A S/M DNA replication checkpoint prevents nuclear and cytoplasmic events of cell division including centrosomal axis alignment and inhibits activation of cyclin-dependent kinase-like proteins in fucoid zygotes

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SUMMARY

S/M checkpoints prevent various aspects of cell division when DNA has not been replicated. Such checkpoints are stringent in yeast and animal somatic cells but are usually partial or not present in animal embryos. Because little is known about S/M checkpoints in plant cells and embryos, we have investigated the effect of aphidicolin, a specific inhibitor of DNA polymerases α and δ, on cell division and morphogenesis in Fucus and Pelvetia zygotes. Both DNA replication and cell division were inhibited by aphidicolin, indicating the presence, in fucoid zygotes, of a S/M checkpoint. This checkpoint prevents chromatin condensation, spindle formation, centrosomal alignment with the growth axis and cytokinesis but has no effect on germination or rhizoid elongation. This S/M checkpoint also prevents tyrosine dephosphorylation of cyclin-dependent kinase-like proteins at the onset of mitosis. The kinase activity is restored in extracts upon incubation with cdc25A phosphatase. When added in S phase, olomoucine, a specific inhibitor of cyclin-dependent kinases, has similar effects as aphidicolin on cell division although alignment of the centrosomal axis still occurs. We propose a model involving the inactivation of CDK-like proteins to account for the S/M DNA replication checkpoint in fucoid zygotes and embryos.

Key words: Fucoid zygote, S/M checkpoint, Aphidicolin, Centrosomes, CDK, cdc25, Olomoucine

INTRODUCTION

DNA damage or alteration of any component of the cell cycle machinery can lead to cell division catastrophes, inducing a loss of reproductive capacity and cell death in unicellular organisms, and aneuploidy or cancer in animal cells (Hartwell and Kastan, 1994; Paulovich et al., 1997). Checkpoints are regulatory mechanisms that help to ensure that the sequence of cell cycle events is correct (Hartwell and Weinert, 1989). For example DNA damage or spindle alteration induces cell cycle delay or arrest in a wide variety of eukaryotes (Murray, 1994). The delay induced by checkpoints ensures the accurate transmission of the genetic information by allowing the repair of DNA before mitosis and the alignment of the chromosomes before the onset of anaphase.

The S/M DNA replication checkpoint has been extensively studied in fission and budding yeasts as well as in animal somatic and embryonic cells. Interestingly the S/M checkpoint displays some variations that are species or developmental stage specific. In yeast and mammalian somatic cells, the inhibition of DNA replication prevents mitosis (Hartwell and Weinert, 1989; Rhind and Russel, 1998). In contrast, during early development in many animal embryos cell division is not dependent upon completion of DNA replication. In Drosophila embryos, for example, the inhibition of DNA replication with aphidicolin, a specific inhibitor of DNA polymerases α and δ (Ikegami et al., 1978), has little effect on either nuclear and cytoplasmic events of cell division or morphogenesis (Raff and Glover, 1988). In this case, the lack of efficiency of the S/M checkpoint is compensated for by other embryo-specific mechanisms that ensure the accuracy of genome transmission (Sullivan et al., 1990).

Cell cycle arrests induced by checkpoints are often mediated by the inactivation of cyclin-dependent kinases (CDKs), a family of serine/threonine kinases that control cell cycle progression in eukaryotes. Besides a positive regulation by binding to cyclins, CDKs can be regulated by phosphorylation or by association with inhibitory proteins (reviewed by Nigg, 1995). Experiments in Schizosaccharomyces pombe indicate that the inactivation of cdc2 by tyrosine phosphorylation is sufficient to prevent entry into mitosis following activation of the S/M checkpoint (Enoch and Nurse, 1990). In contrast, other
additional mechanisms are to be required in both Saccharomyces cerevisiae and mammalian cells (Sorger and Murray, 1992; Jin et al., 1996).

Although cyclins and CDKs, including conventional CDKs with the conserved PSTAIRE motif, have been described in plant cells, their respective roles in controlling cell cycle progression and cell cycle arrest are not well understood (Mironov et al., 1997). Only a few checkpoints, such as an oxidative stress checkpoint in tobacco (Reichheld et al., 1999), have been described. The lack of knowledge in this field is mainly due to the limited number of cell cycle mutants and to the absence of naturally synchronised cells in higher plants. Zygotes of fucoid algae, including the genera Fucus and Pelvetia, are large cells (90 μm in diameter) that are naturally synchronised by fertilisation (Kropf, 1992). In addition, large populations of embryos can be easily obtained for biochemical experiments. This material is, therefore, particularly well suited to investigate developmental processes (Brownlee and Bouget, 1998). Little is known, however, about the regulation of cell cycle events in fucoid embryos and much of our knowledge on the cell cycle in fucoid algae comes from the analysis of microtubules and microtubule organising centres (centrosomes). During the first cell cycle, the separation of the sperm-derived centrosomes is slow, asynchronous and is completed just before mitosis, approximately 16 hours after fertilisation (AF) (Nagasato et al., 1999; Bisgrove and Kropf, 1998; Bisgrove et al., 1997). The centrosomal axis rotates and aligns with the rhizoid/thallus growth axis either just prior to or during mitosis (Bisgrove and Kropf, 1998). As a result, the mitotic spindle, which forms from the centrosomes, is aligned with the growth axis. Cytokinesis completes the first asymmetric division 20-24 hours AF (Bisgrove and Kropf, 1998). In this paper we describe a S/M DNA replication checkpoint in fucoid zygotes, which prevents cell division and alignment of the centrosomal axis until DNA is fully replicated but has no effect on germination or rhizoid elongation. This checkpoint appears to operate in part through the inactivation of CDK-like proteins by tyrosine phosphorylation.

**MATERIALS AND METHODS**

**Culture and inhibitors**

Gametes of Fucus spiralis (collected at Le Dossen, Brittany, France), were released by standard osmotic shock procedures in filtered sea water (FSW) over a 1 hour period (Corellou et al., 2000). Sexually mature receptacles of Pelvetia compressa (J. Agardh) were collected near Pigeon Point Lighthouse, just north of Santa Cruz, California. Receptacles were shipped cold and stored at 4°C in the dark. To near Pigeon Point Lighthouse, just north of Santa Cruz, California.

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Inhibition of DNA replication was performed using aphidicolin (20 μM, Sigma). Olomoucine, a specific inhibitor of CDKs, was chemically synthesised (Vesely et al., 1994). Nocodazole (0.33 μM, Sigma) was used to prevent spindle formation. Aphidicolin, olomoucine and nocodazole were dissolved in DMSO at 10 mg/ml, 300 mg/ml and 1 mg/ml respectively and further diluted in ASW before use. Control experiments were performed in FSW or ASW containing the same final concentrations of DMSO.

**DNA staining and quantification**

Zygotes and embryos of Fucus and Pelvetia were fixed for 12 hours in 0.2 M citric acid and 0.2% Triton X-100, and subsequently stored in 100% methanol. Fixed cells were attached to poly-L-lysine-coated coverglasses and rinsed with 0.04 M citric acid, 0.33 M NaH2PO4 (pH 7). Cell walls were softened by incubation in 1 M LiCl for 20 minutes (Evans, 1962) followed by two rinsing in 50 mM MgCl2. For epifluorescence microscopy, cells were counter-stained with 0.01% Evans Blue for 5 minutes and rinsed twice in 50 mM MgCl2. Cells were subsequently incubated with 50 μg/ml of mithramycin in 50 mM MgCl2 for 2 hours, rinsed twice in 50 mM MgCl2 then mounted and observed by epifluorescence microscopy under blue light excitation. For DNA quantification the counter-staining step was omitted. The procedure was performed only on two-celled and older embryos because of its lack of reproducibility in earlier stages of development. After the staining procedure, the embryos were mechanically disrupted between the slide and the coverglass and mounted. DNA was quantified using a Biorad MRC 600 confocal microscope (excitation at 488 nm and emission wavelength above 605 nm in the red channel). Z series of 0.5 μm were collected (with an average of 15 sections per nucleus) and stacked. Fluorescence in the nuclei was quantified using the Lasershop Analysier software (Biorad). Relative fluorescence of nuclei (1-8 relative units) was divided into seven equal classes and a histogram representing the frequency of nuclei in each class was established (50-75 nuclei).

**Immunofluorescence microscopy of microtubules**

Pelvetia zygotes were processed for microtubule immunolocalisation with monoclonal anti-α-tubulin antibodies (DM1A; Sigma; diluted 1:100 in mPBS), as previously described (Bisgrove and Kropf, 1998). Images were obtained on an MRC-600 laser scanning confocal microscope, using a narrow band pass (578-618 nm) filter set.

**Protein extraction**

Embryos were harvested, briefly centrifuged (3000 g), frozen in liquid nitrogen and stored at −80°C. Frozen samples were ground in liquid nitrogen and resuspended in 1.6 ml of extraction buffer containing 0.5% (w/v) polyvinylpolypyrrolidone and 0.5% (w/v) nonidet P-40 (Corellou et al., 2000). Samples were extracted on ice for approx. 30 minutes, with periodic quick vortexing. The pellet was discarded after centrifugation (10 000 g, 4°C, 10 minutes) and the protein extract (supernatant) was immediately used.

**Purification of CDK-like proteins and histone H1 kinase assay**

CDK-like proteins were purified on p9CKShs1 Sepharose beads and their ability to phosphorylate histone H1 was determined as previously described (Azzi et al., 1994). Briefly, protein extracts were incubated with 10 μl of beads containing 3.9 mg/ml of the recombinant human suc1 homologue p9CKShs1, on a rotary shaker at 4°C for 1 hour (Azzi et al., 1994). Beads were spun down at 3000 g for 1 minute and the supernatant was discarded. The beads were subsequently washed three times in bead buffer (Azzi et al., 1994). Histone H1 kinase activity of proteins bound to p9CKShs1 Sepharose beads was measured at 3°C for 30 minutes. Phosphorylation was initiated by adding to the beads 30 μl of a reaction mixture consisting of 20 μl of buffer C (Azzi et al., 1994), 5 μl of histone H1 at 5 mg/ml (Calbiochem) and 5 μl of 90 μM [γ-32P]ATP (2 μCi). Beads were resuspended every 3 minutes by a pulse of vortexing and the reaction was stopped by addition of 30 μl of 4x denaturing buffer (62.5 mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol). Samples were heated at 100°C for 10 minutes and thoroughly vortexed. Bead supernatants were analysed on a 12% SDS denaturing gel. Gels were stained with Coomassie Blue and the radioactivity of histone H1 was quantified using a STORM.
phosphorimager with the Image QuanT software (Molecular Dynamics). For each lane, a protein-free area of the same surface was used to determine the background according to the object average method.

**Western blot analysis**

40 μl of 4x Laemmli buffer were added to p9CKShs1 beads and proteins were eluted at 90°C for 10 minutes. Eluted proteins were resolved on a 10 or 12% SDS-polyacrylamide denaturing gel and transferred onto a PVDF membrane for ECL or ECL+ detection (Amersham Life Science, Buckinghamshire, UK). Liquid electrophotransfer was performed in 0.025 M Tris and 0.192 M glycine, for 90 minutes at 1 A. Proteins were stained with Ponceau Red to check the homogeneity of the transfer. The membranes were blocked in Tris-buffered saline (TBS) containing Blotto B as described by the manufacturer (Santa Cruz Biotechnology, California) and then incubated either with a monoclonal anti-phosphotyrosine antibody (PY20, Santa Cruz Biotechnology) at a 1/20,000 dilution or with a monoclonal anti-PSTAIRE antibody (Sigma) at a 1/3,000 dilution. The GST-cdc25A fusion protein was overproduced in E. coli, then reprobed with the anti-PSTAIRE antibody, which revealed the presence of PSTAIRE antibody. The anti-phosphotyrosine antibody could not be detected using the classical ECL detection system. Therefore, in some experiments, the blots were probed first with this antibody, revealed in ECL+, then reprobed with the anti-PSTAIRE antibody, which required short times of exposure in ECL detection.

**Dephosphorylation of CDK-like proteins by GST-cdc25A**

The GST-cdc25A fusion protein was overproduced in E. coli and purified by affinity on glutathione-agarose beads as previously described (Borgne and Meijer, 1996). Elution of the GST-cdc25A was performed in buffer B (50 mM Tris-HCl pH 8, 50 mM NaCl, 1 mM EDTA, 1mM DTT) containing 10 mM glutathione. Phosphatase activity of the eluted fraction was 0.62 units/μl (1 unit corresponds to the hydrolysis of 1 nmol of pNPP per minute). Dephosphorylation was initiated by adding to the proteins bound to the p9CKShs1 beads 100 μl (62 units) of the purified GST-cdc25A in buffer B containing 20 mM DTT. Dephosphorylation reactions were performed at 30°C for 1 hour. Control samples were treated in the same conditions with 100 μl of buffer B containing 20 mM DTT but lacking GST-cdc25A.

**RESULTS**

**Aphidicolin treatment inhibits DNA replication and cell division but not morphogenesis**

The effect of aphidicolin on DNA replication, cell division and early development was investigated in *Fucus zygotes* and embryos. Since the incorporation of either [3H]thymidine or 5-bromodeoxyuridine into DNA was not successful, we developed a new DNA staining protocol with mithramycin A. This protocol allowed the visualisation of nuclei and the quantification of DNA by confocal microscopy reliably in 2-celled and older embryos (Fig. 1) but not in zygotes. Embryos were treated with 20 μM aphidicolin for 24 hours beginning either during the first mitosis (Fig. 1A) or a few hours later (Fig. 1C). The first mitosis proceeded normally when cells were treated during mitosis (Fig. 1B). However, the second embryonic division was blocked and the nuclei remained decondensed when aphidicolin was added either at the time of the first mitosis or after cytokinesis (Fig. 1B,D). Cells arrested in mitosis with 0.33 μM nocodazole displayed heavily condensed chromosomes (Fig. 1E) and the relative fluorescence units (RFU) of mithramycin A corresponding to a 4C DNA content (5.7 RFU) was used as an internal reference (Fig. 1H). In aphidicolin-treated embryos, DNA levels were lower following treatments that were applied during mitosis (average of 2.6 RFU, Fig. 1F) than after treatments that were applied to 2-celled embryos (average of 4.0 RFU, Fig. 1G). Together these results indicate that cells treated during the first mitosis were arrested in early S phase of the next cell cycle, whereas a number of cells treated after the first division (either in G1 or S phase) were arrested in S phase with various contents of DNA. They also show that, in *Fucus* embryos, the entry into mitosis is delayed until DNA is replicated. We have taken advantage of this DNA replication checkpoint to estimate the end of S phase in young zygotes, for which quantification of DNA was not technically feasible. Aphidicolin (20 μM) was added at different times after fertilisation and the ability to complete cytokinesis was scored after a 36 hour incubation (Fig. 1). A majority of zygotes proceeded through mitosis into the next cell cycle, only when treated later than 12 hours AF. Since mitosis occurs 16 to 18 hours AF in untreated zygotes, as determined by staining DNA with mithramycin A (Fig. 1I), it follows that G2 phase typically starts at about 10 hours AF and lasts approximately 6 hours.

Next, the effect of aphidicolin on early morphogenesis was investigated (Fig. 2). Aphidicolin (20 μM) was added at 2 hours AF prior to pronuclear fusion and the initiation of DNA replication (Motomura, 1995). Aphidicolin treatments inhibited nuclear envelope breakdown, chromatin condensation and cytokinesis for at least 36 hours (Fig. 2B). Germination, however, occurred in treated zygotes (Fig. 2B), although with a slight delay (not shown). Rhizoid elongation continued in aphidicolin-treated zygotes and, when examined at 48 hours AF (Fig. 2D), treated zygotes had rhizoids that were similar in length to those of multicellular control embryos (Fig. 2E). However, after longer incubations with aphidicolin, the rhizoid often swelled (Fig. 2F) and after a few days in culture it burst (not shown), probably because of the weaker wall at the rhizoid tip.

**Aphidicolin treatment prevents spindle formation and centrosomal axis alignment**

The effects of blocking DNA replication on microtubule-regulated aspects of the cell cycle were investigated in *P. compressa* zygotes, since the protocols for microtubule immunolocalisation have been well established in this species (Bisgrove et al., 1997; Bisgrove and Kroopf, 1998). As in *Fucus* (e.g. Fig. 1B), when *Pelvetia* zygotes were incubated with 25 μM aphidicolin beginning during S phase, the chromatin did not condense (not shown) and cytokinesis did not occur. At the time of aphidicolin treatment (6 hours AF), the centrosomes were positioned close together on the nuclear envelope and microtubules emanated from both the nuclear envelope and, more prominently, from the centrosomes (Fig. 3A). By 16 hours AF, the centrosomes in untreated zygotes had completed their migration to opposite sides of the nuclear envelope and they served as the primary microtubule-organising centers in the cell (Fig. 3B). Mitosis began 15 to 17 hours AF (Fig. 3C) and, by 45 hours AF, untreated embryos were multicellular (Fig. 3D). In aphidicolin-treated zygotes, centrosomal
separation occurred, but metaphase spindles did not form. In zygotes sampled at either 24 or 45 hours AF, only one nucleus was present and microtubules were organised in bipolar centrosomal arrays (Fig. 3E,F).

To determine whether alignment occurs, the angle between the centrosomal axis and the growth axis was measured and the average alignment angles in populations of aphidicolin-treated and control zygotes were determined (Fig. 3I). In a population of young zygotes that have not yet undergone centrosomal alignment, the centrosomal axes are positioned randomly with respect to the growth axes (Bisgrove and Kropf, 1998) and the average alignment angle is about 45°. In aphidicolin-treated zygotes, the centrosomal axes were positioned randomly with respect to the growth axes (average alignment angles were close to 0°, the average angle expected from a population where the centrosomal axes are aligned with the growth axes. These results were also analysed statistically. In the presence of aphidicolin, 48% of zygotes sampled at 24 hours AF and 54% of those sampled at 48 hours AF had centrosomal axes that were aligned within 45°C of the growth axes. These proportions are not statistically different from the 1:1 ratio (aligned/unaligned axis) that is expected if the centrosomal axes were randomly positioned ($P=0.8537$ at 24 hours and $P=0.6122$ at 48 hours by a $\chi^2$ analysis). In contrast, the axes were aligned within 45° in all control zygotes sampled at 24 hours AF ($P<0.001$). When zygotes were released from a 36 hour treatment with aphidicolin, they were able to complete DNA replication and subsequent division, with a normally oriented plane of cleavage (Fig. 3G). In the long term, these embryos displayed a normal apical-basal pattern with normally localised apical hairs and basal rhizoids (Fig. 3H).

PSTAIRE CDK-like proteins remain phosphorylated on tyrosine and inactive following aphidicolin treatment

We, then, monitored the effect of the inhibition of DNA replication on CDK-like activities in *Fucus* zygotes. CDK-like proteins were bound to the human su1 homologue p9CKShs1 and assayed for histone H1 kinase activity (referred to as histone H1 kinase activity below). In control zygotes, a mitotic peak of histone H1 kinase activity was detected before the onset of mitosis (Fig. 4). In contrast, in cells treated with 20 $\mu$M aphidicolin from 4 hours AF, histone H1 kinase activities remained at low levels, similar to those of late S phase, for at least 36 hours.

The presence, in *Fucus* zygotes, of CDK-related proteins with the hallmark PSTAIRE motif was investigated next. Two proteins bound to p9CKShs1 were immunodetected with an anti-PSTAIRE antibody during early development and following treatments with either nocodazole or aphidicolin (Fig. 5A,B). These two proteins migrated at 34 and 32 kDa, respectively, and they are referred to as p34 and p32. PY20, a monoclonal
antibody directed against phosphotyrosine residues (Hamaguchi et al., 1988), revealed two proteins differentially phosphorylated on tyrosine during the first cell cycle (Fig. 5A). These proteins are likely to be the PSTAIRE CDK-like proteins described above for at least three reasons: (i) the proteins recognised by the PY20 antibody also featured molecular masses of 34 and 32 kDa, respectively; (ii) the levels of both PY and PSTAIRE epitopes increased in parallel until 16 hours AF in all samples (Fig. 5A,B); and (iii) at mitosis (18 hours AF), when histone H1 kinase activity is maximal, p34 and p32 were recognised by the anti-PSTAIRE antibody but not by the PY20 antibody.

Cells arrested in S phase with aphidicolin exhibited high levels of tyrosine phosphorylated p34 and somewhat lower levels of tyrosine phosphorylated p32, at the time corresponding to mitosis in control zygotes (18 hours AF) as well as long after (Fig. 5B). The hypothesis that CDK-like proteins are regulated by tyrosine phosphorylation in fucoid
zygotes was further tested using the human protein phosphatase cdc25A (Hoffman et al., 1994). Compared to controls histone H1 kinase activities from zygotes in mitosis (18-hour-old or treated with nocodazole) were not significantly activated upon incubations with GST-cdc25A to dephosphorylate and thereby activate CDK-like proteins (Fig. 5C). In contrast, upon incubation with GST-cdc25A, these activities increased dramatically in protein extracts from both normal G2 cells (14 hours AF) and aphidicolin-treated cells (Fig. 5C). Taken together, the findings shown in Figs 4 and 5 suggest that, in Fucus zygotes, inhibition of DNA replication prevents the activation of mitotic CDK-like proteins, by inhibiting their dephosphorylation on tyrosine residues.

Olomoucine inhibits G2/M CDK like activity in vitro and prevents cell division

CDK-like proteins were also inhibited in Fucus zygotes using olomoucine (Vesely et al., 1994). When applied before the G1/S transition, treatment with 100 μM olomoucine inhibited DNA replication through the inactivation of G1/S CDKs (not shown). To prevent a possible activation of the DNA replication checkpoint olomoucine was, therefore, added after the G1/S transition. Based on the expression of histone H3, a gene expressed at the onset of S phase, the G1/S transition was estimated to occur at about 5 hours AF, i.e. after pronuclear fusion (not shown). When incubated with 100 μM olomoucine beginning either 6 or 10 hours AF (early and late S phase, respectively), zygotes were blocked with a decondensed nucleus and by 45 hours AF they had not yet entered mitosis (Fig. 6A,B). Because olomoucine is a reversible inhibitor, histone H1 kinase activity could be detected in protein extracts from cells arrested in G2 (100 μM added at 10 hours AF). This activity was also monitored in extracts from zygotes in late G2 (16
In vitro inhibition by olomoucine of histone H1 kinase showed a dose-dependent effect, indicating that G2/M CDK-like proteins are sensitive to olomoucine (Fig. 6C). In the presence of olomoucine from 6 hours AF, the same type of arrest was observed in Pelvetia zygotes (not shown). Centrosomes separated normally and microtubules were organised in bipolar centrosomal arrays (Fig. 7A,B). Spindles, however, did not form in zygotes sampled at either 24 or 48 hours after fertilisation. In contrast to aphidicolin-treated zygotes (Fig. 3I), however, the centrosomal axes were capable of aligning with the growth axes in olomoucine-treated zygotes (Fig. 7C). The average alignment angles in populations of olomoucine-treated zygotes were close to 20° at 24 hours AF and 10° at 48 hours AF and in most treated zygotes the alignment angles were smaller than 45° (in 84% of the cells at 24 hours AF and 95% at 48 hours AF), proportions that vary significantly from the 1:1 ratio that is expected if the centrosomal axes are positioned randomly (P<0.001 at 24 hours, P=0.003 at 48 hours). Therefore, activating the DNA replication checkpoint appears to lead to an inactivation of G2/M CDKs, which could account for the inhibition of spindle formation seen in both aphidicolin- and olomoucine-treated zygotes but not for the disruption of the alignment of the centrosomal axis observed in aphidicolin-treated zygotes.

DISCUSSION

A DNA replication checkpoint prevents cell division but has no effect on germination in zygototes of fucoid algae

Virtually no information is available on cell cycle checkpoints in early plant embryos. In this paper we report on the effect of inhibiting DNA replication on cell cycle progression in fucoid algae. Aphidicolin, a specific inhibitor of DNA polymerases α and δ, inhibited DNA replication in fucoid embryos at all tested stages of early development. Both the nuclear and cytoplasmic events characteristic of mitosis, i.e. chromatin condensation and spindle formation respectively, were inhibited when aphidicolin was added prior to and during S phase. When cells were treated after DNA replication, they proceeded normally through mitosis and cytokinesis and became arrested at the beginning of the next S phase. No effects were observed on early development (see below), indicating that aphidicolin specifically inhibits cell division. When applied before S phase, olomoucine, a specific inhibitor of CDKs, inhibited the entry
into S phase and, therefore, DNA replication (not shown). Under these conditions, olomoucine had effects similar to those of aphidicolin, i.e. it prevented all aspects of cell division, including spindle formation and chromatin condensation, and it triggered the inactivation of mitotic CDK-like activities by tyrosine phosphorylation (not shown). The effect on nuclear rotation, however, could not be tested since early treatments with olomoucine blocked germination (not shown). These results confirm the presence of specific mechanisms responsible for a DNA replication checkpoint in fucofid algae.

S/M checkpoints that prevent chromatin condensation, spindle formation and cytokinesis when DNA replication is blocked have been reported in animal somatic cells and in *S. pombe* (Rattner and Philipp, 1973; Byers and Goetsch, 1974). In *S. cerevisiae*, in contrast, short spindles are formed but they do not elongate, therefore preventing the completion of mitosis until DNA is replicated (Stueland et al., 1993). The difference between the two yeasts may be explained by the specificity of their cell cycles (Rhind and Russel, 1998). It should be noted, however, that despite the differences observed between S/M checkpoints in yeasts, they both ultimately prevent the separation of unreplicated DNA between daughter cells. This contrasts with some animal embryos, where the inhibition of DNA replication has little effect on cell division. In *Drosophila* embryos treated with aphidicolin, neither nuclear nor cytoplasmic events are inhibited. Chromatin continues to periodically condense and decondense, and spindles are formed but they are unable to separate chromosomes at telophase (Raff and Glover, 1988). In zygotes of the sea urchin *Sphaerechinus granularis*, inhibition of DNA replication prevents the nuclear envelope breakdown and chromatin condensation but does not fully inhibit cytoplasmic events. Spindles do not form completely but asters move away, eventually leading to cell division with aberrant partitioning of DNA (Genevière-Garrigues et al., 1995). The S/M checkpoint in zygotes of fucofid algae prevents mitosis until DNA is replicated and is therefore more similar to the S/M checkpoints in yeasts and animal somatic cells than to those described in animal embryos.

Aphidicolin had no effect on early morphogenesis. Zygotes germinated and elongated a rhizoid for several days in the absence of cell division. Furthermore, embryos that had recovered from aphidicolin treatment were indistinguishable from control embryos. Uncoupling morphogenesis and cell division has also been reported in *Xenopus* and *Drosophila* embryos treated with aphidicolin (Rollins and Andrews, 1991; Raff and Glover, 1988). These observations suggest that DNA replication is not involved in the control of early morphogenesis in either animal or in plant zygotes.

**The DNA replication checkpoint prevents centrosomal alignment and spindle formation**

During the first cell cycle of *Pelvetia* zygotes, centrosomal separation is slow and asynchronous between fertilisation and mitosis (Bisgrove and Kropf, 1998). Inhibition of DNA replication with aphidicolin had no effect on centrosomal separation during the first cycle. However, alignment of the centrosomal axis was disrupted following inhibition of DNA replication. Zygotes recovering from aphidicolin treatment displayed a normally oriented asymmetric division plane, indicating that the late completion of S phase was sufficient to align the centrosomal axis with the growth axis. The disruption of centrosomal alignment is unlikely to be accounted for by the inhibition of spindle formation since olomoucine prevents spindle formation but not alignment. The biological significance of the control of centrosomal alignment by the DNA replication checkpoint remains unclear. It may be that once zygotes pass the checkpoint they become competent to rotate their axes by a mechanism that does not involve CDKs. In this respect it would be interesting to test the effects of inhibiting DNA replication on nuclear rotation in budding yeast and *Caenorhabditis elegans* zygotes, two other cell types involving a nuclear rotation before an asymmetric cell division (White and Strome, 1996; Palmer et al., 1992).

Our observations indicate that the DNA replication checkpoint controls spindle formation but not the development of a normal premiotic microtubule array. Centrosomes separate and become the primary microtubule-organizing centers in the cell. This checkpoint, therefore, is likely to inhibit spindle formation by preventing the activation of microtubule-associated proteins (MAPs), rather than by acting directly on microtubules as previously described in Hela cells (Blangy et al., 1995).

**G2/M CDK-like proteins are involved in the DNA replication checkpoint**

We have demonstrated the presence of CDK-like proteins in *Fucus*. The mitotic peak of histone H1 kinase activity was abolished following aphidicolin treatment, suggesting that, in zygotes of fucofid algae, mitotic CDK/cyclin complexes are targets of the DNA replication checkpoint. Similarly, inhibition of DNA replication leads to the inhibition of mitotic cdc2 activity in both fission yeast and animal somatic cells (Enoch and Nurse, 1990; Jin et al., 1996) and abnormal activation of cdc2 when DNA is not replicated leads to mitotic catastrophes (Sorger and Murray, 1992). In budding yeast, the mitotic CDK, cdc28, maintains high histone H1 kinase activity following the inhibition of DNA replication (Amon et al., 1992), yet fully activated cdc28 is not sufficient to promote the entry into mitosis when DNA is not replicated (Stueland et al., 1993). In contrast, a normal peak of mitotic cdc2 activity is observed upon the inhibition of DNA replication in *S. granularis* zygotes and the cytoplasmic events of mitosis occur normally (Genevière-Garrigues et al., 1995). Our results strongly support that, as in animal somatic cells and yeasts, CDK-like proteins are a target of the DNA replication checkpoint in fucofid zygotes (Fig. 8). However, we cannot rule out the possibility that other G2/M CDK-like proteins which do not bind to p9CKShs1 are not targets of the DNA replication checkpoint in vivo.

Inhibition of the activity of CDK-like proteins by treatments with olomoucine during S phase mimicked some of the effects of the DNA replication checkpoint. When added after the G1/S transition with the aim of preventing the activation of the DNA replication checkpoint, olomoucine had similar effects as aphidicolin, i.e. it prevented chromatin condensation and spindle formation. In Hela cells and *Xenopus* eggs the kinase activity of cdc2 is required for the nucleation of microtubules at centrosomes, which is required for spindle formation (Blangy et al., 1995; Verde et al., 1992). Therefore it is likely, as proposed in Fig. 8, that in fucofid zygotes the inactivation of G2/M CDK(s), either directly with olomoucine or following
activation of the DNA replication checkpoint, prevented spindle formation. Like aphidicolin, olomoucine did not prevent centrosome separation when added during S phase of the first cell cycle but, in contrast to aphidicolin, olomoucine did not prevent centrosomal alignment. Therefore, CDKs are probably not regulators of centrosomal alignment.

The role of protein phosphorylation in the DNA replication checkpoint

In fission yeast and animal cells, at least two mechanisms have been proposed to account for the inactivation of mitotic CDKs following activation of the DNA replication checkpoint. (1) In S. pombe, the mitotic cdc2 remains phosphorylated on tyrosine and inactive (Rhind and Russel, 1998). This phosphorylation results from the inactivation of the tyrosine phosphatase cdc25 (Enoch and Nurse, 1990) and activation of the tyrosine kinase wee1 (Boddy et al., 1998). (2) In Xenopus egg extracts and human cells, tyrosine phosphorylation is not the only mechanism required to inactivate cdc2 since non phosphorylatable cdc2 cannot override an arrest resulting from the inhibition of DNA replication (Kumagai and Dunphy, 1995; Jin et al., 1996).

In fucoid zygotes, several lines of evidence suggest that the DNA replication checkpoint directly prevents the activation of G2/M CDK-like proteins rather than inhibiting the synthesis of active CDK/cyclin complexes. (i) The level of both PSTAIRE CDK-like proteins continued to increase following activation of the DNA replication checkpoint but histone H1 kinase activity did not. (ii) PSTAIRE CDK-like proteins, in the usual range of molecular masses for CDKs (from 30 to 40 kDa), and more particularly p34, were the main proteins phosphorylated on tyrosine following aphidicolin treatment. (iii) In vitro dephosphorylation of CDK-like proteins by cdc25A phosphatase restored the kinase activity, suggesting that functional CDK/cyclin complexes are present but maintained inactive by tyrosine phosphorylation. In tobacco pith parenchyma cells, such a regulation of mitotic CDK-like proteins occurs following specific hormonal treatments (Zhang et al., 1996) but it remains to be demonstrated that this mechanism is involved during normal cell cycle progression. The presence of tyrosine kinases that inactivate G2/M CDK-like proteins is also indirectly suggested by our experiments since the level of phosphorylation of CDK-like proteins continued to increase until at least 36 hours AF following treatments with aphidicolin. Altogether our results strongly support the idea that, in fucoid zygotes, tyrosine phosphorylation is involved in the regulation of CDK-like proteins (pathway 1 in Fig. 8). In addition, another regulatory mechanism may not involve tyrosine phosphorylation (pathway 2 in Fig. 8). However they do not yet allow us to discriminate between mechanisms (1) and (2) (Fig. 8). Finally, we cannot rule out the possibility that other CDKs, that do not bind to p9CKShs1, are regulated by different mechanisms and/or are not inhibited following activation of the DNA replication checkpoint.

In conclusion the DNA replication checkpoint in fucoid zygotes and embryos, which prevents mitosis until DNA is replicated, resembles the checkpoints described in yeast and animal somatic cells and depart from those described in animal embryos. Therefore, the relative lack of control on genome integrity reported in early cell cycles of several animal embryos does not appear to be a general feature of early embryonic cells. Like in S. pombe and animal somatic cells, the S/M checkpoint of fucoid zygotes induces a down-regulation of CDK-like activity by tyrosine phosphorylation and it would be interesting to determine if such a mechanism also operates in other plants. Finally the S/M checkpoint in embryos of fucoid algae may be involved in regulating other aspects of asymmetric cell division, such as the control of centrosomal alignment but it does not control germination or rhizoid elongation.

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