating cell cycle progression in antheridial filaments of *Chara*.

MATERIAL AND METHODS

Chara vulgaris L. was collected from a pond located in the neighbourhood of the Botanical Garden in Łódź. Apical parts of thalli carrying antheridia were divided onto 9 groups (about 50 plants each). Plants were cultivated in aerated water

from the natural environment for 4 days in complete darkness. The experiments were performed twice during summer (July and August).

Following exposition in the darkness, each group of plants was treated separately. The control samples were transferred into continuous light and were not treated with cycloheximide. These plants were fixed in portions (5-10 plants each) within the 2.5 h intervals, beginning from the 20th up to the 37.5 h of exposure to the light (at 5 am, 7.30 am, 10 am, 0.30 pm, 3 pm, 5.30 pm, 8 pm, and 10.30 pm).

In the portion of illuminated, cycloheximide – treated plants (2.5 µg/ml – 2 h incubation, each sample; the concentration of Cx and the duration of incubation were adopted acc. to the procedure of Olszewska et al. 1982) 8 experimental series were performed. In the series Cx I, the inhibitor was applied between the 0-2 h, i.e. within the first two hours after transferring the plants from darkness to the light; series Cx II – after 2.5 hs; series Cx III – after 5 hs; series Cx IV – after 7.5 hs; series V – after 10 hs; series VI – after 12.5 hs; series Cx VII – after 15 hs; and series Cx VIII – after 17.5 hs. Following each incubation, plants were thoroughly washed with pond water and further kept under the light. At appropriate periods, after the same intervals as in the control groups, the plants were fixed in portions (5-10 plants per sample). A scheme of the experiment is given in Table 1.

The thalli were fixed in Carnoy's mixture (glacial acetic acid and absolute ethanol 1:3, v/v). After 1 h, plants were washed with 96% and 70% ethanol. Following rehydration and 10 min treatment with 50% acetic acid, the plants were stained with saturated orcein for 15 min and with 2% Fast Green FCF for 50-60 sec, and then washed with distilled water. Antheridia localized at the upper branchlets of pleuridia were isolated and squashed onto gelatin-coated microscope slides.

TABLE 1. Schematic presentation of experiments using cycloheximide (Cx).

4 days of darkness	Duration of exposure to light														
	Timing of Cx pulse treatment in particular series of plants								Timing of fixation for particular series of plants						
	2.5	5	7.5	10	12.5	15	17.5	20	22.5	25	17.5	30	32.5	35	37.5
CD															
CL															
I	Cx-I →							•	•	•	•	•	•	•	•
II		Cx-II →						•	•	•	•	•	•	•	•
III			Cx-III →					•	•	•	•	•	•	•	•
IV				Cx-IV →				•	•	•	•	•	•	•	•
V					Cx-V →			•	•	•	•	•	•	•	•
VI						Cx-VI →		•	•	•	•	•	•	•	•
VII							Cx-VII →	•	•	•	•	•	•	•	•
VIII								Cx-VIII →	•	•	•	•	•	•	•
	11 ³⁰	14 ⁰⁰	16 ³⁰	19 ⁰⁰	21 ³⁰	24 ⁰⁰	2 ³⁰	5 ⁰⁰	7 ³⁰	10 ⁰⁰	12 ³⁰	15 ⁰⁰	17 ³⁰	20 ⁰⁰	22 ³⁰

→ – duration of Cx pulse treatment

CL – control; light

CD – control; darkness

Mitotic index for antheridial filaments was calculated according to the formula:

$$MI_{af} = \frac{\text{number of antheridial filaments at mitosis}}{\text{total number of antheridial filaments}} \times 100$$

RESULTS

Since a substantial number of antheridial filaments at later stages of development (in male sex organs containing mixed populations of 16-, 32-, and 64-cell filaments) was found capable to enter mitotic divisions in the absence of light (in response to modified physiological conditions connected with differentiation of spermatids into spermatozooids – Kwiatkowska and Maszewski 1987; Kwiatkowska 1997), the analysis of mitotic activity was limited to the 2-, 4-, 8-, and 16-cell stages of antheridial filaments. The results are presented in Fig. 1.

In the control series (Fig. 1), the appearance of the first, low peak of mitoses was noticed between the 22.5 and 25th h of exposure to the light, i.e. some 2 hs later than it might be expected according to experiments of Maszewski (1979).

The second, apparent peak of mitoses commences 30 hours after the onset of illumination. The amplitude of the wave is not high, as it was the case in earlier experiments carried out using axenic plants cultivated in mineral medium (Maszewski 1977). The lack of mitotic synchronization after transferring the plants into light results from an imbalance between nuclear DNA-division cycle, which is continued up to the early stage of the G2 period, and the growth cycle, which in turn becomes retarded considerably (Maszewski and Kwiatkowska 1982).

The above-mentioned studies have shown that the onset of mitosis is, primarily, restricted to those cells, which attain the sizes characteristic for the late G2 period of a given stage of antheridial filaments (compare Fig. 1; Maszewski and Kwiatkowska 1982).

Administration of cycloheximide during the first 2 hs, i.e. just after transferring the plants from darkness into light (Fig. 1; series Cx I) results in a shift of the mitotic wave by about 5 hs. Its amplitude is similar to that of the second mitotic wave in the control plants. The second wave of mitotic divisions reaches its maximum level some 5 hs later, i.e. 37.5 hs after the onset of illumination.

Within the period elapsing till 5-9.5 hs after the start of exposure to the light, cycloheximide delays the onset of mitotic divisions by 10 and 12.5 hs, respectively.

During 37.5 hs of observations, only the upraising arm of the mitotic wave could be discerned, and hence, the height of its amplitude remains unknown. The slope of the obtained curve may indicate, however, that in the series Cx III the height of the wave is greater than that noticed in the corresponding control samples.

Beginning from the 10th h (Cx V) to the 17th h of light, a low mitotic activity (Cx V) or its complete cessation (Cx VI and Cx VII) has been observed. Most probably, the above critical period (7 hs) comprises these stages of light-induced recovery, during which photo-stimulated synthesis of proteins essential for the resumption of mitoses takes place. After 17.5 hs of exposition to light, further preparations to commence mitotic divisions in antheridial filaments do not require *de novo* synthesis of regulatory proteins. Mitotic activity in the series Cx VIII is similar to that evidenced in the control.

Thus, the obtained results seem to indicate, that the effects induced by Cx depend largely on the timing of pulse treatment. Within the first 10 hs of exposition to the light, incubations with Cx result in the delayed appearance of mitoses. Within the second period – between the 10th and the 17th h of light-induced recovery – the presence of Cx causes cessation of mitotic divisions. During the third period, beginning from the 17.5 h after the transfer of plants into light, no influence of Cx is noticed on mitotic activity in antheridial filament arrested previously by 4-day dark treatment at the G2 phase of the cell cycle.

Fig. 1. Mitotic activity (MI; %) in antheridial filaments from dark – treated plants (4 days) and incubated with cycloheximide (2.5 µg/ml) for 2 h within various periods of light induced mitotic recovery.

DISCUSSION

Ultrastructural studies and quantitative analyses of condensed chromatin in cell nuclei of antheridial filaments at the stages preceding light-induced recovery of cell divisions have shown that the appearance of mitoses is preceded by a number of morphological changes. The initial modifications have been evidenced within the nucleoli. Following prolonged dark-treatment, the nucleoli become considerably smaller and completely deprived of their granular component. The latter become reconstructed in the period preceding mitotic divisions, the extent of this recovery being especially large in nucleoli characterized by the greatest sizes (Kwiatkowska and Maszewski 1980). Furthermore, the measurements performed using silver-stained antheridial filaments viewed under the light microscope (Papiernik, in. prep.) have indicated significant increase in the total nucleolar volumes calculated per cell nucleus. Within the period preceding light-induced resumption of mitotic divisions, the frequency distribution patterns of nucleoli obtained from population of the largest cells (i.e. these, which acquired commitment to enter mitoses) revealed evident similarity to the control samples. Preliminary studies using pulse treatments with cycloheximide during the lag phase preceding resumption of mitotic divisions indicates that this inhibitor severely diminishes the dynamics of nucleolar growth (Malinowski, Kwiatkowska, n. publ.).

It may be supposed then, that within the initial stage preparing the cells to commence mitoses during light-induced recovery, evident retardation of that process caused by cycloheximide may result from blocking of the nucleolar renewal, preventing from restoration to the maximum sizes attained (and required) before the onset of prophase. The analysis of successive cell division cycles in antheridial filaments clearly indicates that there is a close correlation between the sizes of nucleoli at late G2 periods during each interphase and the dynamics of nucleologenesis within the next developmental stage (Kwiatkowska and Maszewski 1985).

This conclusion is supported by the research of Medina et al. (1995), indicating that nucleolar matrix constitutes an element which, at telophase, aggregates around NOR many components, including pre-rRNA, snoRNA, fibrillarin and nucleolin. Resumption of nucleolar activity constitutes a key factor determining growth of these cells which, *via* increase in volume of cytoplasm, must reach appropriate sizes (similar to those in the control) before entering mitosis; these cells may undergo division during the second "mitotic wave" observed in the control samples and in the Cx I series.

After the prolonged dark-treatment all the cells assume a uniform and characteristic structure, typical for the early G2 period (stage VII, during which large, irregular clusters of condensed chromatin become separated by the areas of dispersed chromatin). This type of nuclear arrangement persists within the first 4 hs of exposition to light (Kwiatkowska and Maszewski 1980). According to Murray (1995), the blocking of ubiquitination and ubiquitin-mediated lysis of proteins that should be removed before entering a new stage of the cell cycle, may represent a key factor preventing the cell from transition from one to the other phase of the cycle. It seems also probable, that mechanisms of this kind underlie regulatory mechanisms involved in progression throughout the cell division cycles in antheridial filaments of *Chara*.

Within the ensuing periods of exposure to light, a cascade of nuclear changes has been found indispensable to allow the cell for entering mitotic division. The structural modifications represent a precise repetition of all these transformations

which were evidenced in the control between early G2 phase (stage VII) and prophase (stage VIII – dispersion of condensed chromatin; stage IX – formation of a network-like chromatin; stage X – the appearance of an apparent reticulate form of chromatin with its highly condensed regions adjoined to the nuclear envelope). Nuclear structure typical for stage X appears after 18 hs of exposure to light (Kwiatkowska and Maszewski 1980), i.e. during the period when the sensitivity of antheridial filaments to cycloheximide ceases. Light-induced structural changes of chromatin seem thus to result from signal-mediated activation of new cell cycle-controlled genes. The main signal that the plant receives after providing the light seems associated with phytochrome system rather, than with the products of photosynthesis. In the far-red illumination, which maintains a high-energy state of phytochrome but does not sustain photosynthesis, mitotic activity in antheridial filaments of *Chara* exposed for 3 days is not blocked, reaching even higher values than in the full light with the natural photoperiodic conditions (Maszewski 1979).

There are three other signaling systems specific for the plant cells: auxins, cytokinins and gibberellins. Auxin seems to be the most important information providing factor required for the dark-inhibited antheridial filaments of *Chara* to resume mitotic divisions. Elimination of auxin by applying an inhibitor of its transport, TIBA, results in a delay of light-induced recovery of mitoses by 22 hours (Maszewski 1979). On the other hand, administration of all the above plant hormones at appropriate concentrations brings about acceleration of mitotic divisions by about 4 hours (Maszewski 1979). All these observations clearly fit numerous data indicating an important role of plant growth substances (mainly cytokinins and auxins) in the regulation of the cell division cycles, particularly that referring to the control point localized at the G2 phase (John 1996). It has been suggested that cells arrested at the cytokinin-dependent control point represent a stage preceding immediately the onset of mitosis, since addition of cytokinin results in a rapid activation of p34cdc2 kinase of histone 1 (Zang et al. 1996). A similar location during the G2 phase has been also evidenced for the arrest-point connected with monitoring of the deficiency of auxins. Moreover, in *Arabidopsis*, both cytokinins and auxins have been found influencing the genes for cyclin D (Soni et al. 1995).

In the slime mole, *Physarum*, characterized by the G1-lacking type of the cell cycle, the highest concentration of a proteinaceous MPF-like complex has been evidenced prior to the initiation of mitosis, while its disappearance occurring after the completion of mitosis effected by a product of an anti-MPF gene (Adlakha et al. 1988). In the same species, transcription of genes for histones takes place during the G2 period, coincidentally with the synthesis of histones H2A and H2B. Synthesis of histones H3 and H4 takes place during S phase, and histone 1 is synthesized throughout the entire cell cycle, reaching the maximum level during early G2 phase (Loidl and Grobner 1987). During the G2 period in *Physarum*, the most intensive production of tubulin has been evidenced (Green and Dove 1988).

In the slime mold *Dictyostelium discoideum* (also lacking the G1 period) Michaelis and Weeks (1992, 1993) have identified both the cdc2 gene and proteins homologous to Cdc2 kinases of other organisms, as well as a cdc2-like gene with its product termed as Crp (Cdc2-Related PCTAIRF). Furthermore, from the same species, Luo et al. (1994) have isolated B-type cyclin, which appeared to attain the maximum level during mitotic division. Modification of this gene by depriving of the destruction box, which, after the mitotic division is

finished mediates the ubiquitin-dependent proteolysis, results in a blocking of the cell cycle at mitosis. Understanding of the processes which control the G1-lacking type of the cell cycle in slime molds is relatively poor, when compared with the knowledge relevant to regulatory factors engaged in coordination of complete cell division cycles typical for yeasts, mammals and higher plants (in the latter case, recent investigations have been presented in 3 volumes of "Progress in Cell Cycle Research", 1995-1996, Majer L., Guidet S., Vogel L., eds).

The data reported in our present work clearly indicate that exposure of plants to the light may appear inoperative and unsuccessful in inducing mitotic recovery of antheridial filaments, if cycloheximide pulse treatments were applied within a relatively long period of time (7 hours). Hence, it may be assumed, that during intervals characterized by the highest sensibility of cells to cycloheximide, a number of regulatory proteins become synthesized, functionally linked both with the initiation of mitosis and with the onset of S phase in the forthcoming cell cycle. The identification of these proteins would provide means to get a closer insight into mechanisms connected with the regulation of cell division cycles in antheridial filaments of *Chara*, and thus, to gain more information relevant to the progression of the cell cycles without the G1 periods.

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WPŁYW PULSOWEGO DZIAŁANIA CYKLOHEKSYMIDU NA FOTOINDUKCYJNE WZNOWIENIE MITOZ W NICIACH SPERMATOGENICZNYCH

STRESZCZENIE

W fazie proliferacyjnej spermatogenezy *Chara vulgaris* komórki nici spermatogenicznych zatrzymują się w rozwoju na etapie wczesnej fazy G₂ pod wpływem 4-dobowej ciemności. Wznowienie mitoz następuje po ok. 20 godzinach ekspozycji roślin na światło. W eksperymencie zastosowano 2 godzinną inkubację roślin w cykloheksymidzie (Cx) w st. 2,5 mg/l, podając go w kolejnych 8 seriach, w odstępach 2,5 godzinnych. Cx wywołuje różne efekty w zależności od okresu, w którym traktowano nim rośliny. W okresie od 0 do 10 godzin ekspozycji roślin na światło Cx powoduje opóźnienie mitoz. W drugim okresie – od 10 do 17 godzin Cx blokuje podziały mitotyczne. W trzecim okresie – po 17,5 godzinach – nie wywiera wyraźnego wpływu na fotoindukcyjne wznowienie mitoz.

Wyniki te stanowią punkt wyjścia dla charakterystyki białek syntetyzowanych w fazie G₂ związanych z mechanizmami regulacji cyklu komórkowego typu S+G₂+M w niciach spermatogenicznych.

SŁOWA KLUCZOWE: cykloheksymid, cykl komórkowy S+G₂+M, ciemność, nici spermatogeniczne, *Chara vulgaris*.