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Cell cycle-dependent control of polarised development by a cyclin-dependent kinase-like protein in the *Fucus* zygote

Florence Corellou[1], Colin Brownlee[2], Bernard Kloareg[1] and François-Yves Bouget[1,*]

[1]Station Biologique, UMR 1931 CNRS and Laboratoires Goëmar, 29680 Roscoff, France
[2]Marine Biological Association, The Laboratory, Citadel Hill, Plymouth PL1 2PB, UK
*Author for correspondence (e-mail: bouget@sb-roscoff.fr)

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SUMMARY

Although iterative development can be uncoupled from morphogenesis in plant organs, the relationship between the cell cycle and developmental events is not well established in embryos. Zygotes of fucoid algae, including *Fucus* and *Pelvetia* are particularly well suited for studying the interaction(s) between cell cycle progression and the early morphogenetic events, as the establishment of polarity and its morphogenetic expression, i.e. germination, and the first cell cycle are concomitant. We have previously demonstrated that, in *Fucus* zygotes, various aspects of cell cycle progression are tightly controlled by cyclin-dependent kinase (CDK)-like proteins, including two PSTAIRE CDK-like proteins, p34 and p32, which are synthesised after fertilisation. We show that specific inhibition of CDK-like proteins, either with purine derivatives such as olomoucine and amino-purvalanol or by microinjection of the CDK inhibitor p21^{cip1}, prevents germination and cell division. Whereas direct inhibition of DNA replication by aphidicolin did not affect polarised development, olomoucine, which has previously been shown to prevent entry in S phase, and other purine derivatives also inhibited photopolarisation. Early microinjection of a monoclonal anti-PSTAIRE antibody also prevented germination and cell division. Only p34 had affinity for amino-purvalanol, suggesting that among PSTAIRE CDKs, this protein is the main target of purine derivatives. Models to account for the simultaneous control of early cell cycle progression and polarisation are proposed.

Key words: *Fucus* zygote, Polarisation, Morphogenesis, Cell cycle, CDK

INTRODUCTION

During development, cell division cycle, differentiation and morphogenesis must be finely balanced. A moderate slowdown of the cell cycle rate in developing organs that would uncouple cell division from morphogenesis may, thus, give rise to organisms with larger cells but normal morphogenesis. However, inhibiting the basic cell cycle machinery in unicellular organisms or in zygotes and early embryos can potentially lead to developmental defects by uncoupling increase in cell mass from increase in cell numbers. Whereas several examples have illustrated the tight coupling of cell cycle and morphogenesis in unicellular yeast, little is known about the interactions between cell cycle and morphogenesis in single-celled zygotes.

Several examples in animals and plants have shown that uncoupling cell cycle and morphogenesis has no dramatic effect on development. *Drosophila* wings that overexpress the cell cycle activator *dE2F* display more cells that are smaller compared with wild-type animals, but the size and morphology of territories in mutants are normal (Neufeld et al., 1998). Similarly, overexpressing the retinoblastoma homolog *Rbf* in *Drosophila* wings causes an increase in cell size but has no effect on development (Neufeld et al., 1998). Furthermore, the constitutive expression of dominant negative alleles of the main cell cycle controller, *cdc2*, in *Drosophila* wings gives rise to organs with fewer cells but which are of normal shape (Weigmann et al., 1997). However, in this *Drosophila* mutant, arresting the cell cycle of early stages of wing development prevents growth and morphogenesis (Weigmann et al., 1997).

Several examples in plants also suggest that there is only a weak interaction between cell cycle and morphogenetic events in developing organs. In *Arabidopsis*, overexpression of the gene encoding cyclin B results in an increased number of cells and longer roots, but no major effects on morphogenesis (Doerner et al., 1996). In tobacco, the constitutive expression of dominant negative alleles of *Arabidopsis cdc2a*, *cdc2aN147*, gives rise to organs with fewer cells, but these plants display normal morphogenesis, histogenesis and developmental timing (Hemelerly et al., 1995).

Altering cell cycle progression appears to have more dramatic effects on morphogenesis in unicellular organisms and early embryos than in developing organs with predefined patterns. In single-celled budding yeast, the cyclin-dependent kinase (CDK) cd28 in association with its regulatory cyclins controls cell cycle progression at the G1/S transition as well as morphogenesis (Lew and Reed, 1993; Cvrckova and Nasmyth, 1993). Inhibition of the cyclin/cdc28 complex prevents both

the localisation of F-actin at the budding site and polarised secretions, which are required for budding (Lew and Reed, 1993). Furthermore, the activation of cdc28 by G1 cyclins is sufficient to trigger the localisation of F-actin at the budding site in the absence of protein synthesis (Lew and Reed, 1993). It has recently been shown that, in G1, the guanine-nucleotide exchange factor cdc24 for the GTPase cdc42 is sequestred in the nucleus by the CDK inhibitor Far1. At budding, cdc28-Cln, by triggering the degradation of Far1, allows cdc24 to move to the cytoplasm at the site of heterotrimeric G-protein activation where it binds to cdc42, allowing the localisation of F-actin (Shimada et al., 2000). A reciprocal control of cell cycle by morphogenesis has been characterised, and this morphogenesis checkpoint monitors the actin cytoskeleton in budding yeast (McMillan et al., 1998). Interestingly, in the multicellular ascomycete *Aspergillus nidulans*, defects in basic cell cycle machinery deeply affect morphogenesis by inhibiting the switch from filamentous to budding growth during conidiophore development as well as spore polarisation (Ye et al., 1999; Harris, 1999), suggesting that cell cycle-dependent control of morphogenesis may be a more specific feature of fungi than of animals and land plants.

The possibility that the cell cycle may exert a control on morphogenesis during early embryogenesis is suggested by three lines of evidence: (1) organ size and shape is severely affected in many tobacco seedlings expressing *cdc2aN147* and the mutants produce only a few viable seeds; (2) in *Arabidopsis* plants expressing *cdc2aN147* under the control of the constitutive CaMV 35S promoter, embryo development appears to be particularly affected (Hemerly et al., 1995); and (3) when *cdc2aN147* is expressed under the control of the seed storage albumin promoter which drives specific expression during late embryo development, either germination is abolished or cotyledons and root development are absent or completely abnormal (Hemerly et al., 2000). However, little is known on the interactions between cell cycle and morphogenesis during early embryogenesis, because in land plants, most cell cycle mutants are not viable and zygotes are not amenable to direct manipulation.

Brown algae of the genus *Fucus* and *Pelvetia* are recognised as model systems with which to study embryogenesis in multicellular plants (Brownlee and Bouget, 1998). In these genera, polarity is established after fertilisation (AF) and the polar axis can be reoriented in response to external factors such as light (photopolarisation), before being irreversibly fixed (axis fixation). Microfilaments of F-actin and polarised secretions play a major role in the establishment of polarity (Hable and Kropf, 2000; Shaw and Quatrano, 1996). Much attention has been devoted to the transduction pathways that lead from signal perception and transduction to the anchorage of polarity (Kropf et al., 1999). However, the influence on early development of intracellular factors and, in particular, of cell cycle progression has never been investigated. Fucoid zygotes are well suited to study the relationships between cell cycle and morphogenesis, as polarisation and germination occur during the first cell cycle. We have recently characterised the first cell cycle in *Fucus* zygotes and showed that it resembles a somatic cell cycle, i.e. cell cycle progression is tightly regulated by CDK-like proteins, which are themselves regulated at the level of synthesis and by tyrosine phosphorylation (Corellou et al., 2001). In particular, two CDK-like proteins containing the hallmark sequence PSTAIRE, p32 and p34, are barely detectable in the egg, and their synthesis is triggered by fertilisation in the absence of transcription. CDKs are required for various events of cell cycle progression and the purine derivative olomoucine, a specific inhibitor of CDK, prevents S-phase entry, as illustrated by the inhibition of both DNA replication and transcription of *histone H3* in early S phase (Corellou et al., 2001). An S/M checkpoint prevents all aspects of cell division until DNA is replicated and inhibits centrosomal axis alignment (Corellou et al., 2000b). This checkpoint, however, has no effect on polarisation, germination and rhizoid elongation.

We now report on the link between cell cycle control and the establishment of polarity in *Fucus* zygotes. We show that both the photopolarisation period and the G1/S transition are concomitant. Inhibiting entry in S phase with purine derivatives, such as olomoucine, which specifically inhibits CDKs, or microinjection of the CDK inhibitor $p21^{cip1}$ (Harper et al., 1995) prevented the early expression of morphogenesis, i.e. germination. Olomoucine and amino-purvalanol, another purine derivative, inhibited photopolarisation and germination only when applied before or at the time of sensitivity to light (or S phase entry), suggesting that the inhibition of germination is due to the inhibition of polarisation. A monoclonal anti-PSTAIRE antibody prevented cell division and germination but only if microinjected very soon after fertilisation. Finally, amino-purvalanol (Gray et al., 1998) had affinity only for the PSTAIRE CDK-like protein referred to as p34 suggesting that, among PSTAIRE CDKs, p34 is the main target of purine derivatives.

MATERIALS AND METHODS

Culture and inhibitors

Receptacles of *Fucus spiralis* were collected at Le Dossen (Brittany, France) and stored at 4°C for up to 14 days. Gametes were released by standard osmotic shock procedures in filtered sea-water (FSW) over a 1 hour period (Quatrano, 1980). The time of fertilisation (0 hour) was taken 30 minutes after the first eggs were released. Zygotes and embryos were grown at 14°C in petri dishes. Inhibition of DNA replication and subsequent inhibition of cell division was performed using aphidicolin (20 μM, Sigma). The beginning of G2 phase was determined as the time for which the first cell division was not affected by the addition of aphidicolin (Corellou et al., 2000b). The purine derivatives olomoucine, roscovitine and amino-purvalanol (Gray et al., 1998) were used to inhibit CDK-like proteins in vivo and in vitro. Aphidicolin, olomoucine, roscovitine, amino-purvalanol and methyl-amino-purvalanol were dissolved in DMSO at 10 mg/ml, 30 mg/ml, 15 mg/ml and 0.4 mg/ml, respectively, and further diluted in FSW before use. Control experiments were performed in FSW containing the same concentrations of DMSO. The structural analogs isoolomoucine and methyl-amino-purvalanol had no effect on cell division at the respective concentrations of 400 μM and 30 μM. Photopolarisation and axis fixation were assayed using light vectors, as previously described (Corellou et al., 2000a).

Staining of DNA and cell wall sulphated compounds

Zygotes and embryos were fixed for 12 hours in 0.2 M citric acid, 0.2% Triton X-100 and kept in 100% methanol for long term storage. Fixed cells were attached to poly-L-lysine-coated coverglasses, and DNA was stained with 50 μg/ml of mithramycin A, as described previously (Corellou et al., 2000b).

For the staining of sulphated cell wall compounds, zygotes were frozen in liquid nitrogen and stored at –80°C. Cell walls were isolated by several cycles of sonication in bi-distilled water and attached to coverslips coated with poly-L-lysine (Sigma). Sulphated compounds were specifically stained with a solution of 0.05% Toluidine Blue O solution-HCl, pH 0.5 (TBO) and washed in 0.1 M HCl.

Protein extraction, purification, histone H1 kinase activity and immunodetection of CDK-like proteins

Protocols for protein extraction and western blot analysis have previously been described in detail (Corellou et al., 2000b). Briefly, embryos were harvested, centrifuged (3000 ***g***) to remove excess FSW, frozen in liquid nitrogen and stored at – 80°C until extraction. Frozen samples were ground in liquid nitrogen and proteins were extracted as described previously (Corellou et al., 2000b). Protein extracts were incubated with 10 μl of $p9^{CKShs1}$ Sepharose beads (containing 3.9 mg/ml of proteins), on a rotary shaker at 4°C for 1 hour. Beads were spun at 3000 ***g*** for 1 minute and washed three times in bead buffer. Western blot analysis was performed as following. Forty microliters of 4× Laemmli buffer were added to $p9^{CKShs1}$ beads and proteins were eluted by heating at 90°C for 10 minutes. For affinity purification on amino-purvalanol, proteins were processed the same way as with $p9^{CKShs1}$ beads, using 10 μl of amino-purvalanol Sepharose beads (purvalanol covalently linked to Sepharose beads at a concentration of 20 μM in packed beads; a kind gift from Nathanael Gray).

Proteins eluted from $p9^{CKShs1}$ or amino-purvalanol beads were resolved on a 10 or 12% SDS-polyacrylamide denaturing gel and electro-transferred onto a nitrocellulose membrane (Amersham Life Science, Buckingamshire, UK) for ECL detection (Amersham Life Science). The membranes were stained with Ponceau Red to check the homogeneity of the transfer, blocked in Tris-buffered saline (TBS) containing Blotto B, as described by the manufacturer (Santa Cruz Biotechnology, California) and then incubated with a monoclonal anti-PSTAIRE antibody (Sigma) at a 1/3,000 dilution. The membranes were washed three times in TBS containing 0.1% Tween 20 and the bound antibody was detected with a goat anti-mouse IgG coupled to horseradish peroxidase (BioRad Laboratories Hercules, CA) and then visualised by enhanced chemiluminescence (ECL, Amersham).

The activity of CDK-like proteins was taken as the histone H1 kinase activity of proteins bound to the human suc1 homolog $p9^{CKShs1}$ sepharose beads. This activity was measured at 30°C for 30 minutes using [γ^{32}P] ATP, as previously reported (Corellou et al., 2000b). Quantification of radioactive histone H1 was performed using a phosphorimager STORM with the Image QuanT software (Molecular Dynamics). When investigating the effect of amino-purvalanol or $p21^{cip1}$ on histone H1 kinase, these inhibitors were preincubated with proteins bound to $p9^{CKShs1}$ for 5 minutes before starting the kinase assay.

Microinjection of the anti-PSTAIRE antibody and the CDK inhibitor $p21^{cip1}$

The CDK inhibitor *p21^{cip1}*, cloned in pGEX as a fusion with GST (a kind gift from André Picard), was overproduced in *E. coli* and purified by affinity on glutathione-agarose beads, using the GST Gene Fusion System, as described by the manufacturer (Pharmacia Biotech). GST-$p21^{cip1}$ (final concentration of 360 μM) was concentrated in buffer A, which consisted of 5 mM $MgCl_2$, 10 mM Hepes, pH 7.5, using the centricon YM-10, 10,000 M_r cut off device (Millipore). A control buffer lacking GST-$p21^{cip1}$ was processed identically. A monoclonal anti-PSTAIRE antibody (Sigma) was brought to a final concentration of 2.5 mg/ml of pure IgG1 (16 mg/ml of total proteins) in buffer A, using the centricon YM30-, 30,000 M_r cut off device (Millipore). Ascite Fluid (Sigma), processed identically (final concentration of 16 mg/ml in buffer A), was used as a negative control in microinjection experiments. Finally, GST-$p21^{cip1}$, the anti-PSTAIRE antibody and ascite fluid were brought to concentrations of 180 μM, 0.7 mg/ml (4.4 mg/ml of total proteins) and 4.4 mg/ml respectively, in injection buffer consisting of 2.5% w/v Oregon Green 488 Dextran 70 kDa (Molecular Probes), 200 mM KCl, 550 mM mannitol and 10 mM, Hepes, pH 7.5.

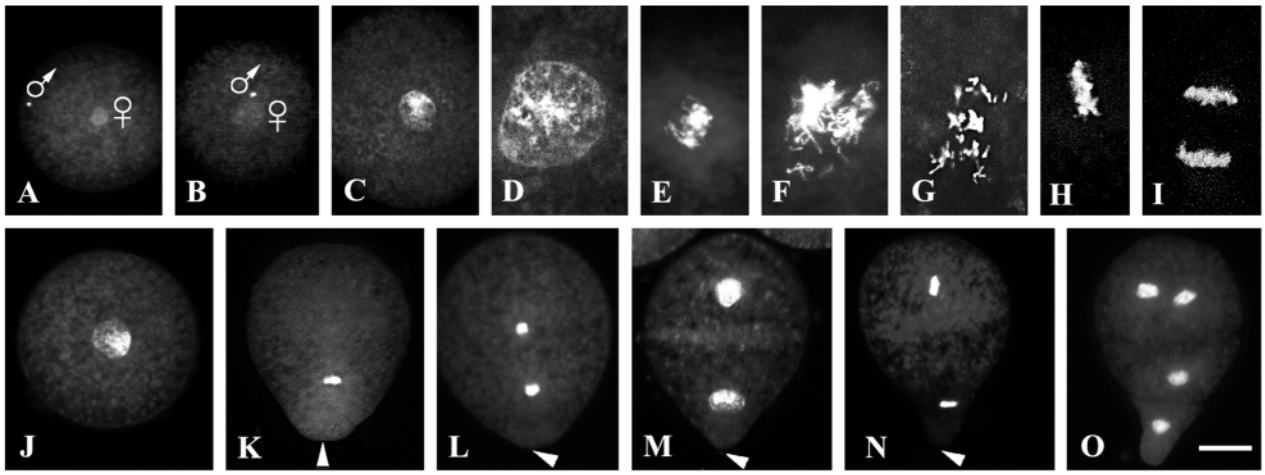
Zygotes were placed in a wedge (Speksnijder et al., 1989) in FSW containing either 0.2 M mannitol (for 1-hour-old zygotes) or 0.6 M mannitol (for 8-hour-old zygotes). Zygotes were loaded with injection buffer, containing either $p21^{cip1}$, anti-PSTAIRE antibody or the control buffers mentioned above, by pressure microinjection (picoinjector PLI-100, Medical System Corp), as previously described (Berger and Brownlee, 1993). The fluorescence of Oregon Green 488 dextran 70 kDa was used to check the loading of the cells. From the average fluorescence, the injection volume was estimated to be approx. 2 to 5% of the cell volume. Oregon Green dextran became localised to the nuclear area and was used to visualise the nuclei of injected cells, using a confocal microscope (Biorad model 1024). Both germination and cell division were monitored after microinjection.

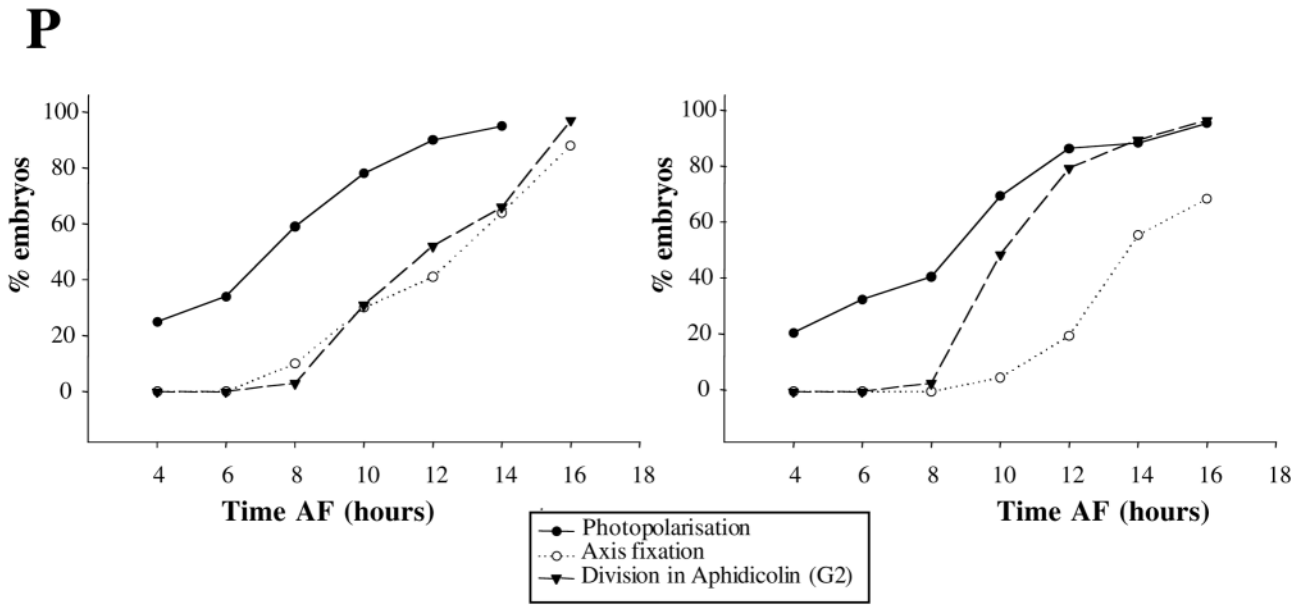
RESULTS

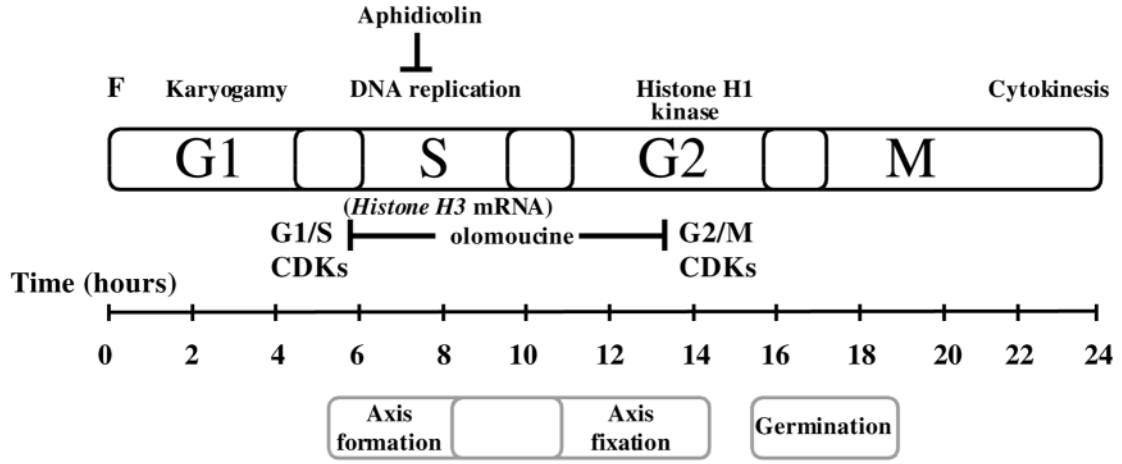
Timing of cell cycle and developmental events in *Fucus* zygotes

Using a new DNA staining protocol (Corellou et al., 2000b), we have investigated the time course of the first cell division relative to early development in *F. spiralis* zygotes (Fig. 1). Soon after fertilisation, the female pronucleus is central and decondensed, whereas the male pronucleus is highly condensed and localised at the periphery of the cell (Fig. 1A). Pronuclei come into contact (Fig. 1B) and fuse at 3-4 hours AF (Fig. 1C). At 14 hours AF, the nucleus is still decondensed (Fig. 1D), and the zygote remains spherical (Fig. 1J). Two to 4 hours later, the nuclear envelope breaks down (Fig. 1E), the nuclear DNA condenses (Fig. 1F) and the chromosomes become clearly visible (Fig. 1G). Soon after, the chromosomes can be seen in metaphase, anaphase or telophase figures (Fig. 1H,I,L). Mitosis occurs at the time of rhizoid outgrowth (Fig. 1K,L,Q) and cytokinesis at 22-24 hours AF (Fig. 1M,Q). Subsequent division of the rhizoid cell involves a rotation of the nucleus, whereas the division of the thallus cell does not (Kropf et al., 1999), and the orientation of metaphase plates predicts the orientation of the division planes (Fig. 1N,O).

We next investigated the timing of cell cycle events with respect to the establishment of polarity. S phase occurs at 5-6 hours AF, as determined by monitoring the transcription of histone H3 (Corellou et al., 2001) (Fig. 1Q). To assay the beginning of G2 phase, we took advantage of the presence of a DNA replication checkpoint, which prevents mitosis and cytokinesis until DNA is replicated (Corellou et al., 2000b). Aphidicolin at 20 μM, inhibits DNA replication, leading to cell cycle arrest during S phase progression (Corellou et al., 2000b). At this concentration, no cell escaped the treatment, (i.e. divided), when treated before 8-9 hours AF (Fig. 1P). When treated at 8-9 hours AF, a few percent of the cells were able to divide, suggesting that they were already in G2 or at least at the end of S phase if the effect of aphidicolin was not immediate. In any case, aphidicolin must be effective within 2 hours, as S phase, which starts at 5-6 hours AF, is likely to last for at least 1 hour (Corellou et al., 2001). The proportion of cells in late S-phase or early G2 phase, at a given time from 0 to 24 hours, was determined as the percentage of zygotes







insensitive to aphidicolin (20 μM) and which underwent cell division when the drug was added at that time. Photopolarisation was then assayed as the percentage of zygotes able to respond to a unidirectional light vector and to germinate accordingly when placed in the dark at that time. (Corellou et al., 2000a). Axis fixation was similarly assayed as

Fig. 1. (A-O) Time course of the first cell cycle and early development in *Fucus spiralis* zygotes. Zygotes were stained with mithramycin A at various times after fertilisation (AF): 1 hour (A); 3 hours, just before pronuclei fusion (B); 4 hours, after fusion (C); 14 hours, decondensed nuclei before germination (D,J); 16-17 hours, nuclear envelope breakdown and chromatin condensation (E,F); 18-20 hours, prophase (G), metaphase in a germinated cell (H,K), anaphase (I), telophase (L); 24 hours, cytokinesis (M). At 26 hours AF, embryo exhibiting two cells in metaphase (N). Note that the metaphase plate is transverse to the polar axis. (O) At 30 hours AF, subsequent parallel and transverse divisions in the rhizoid and thallus cells. Arrowheads in L-N indicate the germination site. Scale bar: 30 μm in A,B,N,O; 25 μm in K,M; 20 μm in C,E,J,L; 10 μm in H,I; 5 μm in D,F,G. (P) Temporal relationships between polarisation and G2 phase. Two representative experiments are shown (100 cells for each time point). The proportion of cells in late S or G2 phase (as determined by their ability to divide in the presence of aphidicolin), as well as the percentage of photopolarised cells (germination in response to a light vector) or those with a fixed axis (irreversible anchoring of polarity), are reported for each time point of development. (Q) Temporal relationships between cell cycle events and stages in zygote polarisation. The beginning of S phase occurs at 5-6 hours AF, as determined by measuring the expression of *histone H3*, a gene expressed at the onset of S phase. A peak of H1 kinase activity, which is representative of CDK activity, is detected at the time of mitosis (Corellou et al., 2000b). The purine derivative olomoucine inhibits cell cycle progression at the G1/S transition (preventing DNA replication and expression of *histone H3*) and at the G2/M transition, and through mitosis, by specifically inhibiting CDK-like proteins. Aphidicolin, by contrast, inhibits DNA replication but not the expression of *histone H3*. Whereas photopolarisation occurs before G2 phase, axis fixation begins in late S phase and ends in G2 phase in the majority of zygotes (as deduced from an analysis of 10 independent populations).

the percentage of zygotes able to respond to a first light vector and to germinate accordingly when exposed to a second opposed light vector from that time (Corellou et al., 2000a). Though variability was observed between developing populations (Fig. 1P) the irreversible anchoring of polarity (axis fixation) started in late S phase and ended in G2 phase, whereas photopolarisation occurred before G2 phase. The timing of G2 phase and polarisation events, as deduced from an analysis of ten independent populations, is represented in Fig. 1Q. Our current knowledge of both the cell cycle and polarisation events is summarised in Fig. 1Q (Corellou et al., 2000b; Corellou et al., 2001).

CDK inhibitors and microinjection of the CDK inhibitor p21^{cip1} inhibit germination and cell division in *Fucus* zygotes

We have tested the effect of olomoucine and of two other purine derivatives, roscovitine and amino-purvalanol, a cell-permeable derivative of purvalanol (Gray et al., 1998), on photopolarisation and germination. These molecules are known to be highly specific for CDKs and more particularly for PSTAIRE CDKs (Meijer, 1995; Gray et al., 1998). When applied at 2 hours AF, 100 μM olomoucine induced a G1/S arrest (Corellou et al., 2001) and mitosis was fully inhibited until at least 36 hours AF (Fig. 2A). The behavior of nuclei in zygotes treated from 2 to 36 hours AF with 30 μM roscovitine or with 5 μM amino-purvalanol was similar to those of nuclei from zygotes treated with 100 μM olomoucine (Fig. 2B,C). The inactive stuctural analogues isoolomoucine and methyl-amino-purvalanol, at the concentrations of 400 μM and 25 μM, respectively, had no effect on cell division (Fig. 2D; Table 1). Remarkably, olomoucine, roscovitine and amino-purvalanol all inhibited rhizoid germination when applied from 2 hours AF (Fig. 2E-G), while isolomoucine and methyl-amino-purvalanol did not (Fig. 2D; Table 1). The uptake of these inactive analogues by the cells has not been quantified. However, it is likely that both olomoucine and its isomer, iso-olomoucine are taken-up to similar extents, as their permeability is related to their hydrophobicity. Arresting the cells in S phase with 20 μM aphidicolin effectively prevented cell division, whereas germination and rhizoid growth were unaffected (Fig. 2I) (Corellou et al., 2000b). Similarly treatments with lower doses of olomoucine (35 μM), which arrested zygotes in mitosis with dispersed chromosomes (Corellou et al., 2001), had no effect on germination (Fig. 2J). In these conditions, three drugs each prevented the localisation, at the rhizoid pole, of sulphated compounds (Fig. 2E-G), a marker of polarity in control zygotes (Fig. 2H) (Shaw and Quatrano, 1996). Zygotes recovering from a treatment with 100 μM olomoucine from 2 to 30 hours AF divided but often lacked a rhizoid (not shown). After 2 weeks in culture, up to 50% of such embryos were formed of thallus tissue only, marked by the presence of thallus-specific apical hairs (Fig. 2K). By contrast, over 80% of zygotes treated with 100 μM olomoucine from 2 to 20 hours AF recovered, divided and displayed polarised growth (data not shown).

We also used the human CDK inhibitor p21^{cip1}, a specific inhibitor of G1/S CDKs in animal cells (Harper et al., 1995), to further test the involvement of CDK-like proteins in the

Table 1. Effect of cell cycle inhibitors on photopolarisation and germination

Treatment	Specificity	Photopolarisation	Germination	Division
Olomoucine (100 μM) from 2 hours AF	G1/S arrest (CDK)	–	–	–
Isolomoucine (400 μM) from 2 hours AF	No (control)	+	+	+
Roscovitine (30 μM) from 2 hours AF	G1/S arrest? (CDK)	–	–	–
Amino-purvalanol (10 μM) from 2 hours AF	G1/S arrest? (CDK)	–	–	–
Methyl-amino-purvalanol (20 μM) from 2 hours AF	No (control)	+	+	+
Aphidicolin (20 μM) from 2 hours AF	S phase arrest (DNA polymerase)	+	+	–
Olomoucine (100 μM) from 8 hours AF	G2/M arrest (CDK)	+	+	–
Nocodazole (0.33 μM) from 2 hours AF	M arrest (microtobules)	+	+	–
Olomoucine (35 μM) from 2 hours AF	M arrest (CDK)	+	+	–

Treatments with cell cycle inhibitors were investigated on photopolarisation and germination, which were monitored, respectively, after a 20 hour and a 36 hour period of contact with the inhibitor, in order to account for a possible delay in development.

+ and – mean that over 90% and less than 10% of the cells, respectively, were not affected by the treatment.

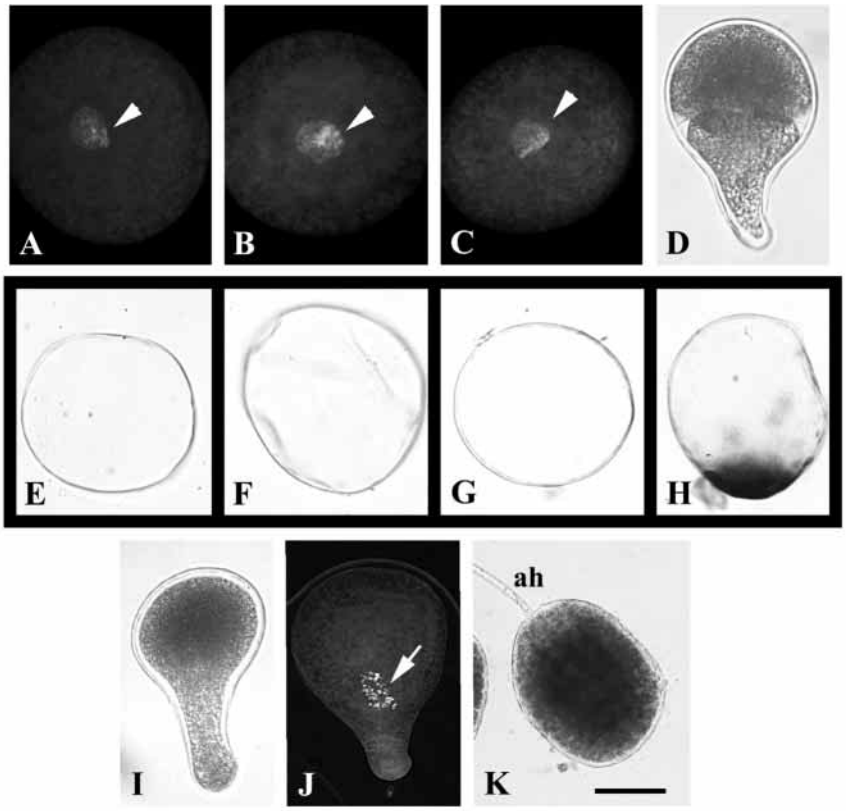


Fig. 2. Effects of CDK inhibitors on early morphogenesis of *Fucus* zygotes. Treatments from 2 hours AF with either 100 μM olomoucine (A,E), 30 μM roscovitine (B,F) or 5 μM amino-purvalanol (C,G) prevent nuclear division, as revealed by staining DNA with mithramycin A (A-C), as well as the polar localisation of cell wall sulphated compounds, as detected by staining isolated walls with TBO (E-G). Arrowheads in A-C point to the decondensed nuclei. (D) Control embryo treated from 2 to 24 hours AF with 400 μM isoolomoucine. (H) Cell wall of 12-hour-old control zygotes exhibiting sulphated compounds localised at the future rhizoid pole. (I,J) Zygotes treated from 2 hours AF with either 20 μM aphidicolin or with lower doses of olomoucine (35 μM) germinated and elongated a rhizoid as observed at 36 hours AF. Note the presence of dispersed chromosomes after treatment with 35 μM olomoucine (arrow in J). (K) Two-week-old embryo recovering from a 28 hour-incubation with 100 μM olomoucine from 2 hours AF. Note that the multicellular embryo is is devoid of any rhizoids. By contrast, an apical hair (ah) is present. Scale bar: 25 μm in A-C; 30 μm in E-H; 40 μm in D,J; 50 μm in I; 80 μm in K.

control of early developmental processes. The effect of GST-p21^{cip1} was first monitored on histone H1 kinase activity bound to p9^{CKShs1} in extracts from cells arrested either at the G1/S transition by olomoucine or in mitosis by nocodazole. Though a full inhibition was never observed, at a concentration of 30 μM GST-p21^{cip1} inhibited 50% and 60% of the activity of G1/S and G2/M CDKs, respectively (data not shown). When microinjected with GST-p21^{cip1} at 1 hour AF, 31 out of 36 zygotes did not germinate and displayed a decondensed nucleus 48 hours later, while the five remaining zygotes germinated and exhibited several nuclei 36 hours later, as in the control injected cells (data not shown). From a Chi square analysis (P<0.0001), the proportion of germinated zygotes after injections with GST-p21^{cip1} (5/36) was significantly different from the proportion of germinated embryos in controls (17/21).

CDK inhibitors prevent germination only when applied before or at the time of photopolarisation

Photopolarisation was efficiently inhibited by olomoucine (100 μM), roscovitine (30 μM) or amino-purvalanol (5 μM) treatments starting at 2 hours AF (Table 1). Olomoucine (100 μM) or amino-purvalanol (5 μM) were then added at various times AF and the germination was scored 36 hours later (Fig. 3). Similarly, aphidicolin was added at various times, to determine the beginning of G2 phase. Photopolarisation and axis fixation were scored in parallel in control populations. Both the photopolarisation period and the period of insensitivity to olomoucine, i.e. the period after which the addition of the drug had no effect on germination, as scored 36 hours later, were concomitant in populations of polarising zygotes (Fig. 3). Although the period of insensitivity to

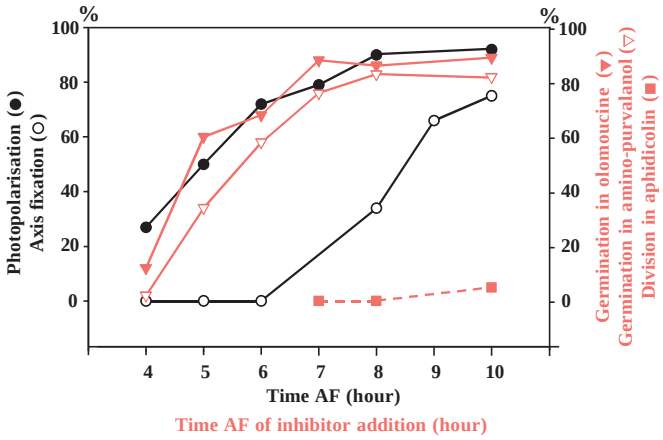


Fig. 3. Time-dependent effect of olomoucine and amino-purvalanol on germination. Olomoucine (100 μM) or amino-purvalanol (5 μM) were added at various times AF (as indicated on the *x*-axis) and the proportion of germinated zygotes was scored 36 hours later (red and white triangles, repectively). To determine the percentage of cells in G2 phase at the time of drug addition (red squares), aphidicolin (20 μM) was added at various times AF (*x*-axis) and the proportion of divided cells was scored 36 hours later. Photopolarisation (black circles) and axis fixation (white circles) are reported for each time point. This graph is representative of three independent experiments.

amino-purvalanol appears to start soon after photopolarisation, this is likely to be due to our experimental protocol, which may lead to an overestimation of photopolarised cells (in this experiment, 25% of photopolarisation corresponds to a random orientation in response to light). For the same reason, zygotes are likely to

become insensitive to olomoucine before they become photopolarisable rather than at the time of photopolarisation as suggested in Fig. 3. More interestingly, neither drug affected germination when added at the time of axis fixation (60% of fixation at 9 hours AF) or germination (16 hours AF, not shown). It is, therefore, likely that the inhibition of germination by olomoucine or amino-purvalanol arose from the prevention of photopolarisation rather than from the inhibition of axis fixation or germination.

Early microinjection of an anti-PSTAIRE antibody inhibits cell division and germination

The hallmark PSTAIRE peptide sequence is involved in the interaction between cyclins and CDKs (Jeffrey et al., 1995). Because both the expression of PSTAIRE CDK-like proteins and the associated histone H1 kinase activities are barely detectable in unfertilised *Fucus* eggs and as their levels markedly increase after fertilisation (Corellou et al., 2001), we have attempted to inhbit the formation of active CDK/cyclin complexes using an anti-PSTAIRE antibody. This antibody was injected at either 1 hour AF or 8 hours AF, i.e. after the G1/S transition. Early injection of the anti-PSTAIRE antibody fully inhibited germination and cell division (Fig. 4A) in 18 out of 20 microinjected cells (Fig. 4F). By contrast, zygotes that were injected at 8 hours AF, germinated and were multicellular 48 hours later (Fig. 4B), like the control zygotes injected with the same concentration of ascite fluid (Fig. 4C,D) or with injection buffer only (Fig. 4E). The proportion of germinated zygotes, upon injection at 1 hour AF (2/20) (Fig. 4F), was statistically different from the proportion of germinated embryos in controls (20/25) (Fig. 4F) by a Chi square analysis ($P<0.001$). By contrast, the proportion of germinated zygotes, following injection at 8 hours AF (19/25), was not statistically different ($P>0.005$) from the proportion of germinated embryos in controls (20/25) (Fig. 4F).

Purvalanol binds only the PSTAIRE CDK-like protein p34

As early treatments with amino-purvalanol prevent cell cycle progression as well as polarisation and morphogenesis (Figs 2, 3) (Table 1), effects of this drug on CDK-like activity were monitored at different stages of cell cycle progression. To this end, histone H1 kinase activity was analysed in extracts from cells previously arrested either at the G1/S transition by olomoucine or in mitosis by nocodazole, in the presence of various concentrations of amino-purvalanol (Fig. 5A). Although G2/M CDK-like proteins were apparently more sensitive to high concentrations of aminopurvalanol than G1/S CDK-like-proteins, at the concentration of 5 μM this drug inhibited approx. 50% of control histone H1 kinase activities at both stages. By contrast, the inactive analogue methyl-amino-purvalanol, at the concentration of 30 μM, had much less or no effect on histone H1 kinase activities.

Amino-purvalanol covalently fixed on Sepharose beads was used to purify CDK-like proteins in *Fucus* zygotes. Proteins extracted from 48-hour-old embryos, i.e. displaying cells at various stages of the cell cycle, were incubated with amino-purvalanol or $p9^{CKShs1}$ Sepharose beads (Fig. 5B). The proteins that were not bound to amino-purvalanol were further purified on $p9^{CKShs1}$ beads. Whereas $p9^{CKShs1}$ bound

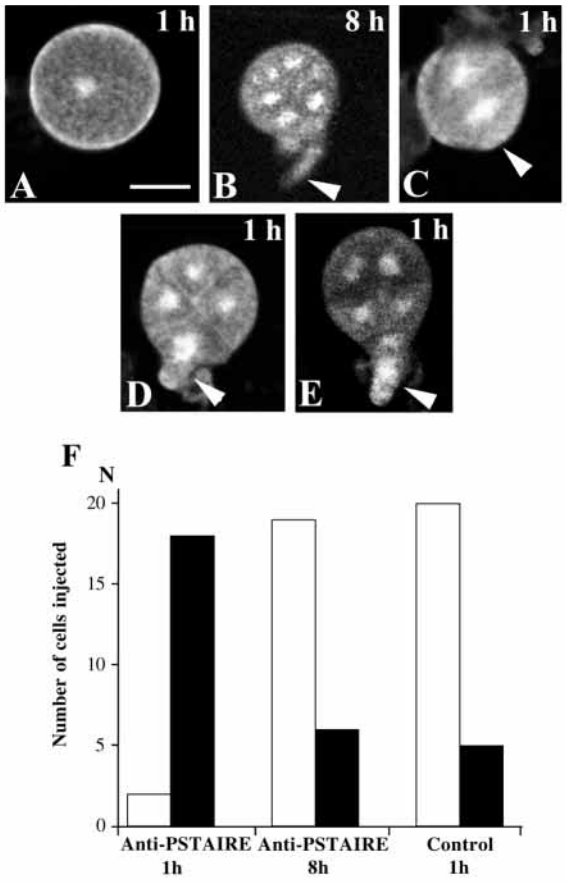


Fig. 4. Effect of microinjection of anti-PSTAIRE antibody on germination. Injection of the anti-PSTAIRE antibody at 1 hour AF inhibited germination and cell division in 18 out of 20 zygotes, as determined 36 hours (black in F) to 48 hours later (zygote in A). By contrast, injections at 8 hours AF allowed 19 out of 25 zygotes to germinate in response to unidirectional light and to divide as determined at 36 hours (white in F) or 48 hours AF (zygote in B). Twenty out of 25 zygotes, injected at 1 hour AF with the same concentration of control ascite fluid germinated and divided, as determined at 30 hours (C), 36 hours (white in F) and 48 hours AF (D,E) though the division planes were sometimes misaligned (D) compared with controls injected with injection buffer only (E). Arrowheads point to the rhizoids. Scale bar: 40 μm in A,C,D,E; 50 μm in B.

both of the two PSTAIRE CDK-like proteins referred to as p34 and p32 (Corellou et al., 2000b) (Fig. 5B), amino-purvalanol retained only p34 (Fig. 5B). However, full depletions of cell extracts from p34 could no be obtained (Fig. 5B), even after several rounds of incubation with amino-purvalanol (data not shown). Among the proteins extracted during the G1 and S phases (from 2 to 10 hours AF), only p34 had affinity for amino-purvalanol (Fig. 5B), suggesting that, of the PSTAIRE CDK-like proteins, p34 was the major target of this drug in vivo. Furthermore, no PSTAIRE CDK-like protein had affinity for the inactive structural analogue methyl-amino-purvalanol covalently fixed on sepharose beads (Fig. 5B). Taken together Figs 2, 3, 4 and 5 suggest that p34 is the main PSTAIRE CDK-like protein involved in the control of early cell cycle progression and morphogenesis in *Fucus* zygotes.

DISCUSSION

CDK-like proteins control both cell cycle progression and early development in *Fucus* zygotes

Although the effect of external factors that influence the establishment of polarity has been extensively studied in fucoid zygotes (Kropf et al., 1999), very little is known about the regulation by intracellular cues. We address the relationship between cell cycle progression and morphogenesis in *Fucus* zygotes. In a previous study, we have shown that arresting the cell cycle in S phase by preventing DNA replication blocks all aspects of cell division, but has no effect on either polarisation, germination or rhizoid elongation (Corellou et al., 2000b). Arresting the progression of the cell cycle in G2 phase or in M phase using olomoucine, a specific inhibitor of CDK, did not prevent either polarisation or morphogenesis (Figs 2, 3). Several lines of evidence, however, support the occurrence of early interactions between the cell cycle and the establishment of polarity. First, the beginning of S phase occurs at about the same time as the formation of an axis of polarity in response to unidirectional light. When applied early, treatments with specific inhibitors of CDKs, including olomoucine, roscovitine and amino-purvalanol, prevent rhizoid germination. Such an inhibition of rhizoid outgrowth is likely to arise from an inhibition of polarisation rather than germination, as neither olomoucine nor amino-purvalanol prevent germination when applied after the photopolarisation period. Interestingly, the period of sensitivity to CDK inhibitors of populations of synchronous zygotes is coincident with the period of photopolarisation (Fig. 3) and these CDK inhibitors prevent photopolarisation, further suggesting a close relationship between polarisation and cell cycle progression. In *Fucus* zygotes, the transcription of *histone H3*, a gene expressed at the onset of S phase, increased from 5-6 hours AF (Corellou et al., 2001). Transcription of *histone H3* was prevented by olomoucine but not by aphidicolin, indicating that, when applied soon after fertilisation, olomoucine induces a cell cycle arrest before aphidicolin, i.e. in G1 or at the G1/S transition (Corellou et al., 2001). Furthermore, like amino-purvalanol, olomoucine inhibits G1/S CDKs in vitro (Corellou et al., 2001). Taken together, the above results indicate that S phase entry and photopolarisation are concomitant and that olomoucine prevents both events. This observation strongly suggests that photopolarisation is controlled by olomoucine-sensitive CDKs. The possibility that olomoucine inhibits key kinases that regulate polarisation independently of the cell cycle can be ruled out by the fact that, when microinjected after fertilisation, $p21^{cip1}$, a specific inhibitor of CDKs, also inhibits germination and cell division.

The nature of CDK-like proteins involved in the control of early development

Compared with animal cells, the first cell cycle of the *Fucus* zygote resembles more a somatic than an embryonic cell cycle. In particular PSTAIRE CDK-like proteins are synthesised after fertilisation, probably from stored maternal mRNAs (Corellou et al., 2001). We show (Fig. 4) that, when injected early, a monoclonal anti-PSTAIRE antibody completely prevents cell division, most likely by competing with cyclins for the PSTAIRE-binding site. The anti-PSTAIRE antibody also inhibits germination, suggesting that the CDK-like proteins

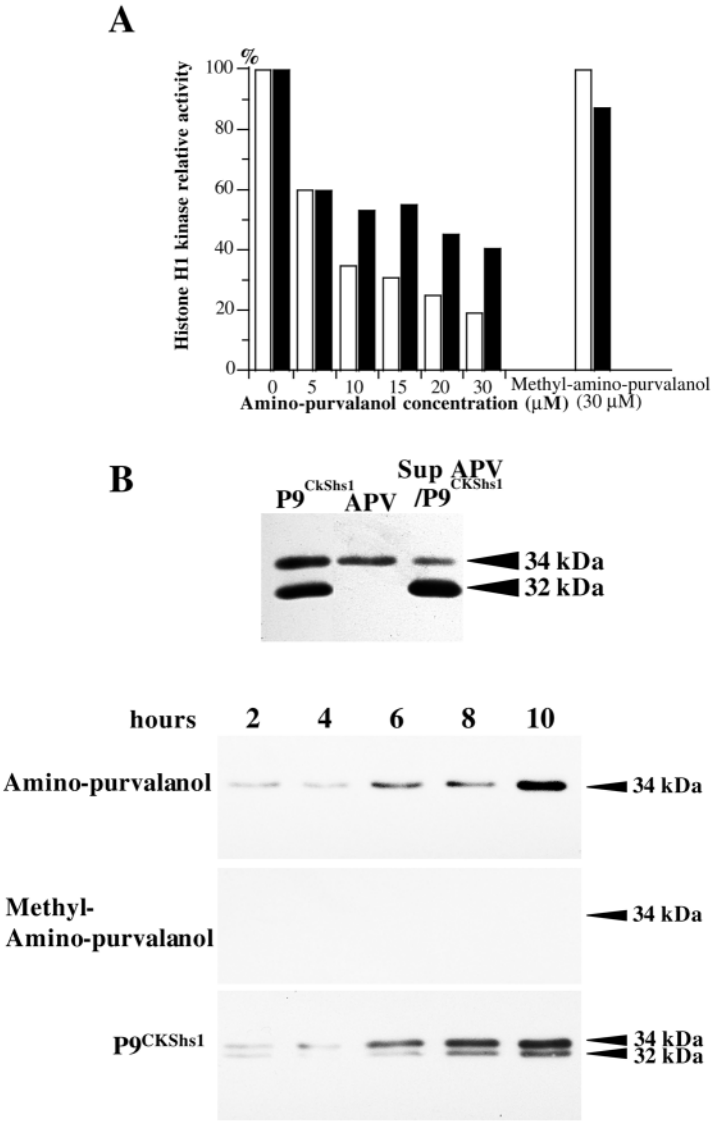


Fig. 5. Affinity purification of p34 on amino-purvanol-sepharose beads. The effect of amino-purvalanol was tested in vitro on the histone H1 kinase activity of extracts from cells arrested at the G1/S transition by a treatment with 100 μM olomoucine from 3 to 36 hours AF (black in A), or in mitosis by a treatment with 0.33 μM nocodazole from 3 to 36 hours AF (white in A). Methyl-amino-purvalanol (30 μM) was used as a control. (B) Although two PSTAIRE CDK-like proteins, p32 and p34, were bound to $p9^{CKShs1}$ ($p9^{CKShs1}$) in extracts from 48-hour-old embryos, only one (p34) was retained on amino-purvalanol-sepharose beads (APV), as detected with the anti-PSTAIRE antibody. Note that p34 could not be completely depleted from cells extracts using amino-purvalanol beads, as the supernatant still contained significant amounts of p34 that could be subsequently fixed on $p9^{CKShs1}$ beads (Sup APV/$p9^{CKShs1}$). Throughout the first cell cycle, from 2 to 10 hours AF, only the p34 PSTAIRE CDK-like protein was retained on amino-purvalanol beads but not on methyl-amino-purvalanol beads, as detected with the anti-PSTAIRE antibody. The data shown are representative of the results of three independent experiments.

involved in the control of early development exhibit the PSTAIRE hallmark. When injected at 8 hours AF, i.e. in S phase, cells divided and germinated normally. It is possible that these cells were not sensitive to the injection at this stage of development. However, it is more likely that the cyclin/CDKs complexes were already formed at the time of injection. In favour of this second hypothesis, preliminary data suggest that the PSTAIRE CDK-like protein p34 also plays a major role in the control of mitosis (Corellou et al., 2001). At all the stages of development investigated in this study, the p34 PSTAIRE CDK-like protein, but not p32, had affinity for amino-purvalanol (Fig. 5). This suggests that, in vivo, p34 is the only PSTAIRE CDK-like protein sensitive to amino-purvalanol and probably to other purine derivatives, such as olomoucine. We therefore propose that p34 is the main PSTAIRE CDK-like protein involved in the control of the progression through the first cell cycle. For the same reason, p34 is likely to be the main PSTAIRE CDK-like protein involved in the control of polarity. Although PSTAIRE CDKs are known to be much more sensitive to purine derivatives compared with non-PSTAIRE CDKs (Meijer, 1995), we do not rule out, however, that other, non-PSTAIRE CDK-like proteins, are involved with the control of cell cycle progression and/or of polarisation in *Fucus* zygotes.

A model for the regulation of early cell cycle and polarisation in *Fucus* zygotes

At least three different pathways can account for the control of polarity by the p34 CDK-like protein (Fig. 6). P34 may directly control both the entry in S phase and the polarisation events (Fig. 6A). This pathway would resemble the cell cycle-dependent control of morphogenesis in budding yeast (Lew and Reed, 1993). A parallel has been established between germination in *Fucus* zygotes and budding in yeast (Goodner and Quatrano, 1993), as both budding and germination require the localisation of F-actin at the budding, or germination, site (Lew and Reed, 1993; Kropf et al., 1989) and polarised secretions (Lew and Reed, 1993; Shaw and Quatrano, 1996; Hable and Kropf, 1998). Once they pass the START point in late G1, budding yeasts are committed to the next cycle (Hartwell, 1974) and inhibiting DNA replication has no effect on budding (Lew and Reed, 1995). Altering the activity of Cdc28 at the G1/S transition prevents polarised secretions and inhibits polar localisation of F-actin (Lew and Reed, 1993). Similarly in *Fucus*, germination occurs when DNA replication is inhibited (Corellou et al., 2000b) but the inhibition, before entry in S phase, of CDK-like proteins prevents photopolarisation and the polar secretion of sulphated compounds. Alternatively p34 may control only the entry in S phase, which in turn positively regulates polarisation events. In this case, the inhibition of polarity would be an indirect consequence of the inhibition of p34 (Fig. 6B). It is also possible that polarisation and the cell cycle are part of two independent pathways (Fig. 6C). In this case, an inhibitory mechanism (checkpoint) would prevent polarisation only before the cell has entered S phase (Fig. 6C).

In conclusion, cell cycle progression and morphogenesis are tightly coordinated in *Fucus* zygotes. Whereas arresting the cells during S phase has no effect on morphogenesis, preventing S phase entry, by inhibiting CDK-like proteins, completely abolishes polarisation, polar secretion of sulphated compounds and subsequent morphogenesis. This raises the possibility that, like in budding yeast, a restriction point in late G1 controls both cell cycle progression and morphogenesis. It remains to be determined whether CDK-like proteins act directly on morphogenesis, in a pathway leading to the activation of 'polarity proteins' or if preventing entry in S phase is sufficient in itself to prevent polarisation and morphogenesis.

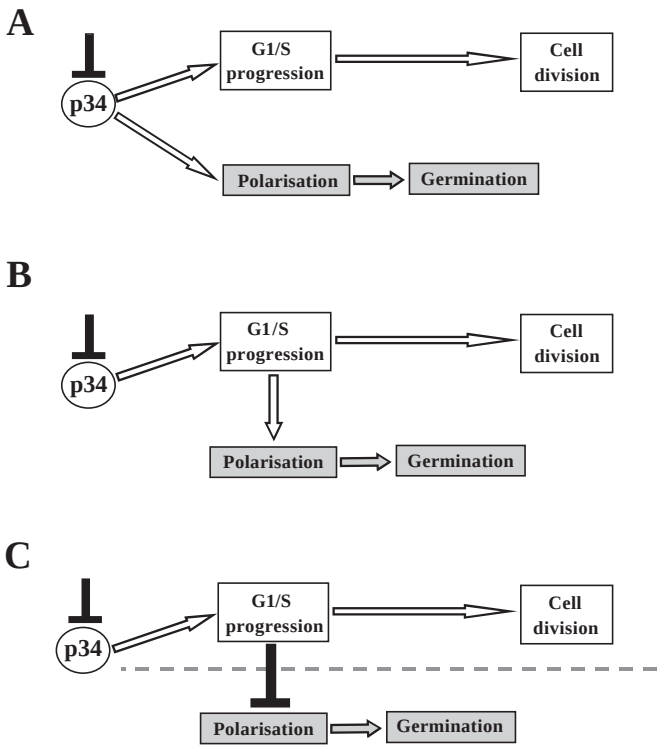


Fig. 6. Possible pathways for the control of early development by p34 in *Fucus* zygotes. (A) P34 positively controls both the entry in S phase and photopolarisation. Inhibition of p34 activity (in black) prevents both events. Alternatively (B), p34 controls the G1/S transition, which itself is required to activate polarisation events. (C) A third possibility is that cell cycle and polarisation belong to two independent pathways, but a checkpoint prevents polarisation when the cell cycle is arrested before S phase.

Until recently, it was assumed that increasing or decreasing the number of cells by affecting the basic cell cycle machinery leads to a modification of cell size without affecting morphogenesis of plant organs (Doerner et al., 1996; Hemelerly et al., 1995). However, a recent study indicates that the constitutive expression of the CDK inhibitor, ICK1, produces *Arabidopsis* plants with defects in growth and morphogenesis (Wang et al., 2000). Furthermore, *Arabidopsis* plants that express constitutively the dominant negative allele *cdc2aN147* do not develop (Hemerly et al., 1995), and the expression of *cdc2aN147* under the control of an embryo specific promoter either prevents germination or induces defects in morphogenesis (Hemerly et al., 2000). These observations suggest that, as in *Fucus* zygotes, early interactions exist between early cell cycle and developmental events in higher plant embryos.

The plasmid carrying the fusion gene GST-P21^{cip1} and amino-purvalanol immobilised on Sepharose beads are kind gifts from André

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