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Research article

A dinoflagellate CDK5-like cyclin-dependent kinase

Thierry Bertomeu, Jean Rivoal and David Morse[1]

Institut de Recherche en Biologie Végétale, Département de Sciences Biologiques, Université de Montréal, 4101 Sherbrooke Est, Montréal, Québec, Canada H1X 2B2

Background information. Mitosis during the dinoflagellate cell cycle is unusual in that the nuclear envelope remains intact and segregation of the permanently condensed chromosomes uses a cytoplasmic mitotic spindle. To examine regulation of the dinoflagellate cell cycle in the context of these unusual nuclear features, it is necessary to isolate and characterize cell cycle regulators such as CDK (cyclin-dependent kinase).

Results. We report the characterization of a CDK from the dinoflagellate *Lingulodinium polyedrum*. This CDK reacts with an anti-PSTAIRE antibody and was identified by protein microsequencing after partial purification. The protein microsequence shows homology toward the Pho85/CDK5 clade of CDKs. Neither the amount nor the phosphorylation state changed over the course of the cell cycle, in agreement with results reported for CDK5 family members in other systems.

Conclusions. We conclude we have probably isolated a dinoflagellate CDK5-like protein. The data reported here support the identification of this protein as a CDK5 homologue, and suggest that dinoflagellates may contain several CDK families.

Introduction

The cell cycle of dinoflagellates is of interest from both health and basic science perspectives. First, many species of dinoflagellates are responsible for causing harmful algal blooms (Wong and Kwok, 2005) and a detailed knowledge of the cell cycle of dinoflagellates could lead to better understanding, monitoring and/or preventing this phenomenon. Secondly, all dinoflagellates show a number of very unusual cell cycle-related characteristics: they are devoid of histones (Herzog and Soyer, 1981), their chromosomes are permanently condensed throughout the cell cycle, their nuclear envelope remains intact during mitosis, and chromosome segregation uses an extra-nuclear spindle that passes through nuclear invaginations and makes indirect contacts with the chromosomes attached to the inside of the nuclear membrane (Bhaud et al., 2000). Finally, since mitosis in dinoflagellates is under circadian control, identifying cell cycle regulators is a necessary prerequisite to uncovering the mechanism by which the circadian clock controls this rhythm.

In eukaryotic cells, the transition between the different phases of the cell cycle is tightly controlled by the activity of different CDKs (cyclin dependent kinases) (Sanchez and Dynlacht, 2005). The yeast *Saccharomyces cerevisiae* has five CDKs, of which only two (Cdc28 and Pho85) are involved in cell cycle control (Toh-e and Nishizawa, 2001). Cdc28 associates with different cell cycle phase-specific cyclins and is required for all the phase transitions throughout the cell cycle (Mendenhall and Hodge, 1998). Pho85 also appears to play a role in the G_1/S transition (Espinoza et al., 1994) and, in particular, has been found to be involved in the degradation of the CDK inhibitor Sic1 (Wysocki et al., 2006), which normally acts to inhibit entry into S phase. In contrast, mammalian cells use many different CDKs at different steps of the cell cycle, such as CDK4 and CDK6 for passage

[1]To whom correspondence should be addressed (email david.morse@umontreal.ca).

Key words: CDK5 (cyclin-dependent kinase 5), cell cycle, circadian rhythm, dinoflagellate.

Abbreviations used: CDK, cyclin-dependent kinase; CIP, calf intestinal phosphatase; DTT, dithiothreitol; EST, expressed sequence tag; FL2-A, fluorescence peak amplitude; FL2-W, fluorescence width; GST, glutathione S-transferase; IPG, immobilized pH gradient; LD, light/dark; LpCyc1, *Lingulodinium polyedrum* cyclin 1; NL, non-linear; PSTAIRE motif, Glu-Gly-Val-Pro-Ser-Thr-Ala-Ile-Arg-Glu-Ile-Ser-Leu-Leu-Lys-Glu motif.

through G_1, CDK2 for entry into and progression through S phase, and CDK1 for the G_2/M transition (Obaya and Sedivy, 2002). CDK1 is a Cdc28 homologue, whereas CDK5 is homologous to Pho85 (Liu and Kipreos, 2000). CDK5 is unrelated to the control of the cell cycle in mammalian cells and appears instead to be involved in neuronal development (Smith and Tsai, 2002) and other diverse functions (Rosales and Lee, 2006).

Most members of the CDK group of serine/threonine protein kinases are themselves regulated post-translationally by phosphorylation. An inhibitory phosphorylation site is located within the P-loop at the N-terminal region of CDKs, while an activating phosphorylation site is located in the T-loop, which otherwise blocks the active site cleft. CDK5 in mammalian cells is a notable exception to this type of regulation, as it does not require phosphorylation in the T-loop for activation (Qi et al., 1995; Tarricone et al., 2001), and phosphorylation of Tyr^{15} in the P-loop increases rather than decreases activity of the enzyme (Zukerberg et al., 2000). This behaviour is also observed for Pho85 in yeast, which is also activated by phosphorylation in the P-loop and does not require T-loop phosphorylation for activity (Toh-e and Nishizawa, 2001).

The activity of CDKs is also regulated by protein–protein interactions. Cyclins, a group of proteins originally isolated because their abundance varied throughout the cell cycle, are absolutely required for CDK activity. They bind CDKs through interactions with their PSTAIRE motif (Glu-Gly-Val-Pro-Ser-Thr-Ala-Ile-Arg-Glu-Ile-Ser-Leu-Leu-Lys-Glu motif) on the CDK. In addition, a group of proteins called CDK inhibitors can bind CDKs, either bound to a cyclin or not, and block their activity.

The cell cycle of dinoflagellates has been the subject of several previous studies using, as a model, the rapidly dividing heterotroph *Crypthecodinium cohnii*. This species contains a 34 kDa protein reacting with an anti-PSTAIRE antibody, and both this signal and histone H1 kinase activity can be enriched using $p13^{suc1}$ immunoprecipitation (Rodriguez et al., 1993). A modest increase (less then 2-fold) in histone H1 kinase activity of proteins immunoprecipitated with $p13^{suc1}$ was found in M phase compared with interphase cell extracts (Bhaud et al., 1994). Interestingly, an antibody raised against a *Schizosaccharomyces pombe* cyclin (Cdc13) was found to cross react with a 56 kDa band in *C. cohnii* extracts and this same antibody was able to immunoprecipitate a higher histone H1 kinase activity (Barbier et al., 1995). Using an anti-(cyclin-box) antibody, four cross-reacting bands were found (Leveson et al., 1997), of which two (at 50 and 65 kDa) accumulated in G_2 and disappeared after M phase. Only the 50 kDa band could be immunoprecipitated using $p13^{suc1}$ binding.

In *Gambierdiscus toxicus*, an anti-PSTAIRE antibody was used to demonstrate the presence of a 34 kDa reacting band whose quantity did not vary over time (Van Dolah et al., 1995). This same antibody used for immunoprecipitation was able to enrich histone H1 kinase activity, and kinase activity was at its maximum when extracts from cells in M phase were used.

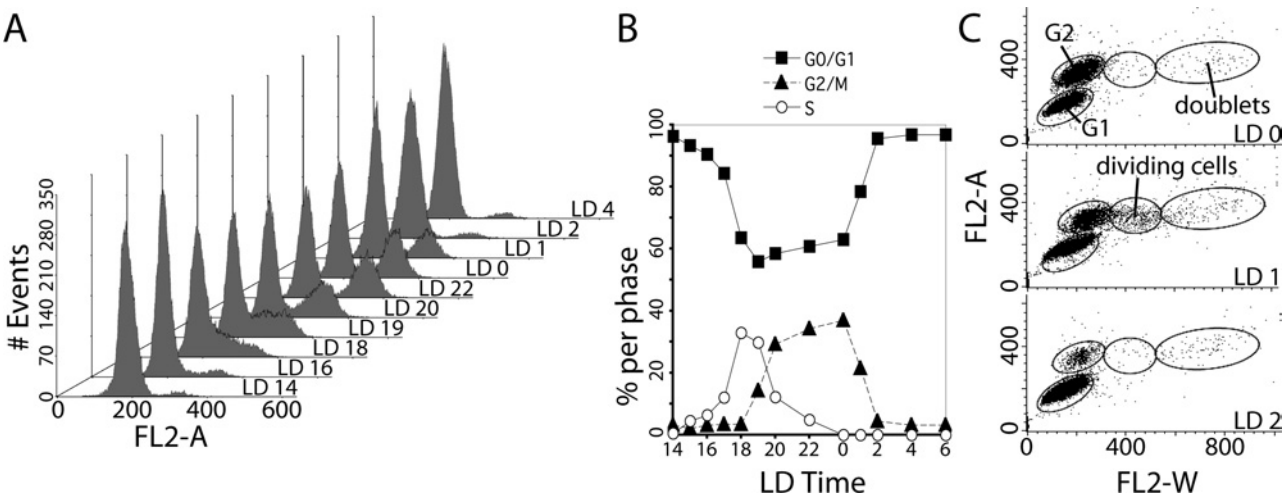
An anti-PSTAIRE reactive protein at around 32 kDa was also found in the dinoflagellate *Lingulodinium polyedrum*, although cloning and analysis of the sequence demonstrated clearly that it was not a CDK (Salois and Morse, 1996). More recently, a cyclin called GpCyc1 has been cloned by functional complementation from this same species (Bertomeu and Morse, 2004). Since *Gonyaulax polyedra* has been renamed as *Lingulodinium polyedrum*, we shall now further refer to this cyclin as LpCyc1. LpCyc1 rescues a mutant of *S. cerevisiae* deficient in the G_1/S cyclins Cln1, Cln2 and Cln3. The presence of a destruction-box in its N-terminal sequence together with the more then 10-fold decrease in amounts from G_2-enriched to G_1-enriched cells, indicated that this clone probably encodes a mitotic cyclin. This protein was the first (and so far the only) cell cycle regulating protein cloned and characterized from a dinoflagellate.

Interestingly, an EST (expressed sequence tag) project of *Karenia brevis* (with 7001 independent ESTs) was found to contain the first potential dinoflagellate CDK sequence (Accession number CO060059) (Lidie et al., 2005). This sequence is clearly a serine/threonine protein kinase with similarity (E value: $2e^{-10}$) to the *Drosophila melanogaster* CDK1-homologue. However, this sequence lacks the first 100 amino acids normally containing the characteristic PSTAIRE motif and its potential identity as a CDK is thus unconfirmed.

We report here the first biochemical isolation of a dinoflagellate CDK and its identification by protein microsequencing. The microsequence data clearly shows the conserved PSTAIRE motif and thus

Figure 1 | Cell cycle analysis of *L. polyedrum* over time

(**A**) DNA content per cell was measured by propidium iodide staining in 20 000 events counted using flow cytometry on cells collected at different times during an LD cycle. (**B**) Percentages of cells in G_0/G_1, G_2/M and S phases, determined using the ModFit software, plotted over time. (**C**) FL2-W versus FL2-A readings of cells in LD 0, LD 1 and LD 2 shows abundant dividing cells at LD 1.



confirms the sequence as a CDK. The sequence has, as expected, high similarity to a *Toxoplasma gondii* CDK based on the phylogenetic relationships of dinoflagellates and apicomplexans. The sequence also appears most similar to the CDK5/Pho85 group of CDKs in phylogenetic analyses. Furthermore, the abundance and phosphorylation state of this CDK is constant over the cell cycle, similar to what is found with CDK5 from other species. As this CDK does not seem to associate with LpCyc1, we conclude that dinoflagellates are likely to express a number of different CDKs, as found for other organisms.

Results

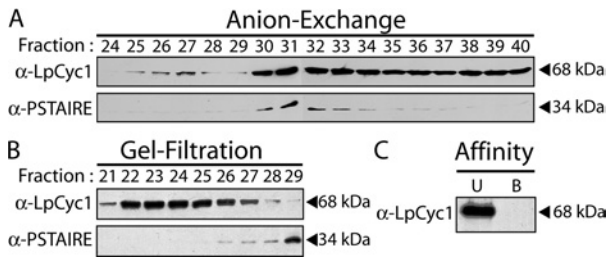
The *L. polyedrum* strain used here is different from that whose cell cycle events were studied previously (Homma and Hastings, 1989). We therefore used flow cytometry to characterize the cell cycle in our strain. For the present study, cells were maintained in exponential phase growth by 2-fold dilution of the cells every 3 days. Under these conditions the cells have a shorter doubling time than reported previously (Bertomeu and Morse, 2004), with approx. 30% of the cells entering S phase during the early night phase (Figures 1A and 1B). In agreement with previous observations, S phase was found to occur within a 4 h period around LD (light/dark) 18 (Homma and Hastings, 1989) and M phase occurs around LD 1 at 1 h after the start of the light phase (Sweeney and Hastings, 1958). M phase can be observed either as the disappearance of cells in G_2/M (Figure 1B) or as the appearance of dividing cells, a population with the same FL2-A (fluorescence peak amplitude) as G_2 cells but an FL2-W (fluorescence peak width) intermediate between G_2 cells and G_1 doublets (Figure 1C).

Interestingly, although most of the G_2 phase cells divided at LD 1, a small percentage of G_2 phase cells can be found at all time points measured between LD 1 and LD 14. Indeed, depending on the particular experiment, up to 8% of G_2 phase cells could be found during the day phase (data not shown). This observation reinforces the idea that M phase is clock-controlled and does not simply occur at a fixed time following S phase.

As an initial approach to isolation of a dinoflagellate CDK, we thought to use binding to the dinoflagellate cyclin LpCyc1 (Bertomeu and Morse, 2004) as a molecular marker that could be monitored following fractionation by column chromatography. The presence of LpCyc1 was determined by an anti-LpCyc1 antibody reaction. A crude protein extract from cells at LD 0.5 was first subjected to ammonium sulfate

Figure 2 | Chromatography of LpCyc1 and an anti-PSTAIRE antibody reactive protein

(**A**) After fractionation by anion-exchange chromatography, LpCyc1 elutes in a single broad peak (fractions 30–41) as detected by Western blotting, whereas a protein reacting with an anti-PSTAIRE antibody co-elutes in the first five of these fractions. (**B**) The gel-filtration chromatography of the partially purified LpCyc1 shows separation of LpCyc1 and the anti-PSTAIRE reacting proteins. (**C**) LpCyc1, partially purified by gel filtration does not bind to a p13^{suc1} affinity chromatography resin. U, unbound; B, bound.

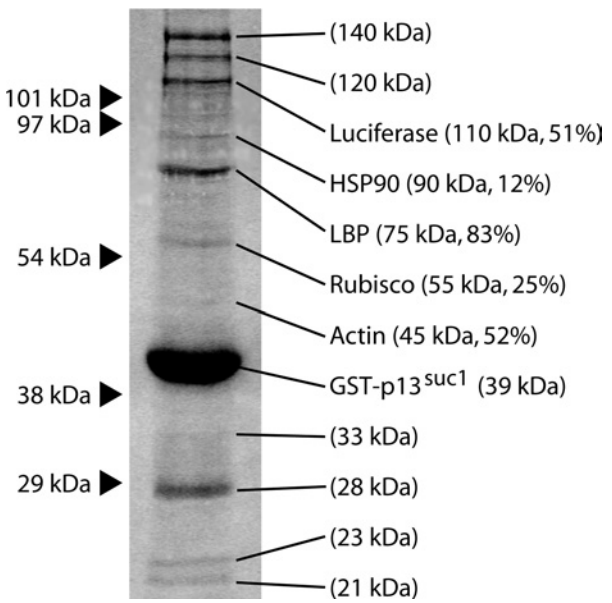


precipitation, with proteins precipitating between 17 and 40% containing all the polypeptides immunoreacting with the anti-LpCyc1 serum (data not shown). The precipitate was resuspended, desalted and subjected to anion-exchange chromatography. A single band at 68 kDa was recognized by the anti-LpCyc1 antibody and the LpCyc1 protein routinely eluted within a major peak on 12 fractions (30–41) (Figure 2A). A non-reproducible and minor LpCyc1-reactive species is occasionally noted at a position prior (25–28) to the main peak. Interestingly, challenging the same fractions with an anti-PSTAIRE antibody identified a single peak of immunoreactive protein whose apparent molecular mass on SDS/PAGE was 34 kDa, although this immunoreactive protein was only found associated with the first several fractions of the major cyclin-containing peak. The majority of the anti-LpCyc1 reactive fractions (30–41) were then pooled and fractionated by gel-filtration chromatography. The cyclin eluted within four fractions (22–25) (Figure 2B) corresponding to an apparent molecular mass of 160 ± 20 kDa (data not shown). However, the anti-PSTAIRE signal was no longer associated with the cyclin, as the immunoreactive protein eluted at a position corresponding to an apparent molecular mass of 34 kDa.

These results suggested that our initial hypothesis of a CDK–LpCyc1 interaction might have been incorrect. To verify if our experimental conditions had disrupted the CDK–cyclin binding, we tested for LpCyc1 binding to p13^{suc1}–Sepharose beads. Cyclin binding to this affinity column is thought to require CDK–cyclin interactions (Vogel et al., 2002), but LpCyc1 failed to bind this resin as judged by Western blot analysis (Figure 2C). We also tested for co-elution of anti-cyclin and anti-PSTAIRE immunoreactivity on gel filtration on Sepharose CL4-B using the crude extract directly, to ensure that the purification procedure itself did not decrease association of the two proteins. The two were again found in different fractions (data not shown), consistent with a lack of interaction between LpCyc1 and the anti-PSTAIRE reactive protein.

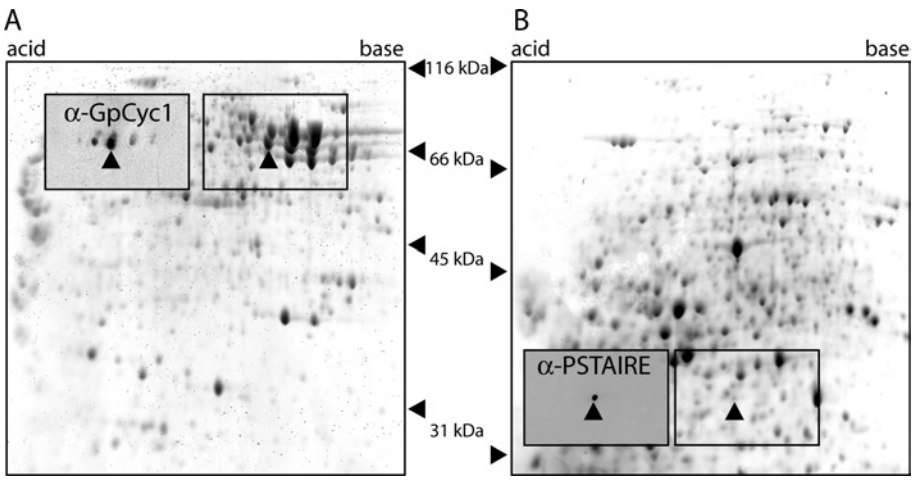
Figure 3 | Microsequence analysis of proteins bound to p13^{suc1}–Sepharose beads

A crude protein extract was bound to p13^{suc1} and the specific eluate was resolved by SDS/PAGE. Shown are the 11 *Lingulodinium* bands that were excised and microsequenced as well as the GST–p13^{suc1}, which was also eluted from the beads. Identity of the protein is written when MS sequencing yielded sufficient homology to known proteins. The molecular mass of the bands and the percentage of sequence coverage by MS are shown in parentheses.



To test if a CDK not identifiable by anti-PSTAIRE immunoreactivity was present in the extracts, p13^{suc1} chromatography was performed on a crude protein extract (Figure 3). The identification of all protein

Figure 4 | Identification of an anti-PSTAIRE reactive protein following two dimensional SDS/PAGE
Partially purified proteins after anion-exchange chromatography and gel-filtration containing LpCyc1 (**A**) or the anti-PSTAIRE signal (**B**) were resolved by two-dimensional SDS/PAGE and stained with Coomassie Blue. Spots indicated by arrowheads represent those reacting to the respective antibodies. The anti-PSTAIRE reacting spot was excised from the two-dimensional SDS/PAGE gel and identified by protein microsequencing.



bands visible by Coomassie Blue staining was then attempted by MS sequencing. Only five of the eleven bands corresponding to *Lingulodinium* proteins could be identified by significant sequence identity. Notably, neither cyclin (around 56 kDa) nor cdc2-like (around 34 kDa) sequences were recovered in this analysis, suggesting that appreciable levels of CDK did not bind to $p13^{suc1}$ under these conditions.

At this point in our analysis, we reasoned that it might be possible to identify the anti-PSTAIRE reactive protein enriched through the two columns in Figure 2 by MS sequencing. Indeed, a Coomassie-Blue-stained spot corresponding to the cyclin, as judged by an anti-LpCyc1 Western blot performed in parallel, was readily detected (Figure 4A). Since a Coomassie-Blue-stained spot was observed at the position of the anti-PSTAIRE signal after two-dimensional SDS/PAGE (Figure 4B), this protein was excised from the gel and microsequenced.

The peptide sequences obtained were very similar to a variety of different CDKs, with the highest similarity (BLASTP E value: 1^{e-37}) obtained with a *Toxoplasma gondii* cdc2-like CDK (see Supplementary Figure S1 at http://www.biolcell.org/boc/099/boc0990531add.htm). This was encouraging, as the apicomplexans (to which *T. gondii* belong) are the closest relatives to dinoflagellates in phylogenetic analyses. Curiously, however, when the protein sequence was compared with the thoroughly sequenced genomes of *Caenorhabditis elegans*, *Danio rerio*, *D. melanogaster*, *S. cerevisiae* and *Schizosaccharomyces pombe*, a higher degree of homology was reproducibly found with the CDK5/Pho85 rather than the CDK1 family of CDKs. This relationship is best illustrated by a phylogenic analysis of selected CDK1 and CDK5 sequences. The inclusion of our *Lingulodinium* sequence within a well-supported CDK5/Pho85 clade (Figure 5) suggested that our protein might correspond to a CDK5-like protein. Unfortunately, exhaustive attempts to clone a DNA fragment of LpCDK5-like using degenerate PCR primers on genomic and complementary DNA, or using radio-labelled degenerate oligonucleotides to screen a cDNA bank were unsuccessful, thus precluding a more detailed analysis of the homology. However, it is important to note that the peptide sequence reported here covers around 35% of the expected amino acid sequence, and includes the characteristic PSTAIRE motif that unambiguously identifies the protein as a CDK.

The reaction of the anti-PSTAIRE antibody with a protein identified as a *bona fide* CDK by microsequence analysis suggested that the antibody could be used to characterize the behaviour of the enzyme. We first tested LpCDK5-like expression over a daily

Figure 5 | Anti-PSTAIRE reactive protein corresponds to a CDK5 isoform

Protein sequences representative of CDK5/Pho85 and CDK1/CDC2/Cdc28 clades from *S. pombe*, *S. cerevisiae*, *D. rerio*, *D. melanogaster* and *C. elegans*, all truncated to match LpCDK5-like microsequence data, were used for phylogenetic analysis. The inclusion of the *Lingulodinium* sequence within the CDK5/Pho85 clade has high bootstrap support.

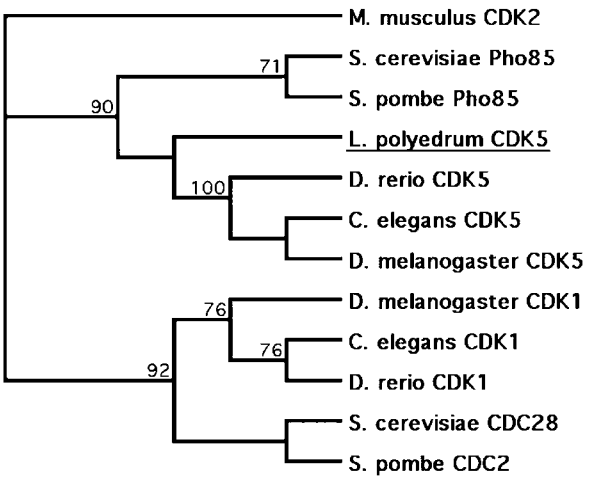
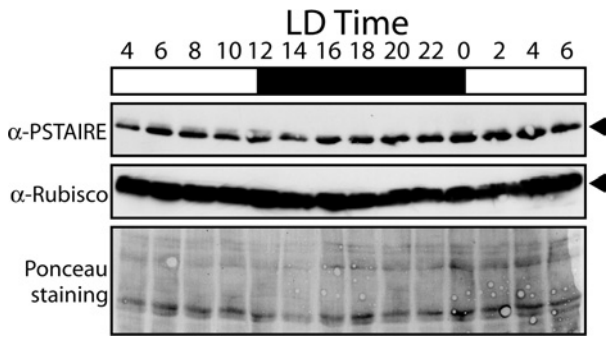


Figure 6 | No variation upon the expression of LpCDK5-like is detected over time

Protein samples of *Lingulodinium* collected at different LD times were resolved by SDS/PAGE and the Western blot was probed with either anti-PSTAIRE or anti-Rubisco antibodies. Arrowheads represent either the 34 kDa or 55 kDa bands for the upper and middle panels respectively. The PVDF membrane stained with Ponceau Red is shown in the lower panel.



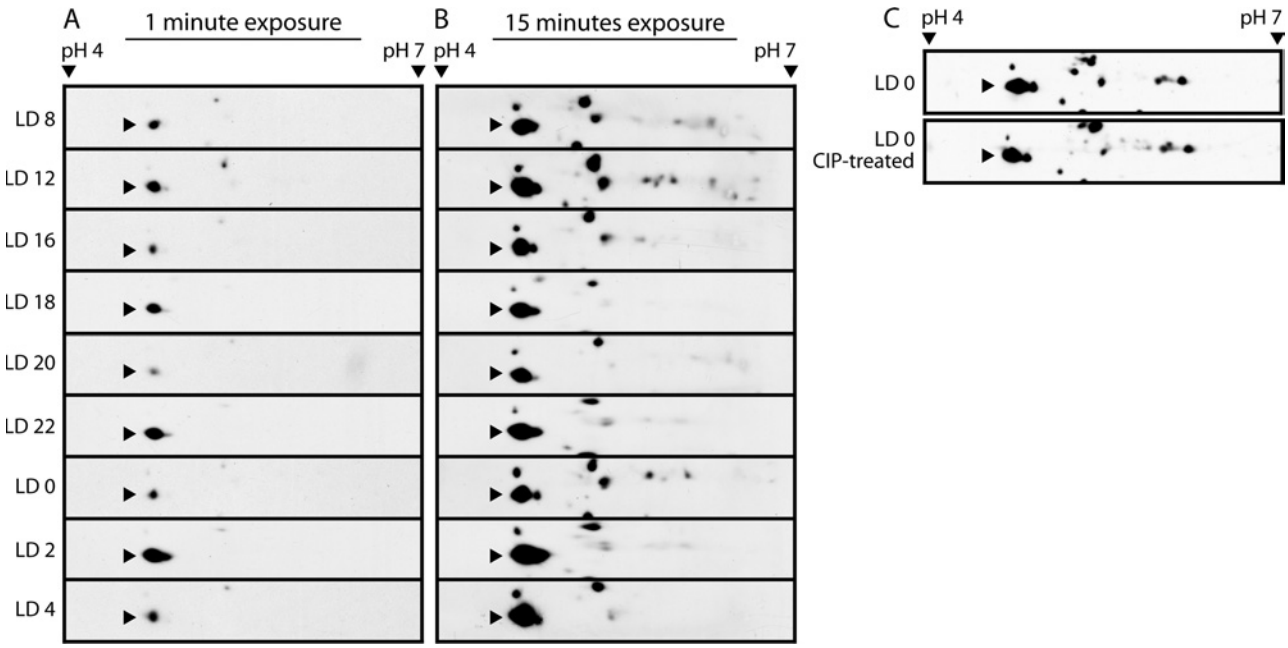
period in *Lingulodinium* cells grown under an LD cycle, where mitosis is clearly rhythmic. No major difference in the expression of LpCDK5-like was detected in unsynchronized exponentially growing cells (Figure 6). Since CDKs can be post-translationally modified by phosphorylation events, the isoelectric point of LpCDK5-like through a cell cycle was also determined. However, no reproducible changes in CDK phosphorylation could be observed. At low exposure times (Figure 7A), antibody reaction with the two-dimensional SDS/PAGE gave rise to a single specific band. Longer exposures of the films revealed several other spots (Figure 7B) at molecular masses compatible with CDKs, mostly at LD 0 and LD 12. However, the mobility of all proteins detected in the over exposed films does not change after incubation with CIP (calf intestinal phosphatase) phosphatase, indicating they do not correspond to differentially phosphorylated isoforms of LpCDK5-like (Figure 7C). We had anticipated that an isoform representing a major part of LpCDK5-like in 30% of the cells entering mitosis at LD 0 would have been readily detected with this technique if this kinase was differentially phosphorylated at mitosis. This observation is in agreement with our assignment of the *Lingulodinium* protein to the CDK5 family, which, unlike its CDK1 relative, does not undergo differential phosphorylation during different phases of the cell cycle.

Discussion

It has long been known that mitosis of *L. polyedrum* grown in a laboratory setting is under circadian control. Under 12 h light/12 h dark (LD::12:12) conditions, mitosis is restricted to 1 h after lights are turned on (LD 1). Since the generation time of a single cell is typically greater then 24 h (Sweeney and Hastings, 1958), the cell cycle is said to be 'gated' rather than controlled directly by the circadian clock. All cells are thus given an opportunity to enter M phase each day, but only the cells in an appropriate position in the cell cycle will do so. Flow cytometric measurements of exponentially growing *Lingulodinium* confirm this, as although most cells in G_2 phase undergo mitosis at this time (Figure 1C), some G_2 phase cells do not divide at LD 1 and can be found later during the day phase. Interestingly, these observations argue against the model of Homma and Hastings (1989), in which M phase was proposed to follow at a fixed time interval from a circadian controlled S phase. G_2 phase cells during day phase suggest they were not ready to

Figure 7 | Isoelectric point of LpCDK5-like is invariant over time

Chemiluminescence of Western blots of two-dimensional SDS/PAGE (pH 4–7, linear) using proteins taken each 2 h from LD 8 up to LD 4 were hybridized to an anti-PSTAIRE antibody and exposed to film for either 1 min (**A**) or 15 min (**B**). Arrowheads indicate the major 34 kDa reacting spot. An LD 0 protein sample treated with CIP does not affect the electrophoretic mobility of the LpCDK5-like protein. Gels from two different experiments (one from times 0,4,8,16 and 20, and the other from times 2,12,18 and 22) are shown.



undergo mitosis at LD 1 and were thus obligated to wait for the subsequent mitotic window.

With respect to S phase, our data showing onset of S phase approx. 9 h prior to mitosis and lasting for approx. 4 h confirms previous observations (Homma and Hastings, 1989). Additional flow cytometry measurements of cells grown in different lighting conditions will be required to assess definitively if there is a circadian control over both S and M phases.

To date, only one study has identified a dinoflagellate cDNA as a CDK (Lidie et al., 2005) and the identification is based on an incomplete EST sequence. Curiously, if this sequence were completed using the N-terminal region of the *Drosophila* CDK1 sequence, the predicted protein would have a molecular mass of 43 kDa, much larger than the 34 kDa expected for CDKs. Moreover, this kinase lacks the RD sequence in subdomain VIb present in all CDKs (Ferrari, 2006). Thus it seems unlikely that this sequence corresponds to a true CDK. There are, however, three other potential CDK sequences that can be found by screening the dinoflagellate EST databanks. One, in *Karenia brevis* (CO061088), contains a PLTAIRE motif with some similarity with CDK9. Two others, one in *Oxyrrhis marina* (EG741876) and the other in *Karlodinium micrum* (KME00004803), contain a PSTAIRE motif with some similarity with CDK1. These sequences thus support our contention that dinoflagellates have many different CDKs as do other eukaryotes.

We report here protein microsequence data that confirms the identity of an anti-PSTAIRE reactive protein as a CDK from a dinoflagellate. Our results do not support an interaction of this CDK with the cyclin LpCyc1 reported previously from this organism. LpCyc1 and LpCDK5-like do not co-elute during gel-filtration, either before or after partial purification on anion-exchange chromatography (Figure 2B

and data not shown). The apparent molecular mass of the cyclin following gel filtration (around 160 kDa) suggests that LpCyc1 may be part of a protein complex, possibly encompassing a CDK interacting with LpCyc1, as the cyclin monomer is expected to be 68 kDa. However, no other protein in the mixture reacts with the anti-PSTAIRE antibody and LpCyc1 does not bind to $p13^{suc1}$ affinity resin (Figure 2C).

The protein identified here by microsequence data appears most similar to the Pho85/CDK5 family members. This conclusion is supported by phylogenetic analysis with the sequence, albeit incomplete, that is currently available, as LpCDK5-like is found in a CDK5/Pho85 clade with high bootstrap support. Furthermore, the invariance of the phosphorylation state at important cell cycle transitions times is in agreement with a CDK not implicated in cell cycle control, such as CDK5, whereas CDK1 is the recognized key regulator of entry into M phase. Since CDK1 and CDK5 types of cyclins are very closely related (Manning et al., 2002), a definite classification of dinoflagellate CDKs will require a more complete catalogue of dinoflagellate sequences.

The anti-PSTAIRE antibody used in the present study recognizes a single major protein of 34 kDa that elutes as a single peak after anion-exchange chromatography and gel-filtration. This immunoreactive protein thus differs from that found in a previous study (Salois and Morse, 1996) in which an anti-PSTAIRE antibody (from another supplier) was found to react with a 32 kDa band. This discrepancy may be due to differences in the amino acids flanking the PSTAIRE antigen used for raising the different antibodies. This suggests that care should be exercised in use of these antibodies to assess the dinoflagellate cell cycle (Rodriguez et al., 1993; Van Dolah et al., 1995) unless independent confirmation of the identity of the immunoreactive protein can be obtained. It is unfortunate that we were unable to recover a cDNA or the genomic sequence corresponding to our protein microsequence. It is possible that the gene is of low abundance in the RNA complement of the cell and thus may be absent from our cDNA library. Furthermore, the genomic copy may contain a large number of intervening sequences precluding amplification by PCR. We note, however, that the lack of a complete LpCDK5-like sequence does not preclude use of the antibody to assess the levels and phosphorylation state of the protein.

Materials and methods

Cell culture

Cultures of *L. polyedrum* (formerly *G. polyedra*) CCMP 1936 were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Booth Bay Harbor, Maine, U.S.A.) and grown in F/2 medium under 12 h light (40 $\mu mol/m^2 \cdot s$ cool white fluorescent light) and 12 h dark at a temperature of $18 \pm 1^{\circ}C$. The timing of cell samples taken under this light regime is termed LD, with LD 0 corresponding to lights on and LD 12 to lights off.

Flow cytometry analysis

100 ml of cell cultures (approx. 8000 cells/ml) were centrifuged at 1000 *g* for 1 min and resuspended in 50 ml of 70% (v/v) ethanol. This centrifugation and ethanol wash was repeated twice with the cells in the last wash exposed to culture room lights for 30 min with gentle shaking to bleach residual pigments. Cell samples were stored at 4°C until use, when they were washed twice with water, treated with 40 mg/ml RNase A for 30 min at 37°C and stained for 1 h in the dark with 50 mg/ml propidium iodide (Sigma). For each time point, 20 000 events were read for exponentially growing cells using a FACScan (BD BioSciences) equipped with a 488 nm laser and a 586 nm filter for the FL2 channel. Data were acquired using the CellQuest Pro software (BD BioSciences). Cellular debris was excluded from our analysis using a gating window in Forward and Side Scatter plots and cell doublets were excluded using a gating window in FL2-area (FL2-A) versus FL2-width (FL2-W) plots. The percentage of cells in each phase of the cell cycle was calculated using the ModFit software (BD BioSciences).

Western blot analysis

Protein samples were boiled for 5 min in five packed cell volumes of 2% (w/v) SDS, 0.7 mM 2-mercaptoethanol, 62.5 mM Tris/HCl (pH 6.8) and 10% (v/v) glycerol and resolved by SDS-PAGE at 150 V on 12% gels. The proteins were then transferred electrophoretically on to PVDF membranes for 20 min at 20 V using a semi-dry transfer cell (BioRad) and stained with Ponceau Red to confirm transfer. Membranes were blocked for 10 min in TBS-T (Tris-buffered saline containing Tween 20; 5 mM Tris/HCl, pH 8.0, 137 mM NaCl, 2.5 mM KCl and 0.05% Tween 20) containing 5% (w/v) non-fat dried milk and incubated with antibodies overnight at 4°C diluted in fresh buffer. The antibodies used in this study were a previously described polyclonal antibody against the N-terminal region of LpCyc1 (Bertomeu and Morse, 2004), a 1/4000 dilution of a commercially available rabbit polyclonal antibody raised against a 15 amino acid epitope of human CDK1 encompassing the PSTAIRE sequence (Santa Cruz Biotechnology; SC-53) and a 1/8000 dilution of anti-(form II Rubisco) (Nassoury et al., 2001) were used as primary antibodies. Membranes were washed 3 times for 10 min each in TBS-T, incubated with 1/4000 goat anti-rabbit antibody coupled to peroxidase (Sigma), washed three times and the position of antibody binding detected with the ECL® Plus substrate (GE Healthcare). Films were exposed for between 20 s to 15 min depending upon the intensity of the signal.

Biochemical purification of a CDK

Lingulodinium cells at LD 0.5 were recovered from culture medium by filtration on Whatman 541 paper and were frozen in

liquid N_2 for later use. All procedures were carried out at 4°C. The cells (10 g from approx. 50 l of actively growing cultures) were broken in 35 ml of extraction buffer {20 mM Hepes, pH 8.0, 20 mM β-glycerophosphate, 25 mM NaF, 2 mM EGTA, 1.5 mM $MgCl_2$, 300 mM PMSF, 3 mg/ml leupeptin, 1 mM DTT (dithiothreitol), 10 mg/ml cytochalasin B and 1 mM [γ-S]ATP} by two runs through a French Press (Thermo Electron Corporation) at 16000 psi in a 40 K cell. The extract was clarified by centrifugation at 30000 *g* for 10 min, and the supernatant mixed with 0.2 volume of 100% saturated $(NH_4)_2SO_4$ solution to yield a 17% solution. Following a 10 min incubation on ice, the solution was centrifuged for 10 min at 30000 *g*, and the supernatant mixed with 0.38 vol. of 100% saturated $(NH_4)_2SO_4$ to yield a 40% solution. Following a 10 min incubation on ice, the solution was centrifuged at 30000 *g* for 10 min and the pellet resuspended in 2 ml of buffer A (20 mM Hepes, pH 8.0, 2 mM EGTA, 1.5 mM $MgCl_2$, 300 μM PMSF, 3 mg/ml leupeptin and 1 mM DTT). The sample was desalted in 1 ml aliquots through PD-10 desalting columns (GE Healthcare) pre-equilibrated in buffer A. The desalted fraction was loaded on to an 8 ml Fractogel EMD DEAE-650 (S) (VWR) column equilibrated with buffer A. Chromatographic separation was performed with a FPLC (GE Healthcare) running at 0.5 ml/min using a 0–50% linear gradient of buffer B (buffer A containing 20 mM β-glycerophosphate, 25 mM NaF and 1 M KCl). A total of 77 1 ml fractions were collected, with LpCyc1 detected by Western analysis in a major peak of 12 ml centred at around fraction 35. This chromatographic step resulted in an 8-fold enrichment of the antibody reactive signal compared with the total protein content in the sample. The fractions containing this peak was then concentrated to 500 μl using an Amicon Ultra-15 10000 (Millipore) and loaded on to a pre-packed Superdex 200 high resolution 10/30 gel filtration column equilibrated with buffer A containing 100 mM KCl at a flow rate of 0.4 ml/min. Fractions of 0.5 ml were collected from the start of loading and resulted in a further 5-fold enrichment of cyclin.

p13^{suc1} affinity purification

Partially purified LpCyc1 was loaded on to a Tricorn 5/20 column (GE Healthcare) containing 200 μl of GST (glutathione S-transferase)–p13^{suc1} coupled to Sepharose beads (Upstate) at 0.1 ml/min and washed with 20 column volumes of solution A with 100 mM KCl. Fresh *L. polyedrum* (2 g) collected at LD 0.5 were broken in 8 ml of extraction buffer, cleared and precipitated with $(NH_4)_2SO_4$ as determined above, then resuspended in 1 ml of PBS (pH 7.4) and loaded on to a PD-10 column equilibrated with PBS (pH 7.4) to remove residual $(NH_4)_2SO_4$. The resulting 1.3 ml sample was rocked gently with 200 μl of GST–p13^{suc1} beads for 3 h at 4°C. The beads where washed four times for 10 min with PBS (pH 7.4). The protein was eluted by a 10 min incubation in Western denaturing solution at 95°C.

Two-dimensional gel electrophoresis

Partially purified protein samples from gel filtration chromatography (fractions 22–25, which contained the majority of the LpCyc1 signal, and fractions 28–31, which contained the anti-PSTAIRE signal) were precipitated with 3 vol. of acetone and the pellets dried and resuspended in 265 μl of 8 M urea, 4% (w/v) Chaps, 20 mM DTT and 0.5% pH 3–10 NL (non-linear) IPG (immobilized pH gradient) buffer. Most (250 μl) of this sample was used to rehydrate a 13 cm IPG strip pH 3–10 NL and was electrophoresed using an IPGphor isoelectric focusing system (GE Healthcare) for 20000 Volt-hours. For the second dimension, strips were incubated 20 min in SDS equilibration buffer [50 mM Tris/HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol and 2% (w/v) SDS] and then resolved by SDS/PAGE (12% gel). The gels were stained with Coomassie Blue and scanned. The rest of the protein sample (15 μl) was diluted to 250 μl using the same buffer and treated similarly, except that the second dimension was transferred on to a PVDF membrane for immunoblotting instead of Coomassie Blue staining. The single protein reacting with the anti-PSTAIRE was microsequenced after identification of the corresponding spot on the Coomassie-Blue-stained gel. To determine the isoelectric points of the CDK as a function of time, 200 ml of *Lingulodium* cultures (approx. 8000 cells/ml) from each time point were centrifuged and frozen in liquid N_2. The cell pellets were broken in 1 ml of buffer [80 mM β-glycerophosphate, 25 mM NaF, 20 mM Hepes, pH 8.0, 5 mM EGTA, 300 mM PMSF, 3 mg/ml leupeptin, 1 mM DTT, and 1% phosphatase inhibitor cocktail 1 and 2 (Sigma)] with approx. 200 μl of 0.5 mm zirconium beads in a Bead Beater for three times 1 min at 4°C. After centrifugation at 20000 *g* for 10 min at 4°C, the supernatant was desalted by sequential addition of 4 vol. of methanol, 1 vol. of chloroform and 3 vol. of water, with vortexing between each addition. The samples were then centrifuged for 2 min at 10000 *g* and the supernatant, containing the methanol, water and salts was discarded. An additional 4 vol. of methanol was added to the chloroform and denatured proteins, vortexed and centrifuged again. The dried pellets were resuspended in 400 μl of 8 M urea, 4% (w/v) Chaps, 20 mM DTT and 0.5% pH 4–7 IPG buffer and 125 μl were resolved on 7 cm IPG strips pH 4–7. For the dephosphorylation experiment, 0.5 g of cells at LD 0 were broken either in the same buffer described above and kept on ice or into 1 ml of Promega's 1× CIP buffer containing 300 mM PMSF and 3 mg/ml leupeptin and centrifuged 5 min at 20000 *g*. The supernatant was incubated for 1 h at 37°C with 100 units of CIP (Promega) and then analysed by two-dimensional gel electrophoresis. The SDS/PAGE gels were all run simultaneously using a Mini-PROTEAN 3 Dodeca Cell (BioRad).

Protein microsequencing

Proteins were excised from the Coomassie-Blue-stained gel, extensively destained, subjected to trypsin digestion and the tryptic fragments sequenced by MS. Sample preparation and analysis was performed by the Harvard Microchemistry Facility (Cambridge, MA, U.S.A.) for LpCDK5-like and the Institut de Recherche en Immunologie et en Cancérologie (Montreal, Qc, Canada) for all others.

Phylogenic tree

Sequences were analysed using the MacVector software (Accelrys). The sequences used for the phylogenic tree were: *S. pombe* (Pho85: 074456 and CDC2: P04551), *S. cerevisiae* (Pho85: NP_015294 and Cdc28: NP_009718), *D. rerio* (CDK5: AAH85381 and CDK1: NP_997729), *C. elegans* (CDK5: AAD37121 and CDK1: AAC60520), *D. melanogaster* (CDK5: AAA63754 and CDK1: NP_476797) and *M. musculus* (CDK2: NP_904326). Sequences were aligned using Clustal W and amino acids for which we had no LpCDK5-like

sequence data were removed before the neighbour-joining tree was performed (Poisson corrected).

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