Regulation by Polyamines of Ornithine Decarboxylase Activity and Cell Division in the Unicellular Green Alga *Chlamydomonas reinhardtii*¹

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Polyamines are required for cell growth and cell division in eukaryotic and prokaryotic organisms. In the unicellular green alga *Chlamydomonas reinhardtii*, biosynthesis of the commonly occurring polyamines (putrescine, spermidine, and spermine) is dependent on the activity of ornithine decarboxylase (ODC, EC 4.1.1.17) catalyzing the formation of putrescine, which is the precursor of the other two polyamines. In synchronized *C. reinhardtii* cultures, transition to the cell division phase was preceded by a 4-fold increase in ODC activity and a 10- and a 20-fold increase, respectively, in the putrescine and spermidine levels. Spermine, however, could not be detected in *C. reinhardtii* cells. Exogenous polyamines caused a decrease in ODC activity. Addition of spermine, but not of spermidine or putrescine, abolished the transition to the cell division phase when applied 7 to 8 h after beginning of the light (growth) phase. Most of the cells had already doubled their cell mass after this growth period. The spermine-induced cell cycle arrest could be overcome by subsequent addition of spermidine or putrescine. The conclusion that spermine affects cell division via a decreased spermidine level was corroborated by the findings that spermine caused a decrease in the putrescine and spermidine levels and that cell divisions also could be prevented by inhibitors of 5-adenosyl-methionine decarboxylase and spermidine synthase, respectively, added 8 h after beginning of the growth period. Because protein synthesis was not decreased by addition of spermine under our experimental conditions, we conclude that spermidine affects the transition to the cell division phase directly rather than via protein biosynthesis.

Polyamines are ubiquitous cell components essential for normal growth of both eukaryotic and prokaryotic cells (Tabor and Tabor, 1984; Marton and Pegg, 1995). In higher plants, polyamines also influence developmental processes and play an important role in the response to abiotic stress (Galston et al., 1997). The three commonly occurring polyamines (putrescine, spermidine, and spermine) are synthesized from Orn and/or Arg, putrescine being the first polyamine in these biosynthetic pathways (Slocum, 1991). Spermidine and spermine are generated from putrescine by the addition of aminopropyl groups derived from decarboxylated 5-adenosyl Met (Slocum, 1991). The rate-limiting step in the formation of putrescine in animals and most fungi is the decarboxylation of orn by Orn decarboxylase (ODC; for references, see Marton and Pegg, 1995). In some plants, putrescine is also synthesized via the decarboxylation of Arg by Arg decarboxylase and subsequent degradation of the generated agmatine (Pegg, 1986; Minocha and Minocha, 1995; Kumar et al., 1997; Walden et al., 1997; Andersen et al., 1998). As previously reported, putrescine formation in the unicellular green alga *Chlamydomonas reinhardtii*, however, is controlled by ODC rather than by Arg decarboxylase activity (Voigt et al., 2000a).

ODC activity is regulated both at the translational level (Tabor and Tabor, 1984; Marton and Pegg, 1995) and via controlled, ATP-dependent proteolysis by the 26S proteasome, at least in mammalian and yeast (Saccharomyces cerevisiae) cells (Bercovich et al., 1989; Mamroud-Kidron and Kahana, 1994). ODC is an enzyme with rather short half-life, varying between 30 and 120 min in all eukaryotic organisms studied so far (Voigt et al., 2000a) with the exception of Trypanosoma brucei (t1/2 = 6 h; Phillips et al., 1987). In synchronized cultures of the unicellular green alga *C. reinhardtii*, a 2.5- to 3-fold increase of the ODC half-life was observed during transition to the cell division phase, accompanied by a 3-fold increase of ODC activity (Voigt and Bohley, 2000). For mammalian cells, it has been reported that degradation of ODC is not mediated by ubiquitination (Bercovich et al., 1989), but by binding of ODC monomers to an inhibitory protein named antizyme (Murakami and Hayashi, 1985; Hayashi and Murakami, 1995; Hayashi et al., 1996). At least two different pathways for degradation of ODC in mammalian cells are known as yet, a constitutive and a polyamine-dependent pathway (Li and Coffino, 1993). The C-terminal domain is necessary in both cases and sufficient to make an ODC molecule constitutively unstable. Surface hydrophobicity and PEST sequences (sequences with...
high proportions of Pro, Glu/Asp, Ser, and Thr and lacking basic amino acid residues) are actually considered as signal structures for the rapid and selective degradation of specific proteins by the proteasome (Bohley, 1996; Rechsteiner and Rogers, 1996). For several years, a PEST sequence in the C-terminal domain of mammalian ODCs (PEST2) was assumed to be responsible for the rapid degradation of the ODC subunits because this particular PEST sequence is missing in the long-living ODC of T. brucei (Phillips et al., 1987; Rechsteiner and Rogers, 1996). However, stabilization of mammalian ODC was achieved by deletion of the C-terminal pentapeptide Ala Arg Ile Asn Val, whereas deletions affecting the neighboring PEST2 sequence did not increase ODC stability (Ghoda et al., 1992; Li and Coffino, 1992, 1993). Although ODC is also a short-living enzyme in plant cells (Hiatt et al., 1986; Voigt and Bohley, 2000; Voigt et al., 2000a), there are still no published data concerning the reason for its rapid degradation in plant cells. The control of ODC degradation by the intracellular polyamine level and the antizyme level (Murakami and Hayashi, 1985; Hayashi and Murakami, 1995; Hayashi et al., 1996) is proposed to assure homeostatic regulation of both the ODC activity and the polyamine concentrations in mammalian cells. However, elevated polyamine levels have been found in proliferating plant and mammalian cells as well as in cancer cells (Hibshoosh et al., 1991; Auvinen et al., 1992; Moshier et al., 1993; Daoudi and Biondi, 1995; Marton and Pegg, 1995; Ben Hayyim et al., 1996; Fowler et al., 1996; Cvikrova et al., 1999). A rapid increase in ODC activity was measured when growth-arrested mammalian or plant cells were transferred to fresh culture medium enabling cell proliferation (Manzella et al., 1991; Fowler et al., 1996; Graff et al., 1997). This increase in ODC activity was found to be mediated by an increased translation of preexisting ODC mRNA (Manzella et al., 1991; Graff et al., 1997). A rapid up-regulation of ODC activity was also observed when dark-adapted (starved) C. reinhardtii cells were transferred to the light (Voigt et al., 2000a). This light-induced increase in ODC activity was abolished by the PSII inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea and could be prevented by inhibition of protein biosynthesis, but not by inhibition of RNA synthesis (Voigt et al., 2000a).

An increase in ODC activity was also observed when (partially) synchronized mammalian cells entered the cell division phase (Koza and Herbst, 1992; Fredlund et al., 1995). However, the mechanism of this putative cell cycle control of ODC is completely unknown. Unicellular green algae like C. reinhardtii and Scenedesmus obliquus can be easily synchronized by cultivation under a constant light-dark regime (Voigt and Münzner, 1987; Krupinska and Humbeck, 1994). Analyses of synchronized cultures of Scenedesmus revealed that the polyamine levels increased during the growth period and decreased after the cell division phase (Kotzabasis and Senger, 1994). In synchronized cultures of the unicellular green alga C. reinhardtii, a 3-fold increase in ODC activity was observed during the transition to the cell division phase that correlated with a 3-fold increase of the ODC half-life (Voigt and Bohley, 2000). Therefore, we have investigated the effects of polyamines on ODC activity and cell cycle progression in synchronized cultures of C. reinhardtii.

RESULTS

Cell Cycle-Dependent Alteration of the Polyamine Levels

In synchronized C. reinhardtii cultures growing under a 14-h-light/10-h-dark regime, the intracellular levels of putrescine and spermidine strongly increased during the light phase (growth period) as shown in Table I. A 10-fold increase of putrescine and 20-fold increase of spermidine was measured 15 h after onset of illumination (Table I). The spermidine level was much lower (3%–10% of total polyamines) than the putrescine level (90%–97% of total polyamines; Table I). Spermine could not be detected. Therefore, the question arose whether or not this cell cycle-dependent alteration of the polyamine level affects cell cycle progression.

Down-Regulation by Polyamines of ODC Activity in C. reinhardtii Cells

Because ODC is the key enzyme of polyamine biosynthesis in C. reinhardtii (Voigt et al., 2000a), we have investigated the response of this particular enzyme to different exogenous concentrations of putrescine, spermidine, and spermine, respectively. When added 8 h after beginning of the light period, all these commonly occurring polyamines caused a decrease in ODC activity after 5 h (Table II). In the case of putrescine, however, a considerably higher division phase (Kotzabasis and Senger, 1994). In synchronized cultures of the unicellular green alga C. reinhardtii, a 3-fold increase in ODC activity was observed during the transition to the cell division phase that correlated with a 3-fold increase of the ODC half-life (Voigt and Bohley, 2000). Therefore, we have investigated the effects of polyamines on ODC activity and cell cycle progression in synchronized cultures of C. reinhardtii.

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Table I. Cell cycle-dependent changes of the levels of intracellular polyamines

Cultures of the wall-deficient strain C. reinhardtii cw-15 were synchronized by growth under a constant 14-h-light/10-h-dark regime. Cells were harvested at the indicated time intervals after onset of illumination and analyzed for free polyamines as described in “Materials and Methods.” Mean values of three individual experiments ± SD are given.

<table>
<thead>
<tr>
<th>Time after Onset of Illumination</th>
<th>Putrescine</th>
<th>Spermidine</th>
<th>Spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td>h</td>
<td>nmol/10^9 cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>14 ± 3</td>
<td>0.4 ± 0.05</td>
<td>nd*</td>
</tr>
<tr>
<td>5</td>
<td>32 ± 5</td>
<td>1.1 ± 0.22</td>
<td>nd</td>
</tr>
<tr>
<td>10</td>
<td>91 ± 11</td>
<td>3.3 ± 0.5</td>
<td>nd</td>
</tr>
<tr>
<td>15</td>
<td>168 ± 18</td>
<td>7.8 ± 1.1</td>
<td>nd</td>
</tr>
<tr>
<td>20</td>
<td>135 ± 16</td>
<td>6.4 ± 1.2</td>
<td>nd</td>
</tr>
</tbody>
</table>

* nd, Not detected.
Cultures of the wall-deficient strain *C. reinhardtii* cw-15 (5 L) were grown under a 14-h light/10-h dark regime to a cell density of 0.5 to 1.0 × 10^6 cells mL^-1. At the day of the experiment, 200-mL aliquots were taken from these cultures 8 h after onset of illumination and further incubated in the light in the presence of different concentrations of putrescine, spermidine, and spermine, respectively. After 5 h, cells were harvested by centrifugation and cell lysates prepared and analyzed for DNA polymerase activity and protein as described in "Materials and Methods." Specific ODC activities are shown as percentage of the maximal activity of each experiment. Mean values of five separate experiments ± SD are given. *P* values were calculated by Student's *t* test.

<table>
<thead>
<tr>
<th>Polyamine</th>
<th>Concentration (mmol L^-1)</th>
<th>ODC Activity</th>
<th>Student's <em>t</em> Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Putrescine</td>
<td>0.05</td>
<td>98.0 ± 3.4</td>
<td>Nonsignificant</td>
</tr>
<tr>
<td>Putrescine</td>
<td>0.10</td>
<td>97.1 ± 3.2</td>
<td>Nonsignificant</td>
</tr>
<tr>
<td>Putrescine</td>
<td>0.20</td>
<td>91.4 ± 7.6</td>
<td><em>P</em> &lt; 0.05</td>
</tr>
<tr>
<td>Putrescine</td>
<td>0.50</td>
<td>82.5 ± 10.2</td>
<td><em>P</em> &lt; 0.0005</td>
</tr>
<tr>
<td>Putrescine</td>
<td>0.75</td>
<td>69.8 ± 13.1</td>
<td><em>P</em> &lt; 0.00001</td>
</tr>
<tr>
<td>Spermidine</td>
<td>1.00</td>
<td>62.4 ± 6.9</td>
<td><em>P</em> &lt; 0.00001</td>
</tr>
<tr>
<td>Spermidine</td>
<td>0.02</td>
<td>78.1 ± 16.7</td>
<td><em>P</em> &lt; 0.005</td>
</tr>
<tr>
<td>Spermidine</td>
<td>0.05</td>
<td>61.3 ± 12.3</td>
<td><em>P</em> &lt; 0.0005</td>
</tr>
<tr>
<td>Spermidine</td>
<td>0.07</td>
<td>50.9 ± 10.5</td>
<td><em>P</em> &lt; 0.0001</td>
</tr>
<tr>
<td>Spermidine</td>
<td>0.10</td>
<td>44.7 ± 5.9</td>
<td><em>P</em> &lt; 0.0001</td>
</tr>
<tr>
<td>Spermine</td>
<td>0.02</td>
<td>79.5 ± 18.4</td>
<td><em>P</em> &lt; 0.0025</td>
</tr>
<tr>
<td>Spermine</td>
<td>0.05</td>
<td>67.1 ± 16.3</td>
<td><em>P</em> &lt; 0.00025</td>
</tr>
<tr>
<td>Spermine</td>
<td>0.07</td>
<td>54.7 ± 9.8</td>
<td><em>P</em> &lt; 0.0001</td>
</tr>
<tr>
<td>Spermine</td>
<td>0.10</td>
<td>48.2 ± 5.3</td>
<td><em>P</em> &lt; 0.0001</td>
</tr>
</tbody>
</table>

Effects of Exogenous Polyamines on Cell Cycle-Dependent Alteration of ODC Activity and Cell Division

In synchronized cultures of the unicellular green alga *C. reinhardtii*, a 4-fold increase in ODC activity was measured during the transition from the cell enlargement to the cell division phase (Fig. 1D) preceding the increase in aphidicolin-sensitive DNA polymerase activity (S-phase; Fig. 1C) and the accumulation of dividing cells (Fig. 1B). This cell cycle-dependent increase in ODC activity was abolished when exogenous polyamines were added 7 to 8 h after beginning of the light period (growth phase; Fig. 1D). After addition of spermine, a transient decrease of ODC activity was measured (Fig. 1D). To minimize effects on cell growth, polyamines were added 7 to 8 h after beginning of the light period when the cells had already doubled their cell mass and therefore were able to divide (Voigt and Münzner, 1987, 1989). Addition of putrescine or spermidine, however, caused the same effect at concentrations of about 0.05 mmol L^-1 (Table II). Cytotoxic effects were observed when putrescine was applied at concentrations above 1.5 mmol L^-1 (data not shown). No cytotoxic effects were observed in the presence of up to 0.3 mmol L^-1 of spermidine or spermine. In the following experiments, therefore, the effects of putrescine were studied at an exogenous concentration of 1 mmol L^-1, and the effects of spermidine and spermine at concentrations of 0.1 mmol L^-1.
The Spermine-Induced Cell Cycle Arrest Is Due to a Lack of Spermidine

Because exogenous polyamines affected ODC activity (Table II; Fig. 1D), the spermine-induced cell cycle arrest might be caused by a lack of putrescine and/or spermidine. Comparative analyses of the intracellular polyamine levels in spermine-treated and untreated cultures revealed that spermine caused a decrease in the putrescine and spermidine levels and a reduced cell cycle-dependent increase of both polyamines (Table III). For this reason, we have investigated whether or not the spermine-induced cell cycle arrest can be overcome by a subsequent addition of putrescine or spermidine. Cell divisions were induced in spermine-treated cells by addition of spermidine and to a lower extend also by addition of putrescine (Fig. 2). Furthermore, we studied the effects of inhibitors of spermidine synthesis on cell division in synchronously growing Chlamydomonas reinhardtii cultures. The inhibitors were applied 8 h after beginning...
The effects of inhibitors of the polyamine metabolism on cell divisions in synchronized *C. reinhardtii* cultures. Cultures of the wall-deficient *C. reinhardtii* strain cw-15 were synchronized by growth under a constant light-dark regime of 14 h of light and 10 h of darkness for 4 d. On the day of the experiment, the cultures were divided and different inhibitors of the polyamine metabolism were added to the subcultures 8 h after beginning of the light period. The cultures were analyzed for cell densities (solid lines) and dividing cells (dashed lines) at the time periods after onset of illumination indicated in the figure. A, Untreated control (○) and addition of the spermidine synthase inhibitor 4-trans-methyl-cyclohexyl-amine (1 mmol L^{-1}; ■); B, addition of the S-adenosyl-Met decarboxylase (SAMDC) inhibitor methylglyoxal-bis-(guanyl-hydrazone) (MGBG) to final concentrations of 50 μmol L^{-1} (△) and 200 μmol L^{-1} (▼), respectively; C, addition of the ODC inhibitor difluoromethylornithine (DFMO; 2 mmol L^{-1}) in the absence (▽) or presence (▲) of 200 μmol L^{-1} of the SAMDC inhibitor MGBG.

Table IV. Inhibitors of spermidine synthesis affect ODC activity

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>ODC Activity</th>
<th>Student’s t Test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mmol L^{-1}</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>--</td>
<td>2</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>DFMO</td>
<td>0.05</td>
<td>380 ± 110</td>
<td>*P &lt; 0.0001</td>
</tr>
<tr>
<td>MGBG</td>
<td>0.20</td>
<td>700 ± 130</td>
<td>*P &lt; 0.0001</td>
</tr>
<tr>
<td>DFMO</td>
<td>0.20</td>
<td>1.9 ± 0.3</td>
<td>*P &lt; 0.0001</td>
</tr>
<tr>
<td>MGBG</td>
<td>2</td>
<td>210 ± 65</td>
<td>*P &lt; 0.00025</td>
</tr>
<tr>
<td>4-Me-CHA</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

of the light period when most of the cells had doubled their cell mass (Voigt and Münnzner, 1987, 1989). Cell divisions were abolished by addition of 4-methyl-cyclohexylamine (4-Me-CHA; Fig. 3A), an inhibitor of spermidine synthase (Marton and Pegg, 1995). Because spermidine is generated from putrescine and decarboxylated S-adenosyl-Met, which are formed by ODC and SAMDC, respectively, the effects of inhibitors of these enzymes were also studied (Fig. 3, B and C). Cell divisions were largely abolished when the SAMDC inhibitor MGBG was added to a final concentration of 200 μmol L^{-1} (Fig. 3B). At a concentration of 50 μmol L^{-1}, MGBG caused a slight increase of dividing cells and a delay in the increase of the cell density (Fig. 3B). Addition of the ODC inhibitor DFMO at a concentration where a quantitative inhibition of ODC was measured after 5 h (Table IV), however, did not abolish cell divisions, but caused an early accumulation of dividing cells (Fig. 3C). Cell density increased, however, at the same time as in the untreated cultures (Fig. 3, A and C). In the presence of both DFMO and MGBG, cell divisions were abolished (Fig. 3C). Significantly increased ODC activities were measured 5 h after addition of the SAMDC inhibitor MGBG or of the spermidine synthase inhibitor 4-Me-CHA (Table IV) as already reported for mammalian cells (Marton and Pegg, 1995).

The Spermine-Induced Cell Cycle Arrest Is Not Due to a Decreased Rate of Protein Synthesis

Because spermidine is required for the activation of eIF-5A (Jakus et al., 1993), we have investigated whether or not the spermine-induced cell cycle arrest mediated by a decreased spermidine level is caused by a down-regulation of protein synthesis. To this end, aliquots were taken from synchronized cultures at different time intervals after onset of illumination, pulse labeled with \([^{3}H]\)Arg, and subsequently analyzed for radioactively labeled protein. In untreated cultures, incorporation of \([^{3}H]\)Arg into protein increased during the growth period (light phase) up to 13 h after onset of illumination (Fig. 4) and declined...
subsequently during cell division (Figs. 1 and 4). Addition of spermine 8 h after beginning of the light period did not cause a decrease in protein biosynthesis (Fig. 4). Instead, the decrease in the incorporation of [3H]Arg into protein observed in the untreated cultures between 15 and 21 h after onset of illumination was not observed in the presence of spermine unless spermidine was additionally applied 13 h after beginning of the light phase (Fig. 4).

**DISCUSSION**

In *C. reinhardtii* cells, an almost constant putrescine:spermidine ratio of about 10:1 was found at all cell cycle stages (Table I). Spermine could not be detected in *C. reinhardtii* cells (Table I). In synchronously growing *C. reinhardtii* cells, a 10- and a 20-fold increase, respectively, in the putrescine and spermidine levels was observed during the light period the maximal level being reached 15 h after onset of illumination (Table I) as already reported for the green alga *S. obliquus* (Kotzabasis and Senger, 1994). The increase in the polyamine level was accompanied by a 4-fold increase in the activity of ODC (Fig. 1D), the key enzyme of polyamine synthesis in *C. reinhardtii* (Voigt et al., 2000a). This cell cycle-dependent increase in ODC activity preceded the transition to the cell division phase (Fig. 1, B and C) and was found to be caused by an increased ODC half-life (Voigt and Bohley, 2000). Therefore, the question arose whether or not this increase in ODC activity and polyamine levels has any significance for cell division. Increased ODC activities and polyamine levels were also observed in proliferating mammalian and higher plant cells as compared with resting cells (Manzella et al., 1991; Daoudi and Biondi, 1995; Marton and Pegg, 1995; Ben Hayyim et al., 1996; El Ghachtouli et al., 1996; Fowler et al., 1996; Graff et al., 1997; Chattopadhyay and Ghosh, 1998; Cvikrova et al., 1999). Furthermore, ODC activities and polyamine levels were found to be elevated in cancer cells (Hibshoosh et al., 1991; Auvinen et al., 1992; Moshier et al., 1993; Marton and Pegg, 1995). As previously reported, inhibition of ODC not only caused a decrease in the polyamine levels, but also a decreased rate of cell divisions (Koza and Herbst, 1992; Fredlund et al., 1995). On the other hand, it has been shown that in eukaryotic cells, spermidine is required for the activation of initiation factor eIF-5A (Jakus et al., 1993) and, therefore, necessary for protein biosynthesis and cell growth. For this reason, the published data indicating a correlation between polyamine level and cell proliferation (Hibshoosh et al., 1991; Auvinen et al., 1992; Moshier et al., 1993; Marton and Pegg, 1995) and the findings that inhibition of of polyamine synthesis impaired division of eukaryotic cells (Koza and Herbst, 1992; Fredlund et al., 1995) could be referred to as a dependence of protein biosynthesis on the spermidine level. Thus, assumptions that polyamines might be directly involved in the regula-

![Figure 4. Effects of spermine and spermidine on cell cycle-dependent variation of the incorporation of [3H]Arg into protein. Cultures of the wall-deficient strain *C. reinhardtii* cw-15 were synchronized by growth under a constant 14-h-light/10-h-dark regime. At the day before the experiment, the cultures were divided and diluted with fresh culture medium to a density of 0.5 × 10⁶ cell mL⁻¹. Spermine and spermidine were added 8 and 13 h, respectively, after beginning of the light period (final concentrations: 0.1 mmol L⁻¹). Aliquots of 1 mL were taken at the indicated time intervals after onset of illumination and pulse labeled with 370 kBq of [3H]Arg for 30 min. After pulse labeling, the cells were harvested, washed, and analyzed for radioactively labeled protein as described in “Materials and Methods.” Data are expressed in dpm [3H]Arg incorporated into protein per 10⁶ cells. Mean values of four separate experiments ± so are given. ○, Control culture; ▽, culture treated with spermine (0.1 mmol L⁻¹) 8 h after beginning of the light period; ▼, culture treated with spermine (0.1 mmol L⁻¹) 8 h after beginning of the light period and with spermidine (0.1 mmol L⁻¹) after 13 h.
tion of cell division (Koza and Herbst 1992; Fredlund et al., 1995) have not, up to now, been corroborated by satisfying experimental proofs.

Unicellular green algae like C. reinhardtii are particularly suitable for such studies because they can be easily synchronized by growth under a constant light-dark regime (Surzycki, 1971; Voigt and Münzner, 1987; Krupinska and Humbeck, 1994). Furthermore, they do not enter the cell division phase upon doubling their cell mass. Under optimal growth conditions, C. reinhardtii cells can multiply their cell mass during the light phase (growth period, G1 phase; for references, see Schlösser, 1966; Mihara and Hase, 1971; Voigt and Münzner, 1987; Harris, 1989). During the subsequent cell division phase that normally occurs during the dark period unless the cells have reached a strain-specific maximal size already during the light phase (Voigt and Münzner, 1987), these “mother cells” undergo several cell divisions without additional G1 phases and without measurable G2 phases (Harris, 1989). Direct effects on cell division of compounds influencing both cell growth and cell division can be studied in this experimental system when applied after most of the cells have doubled their cell mass and, therefore, attained the commitment to divide (e.g. 7–8 h after onset of illumination; for references, see Voigt and Münzner, 1989). For this reason, we have investigated the influence of both exogenous polyamines and inhibitors of polyamine metabolism on ODC activity and cell divisions in synchronized C. reinhardtii cultures when added 7 to 8 h after beginning of the light period. Addition of spermine, but not of putrescine or spermidine, inhibited the transition to the cell division phase (Fig. 1, B and C). In untreated C. reinhardtii cultures, the transition to the S-phase (Fig. 1C) was preceded by a 4-fold increase in ODC activity (Fig. 1D). Addition of polyamines prevented this up-regulation of ODC activity (Fig. 1D), indicating that the spermine-induced cell cycle arrest (Fig. 1, A–C) might be due to a lack of putrescine and/or spermidine. This conclusion was corroborated by our observations that the levels of putrescine and spermidine were decreased after addition of spermine (Table III) and that the spermine-induced cell cycle arrest was overcome by subsequent addition of spermidine or, to a lesser extent, also by addition of putrescine (Fig. 2). Furthermore, cell divisions could also be prevented by addition of inhibitors of spermidine synthesis (Fig. 3) with the exception of the ODC inhibitor DFMO. Because the ODC activity was shown to be completely inhibited under these experimental conditions (Table IV), the putrescine level must have been high enough for the formation of amounts of spermidine sufficient for the transition to the cell division phase under these conditions. On the other hand, it has been shown that inhibition of ODC by DFMO causes an increase in SAMDC activity, thus resulting in an increased formation of spermidine (Marton and Pegg, 1995).

The question arose whether the lack of spermidine affects cell division directly or via a decrease in the rate of protein biosynthesis caused by a decreased spermidine-dependent activation of eIF-5A (Jakus et al., 1993). As shown in Figure 4, no decrease in protein synthesis could be observed after addition of spermine under conditions where spermine caused a cell cycle arrest (Figs. 1 and 2). Addition of spermine under these experimental conditions prevented the decrease in protein biosynthesis normally observed when the cells entered the division phase (Fig. 4; for references, compare with Voigt et al., 2000b). This finding was in agreement with the observation that the loss of cell motility that accompanies the transition to the cell division phase was also prevented by treatment with spermine (data not shown). In accordance, a decrease in protein biosynthesis (Fig. 4) and a loss of cell motility (data not shown) were observed when spermidine was added to cultures that were cell cycle arrested by spermine treatment. These findings indicate that, at least in the case of C. reinhardtii, spermidine is required for the transition from G1 to the S phase and that, under our experimental conditions, spermidine does not affect cell division by its effects on the activation of eIF-5A (Jakus et al., 1993) and, therefore, via protein synthesis and cell growth. When added at the beginning of the light period, however, polyamines caused an increase in protein biosynthesis (data not shown) that can be explained by effects on the activation of eIF-5A (Jakus et al., 1993).

**MATERIALS AND METHODS**

**Strains and Growth Conditions**

The cell wall-deficient strain CW15 of Chlamydomonas reinhardtii (Davies and Plaskitt, 1971) was obtained from the Sammlung von Algenkulturen at the University of Göttingen (Germany). Cells were grown at 24°C under a photon fluence rate of 40 μmol m⁻² s⁻¹ in a high-salt medium supplemented with 0.2% (w/v) sodium acetate as described previously (Voigt and Münzner, 1987). Synchronized cultures were obtained by light-dark cycling under a 14-h-light/10-h-dark regime (Voigt and Münzner, 1987). Cell concentrations were determined by duplicate hemocytometer counting.

**Preparation of Cell Lysates**

Cells were harvested by centrifugation at 6,000g for 10 min at 4°C. All subsequent steps were performed at 0°C to 4°C. The cells were washed with and resuspended to a final cell density of 0.5 to 1 x 10⁸ cells mL⁻¹ in ice-cold homogenization buffer A consisting of 25 mmol L⁻¹ Tris-HCl, pH 7.0, 2 mmol L⁻¹ dithiothretiol, and 0.1 mmol L⁻¹ EDTA. After addition of the protease inhibitors phenylmethylsulfonyl fluoride (final concentration: 0.1 mmol L⁻¹)
and chymostatin (final concentration: 5 μg mL⁻¹), cell lysis was performed by addition of Triton X-100 to a final concentration of 0.5%–1% (w/v)). After 5 min, efficiency of lysis was checked microscopically and the particulate constituents removed by centrifugation for 15 min at 400,000g in a Beckman (Munich, Germany) ultracentrifuge rotor TLA 100.2. The supernatants were stored at −75°C.

ODC Assay

ODC activity was determined by measuring the release of $^{14}$CO₂ from L-[1-¹⁴C]Orn (Schulz et al., 1985). No release of $^{14}$CO₂ from L-[1-¹⁴C]Orn was detected when the *Chlamydomonas* sp. lysates were pre-incubated in the presence of the ODC inhibitor DFMO at a concentration of 0.1 mmol L⁻¹ for 10 min at 4°C before incubation with L-[1-¹⁴C]Orn. One unit of ODC activity catalyzed the decarboxylation of 1 μmol of Orn min⁻¹ at 37°C.

Determination of Protein

Quantitation of protein was performed by the method of Minamide and Bamburg (1990) using bovine serum albumin as standard.

Isolation of Nuclei

Nuclei were prepared as recently described (Voigt and Bohley, 2000) and stored at −75°C as a suspension (1 × 10⁸ nuclei mL⁻¹) in a buffer containing 2.5% (w/v) Ficoll, 0.002% (w/v) spermidine, 1 mmol L⁻¹ dithiothreitol, 5 mmol L⁻¹ MgCl₂, and 50% (v/v) glycerol.

DNA Synthesis

DNA synthesis was measured in the absence and presence of aphidicolin (70 μmol L⁻¹), an inhibitor of DNA polymerase-α, as previously described (Voigt and Münzner, 1987). The reaction mixture (20 μL) contained 55 mmol L⁻¹ Tris-HCl (pH 8.0), 150 mmol L⁻¹ KCl, 2.5 mmol L⁻¹ MgCl₂, 0.002% (w/v) spermidine, 125 mmol L⁻¹ sorbitol, 0.52% (w/v) Ficoll, 12.5% (v/v) glycerol, 2.5 mmol L⁻¹ dithiothreitol, 2.5% (v/v) dimethylsulfoxide, 1 mmol L⁻¹ dCTP, 1 mmol L⁻¹ dGTP, 1 mmol L⁻¹ dTTP, 37 kBq [α-³²P] dATP (110 Tbp/μmol), and 5 × 10⁹ nuclei. After addition of nuclei, the reaction mixture was incubated at 25°C for 1 h. After incubation, 5 μL of stop mix, containing 5% (w/v) SDS and 50 mmol L⁻¹ EDTA, was added and the reaction mixture plated onto 3-mm filters (Whatman Ltd., Maidstone, UK). The filters were washed once with 10% (w/v) trichloroacetic acid (TCA), twice with 5% (w/v) TCA, and twice with methanol, dried, and measured for radioactivity after addition of 4 mL of UltimaGold (Packard Instruments, Groningen, The Netherlands). Blank values (reaction mixtures without incubation) were subtracted.

Determination of Protein Biosynthesis

Biosynthesis of proteins was analyzed in vivo by pulse labeling with [³H]Arg and measuring the radioactivity incorporated into protein. Aliquots (1 mL) were taken from the cultures and incubated for 30 min in the presence of 370 kBq of [³H]Arg (specific radioactivity 1.7 Tbp mmol⁻¹; Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). After pulse labeling, the cells were rapidly cooled to 0°C and centrifuged through a 0.4-mL cushion of 40% (w/v) Percoll in phosphate-buffered saline. The cells were washed twice with 1 mL of phosphate-buffered saline and finally dissolved in 100 μL of urea-SDS buffer containing 8 mol L⁻¹ urea, 2% (w/v) SDS, 10 mmol L⁻¹ EDTA, 200 mmol L⁻¹ 2-mercaptoethanol, and 20 mmol L⁻¹ Tris-HCl, pH 7.5. Aliquots (50 μL) were plated onto glass microfiber filters (GF/C, Whatman Ltd.) and boiled for 15 min in 10% (w/v) TCA to hydrolyze the aminoacyl-tRNAs. The filters were then washed twice for 1 min with 5% (w/v) TCA, twice for 1 min with 95% (v/v) ethanol, and finally with diethylether. The dried filters were then measured for radioactivity.

Analysis of Polyamines

Lyophilized samples were extracted in 5% (w/v) TCA for 1 h in an ice bath and centrifuged for 30 min at 4°C and 20,000g in a Sorvall SS34 rotor. The supernatants were evaporated at 70°C to 80°C. Dried samples were redissolved in 200 μL of 5% (v/v) perchloric acid and the polyamines benzoylated according to Flores and Galston (1982). Aliquots were analyzed by reversed-phase HPLC according to Kotzabasis et al. (1993) using the HPLC system Gold (Beckman Instruments, San Ramon, CA) equipped with an Ultrasphere C18 column, 4.6 × 250 mm, 5-μm particle size (Beckman, Mereoue, Galway, UK). Elution of the benzoyl-polyamines was performed at 25°C and a flow rate of 1.0 mL min⁻¹ with 60% (v/v) methanol and monitored at 254 nm.

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LITERATURE CITED


reticulocyte lysate is ATP-dependent but ubiquitin-independent. J Biol Chem 264: 15949–15952


