# A Type-1 Phosphoprotein Phosphatase from a Dinoflagellate as a Possible Component of the Circadian Mechanism

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*Abstract* Indicative of the importance of protein phosphorylation in the core circadian clock mechanism, chronically applied inhibitors of both protein kinases and phosphoprotein phosphatases have significant effects on the period, phase, and light-dependent regulation of circadian rhythms in the dinoflagellate *Lingulodinium polyedrum*. This study was aimed at identifying the presence of the affected phosphatase(s). Dephosphorylation of a PP1/PP2A-specific substrate by *L. polyedrum* extracts was inhibited by okadaic acid only at concentrations greater than 100 nM, as in vivo, by mammalian inhibitor-2 (I-2), and by an endogenous inhibitor with properties similar to I-2, indicating that a type-1 protein phosphatase (PP1) was predominant. A cDNA encoding a highly conserved PP1 was isolated, the 1st such signaling molecule identified in dinoflagellates. Antisera specific for this type of phosphatase recognized a 34 kDa protein in *L. polyedrum* extract, this being the same size as the PP1 encoded by the isolated cDNA. These findings are consistent with the suggestion that the *L. polyedrum* PP1 may be a part of the clock mechanism in this species.

*Key words* protein phosphorylation, circadian clock, phosphoprotein phosphatase inhibitors, dinoflagellate, okadaic acid, cantharidin, calyculin

Protein phosphorylation has been shown in many diverse organisms to be of central importance in the functioning of the core circadian clock, notably by modifying clock proteins involved in feedback repression and by affecting the period of the cycle (Allada et al., 2001; Dunlap, 1999; Young and Kay, 2001).

In *Arabidopsis*, the protein kinase CK2 was shown to phosphorylate the circadian clock-associated-1 (CCA-1) and late elongated hypocotyl proteins, both of which are identified as core clock proteins, while overexpression of CKB3, a regulatory subunit of CK2, shortened the circadian periods, as measured by gene transcription (Sugano et al., 1999). Control of the timing of the circadian cycle in the cyanobacterium *Synechococcus* has been attributed to autophosphorylation of the clock protein Kai C (Iwasaki et al., 2002; Williams et al., 2002). The *Neurospora* clock protein FRQ is progressively phosphorylated over the course of the circadian cycle by homologs of casein kinase (Garceau et al., 1997; Liu et al., 2000), and absence of this kinase resulted in hypophosphorylation of FRQ and the loss of overt circadian rhythms (Yang et al., 2002).

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In *Drosophila*, the PER protein, which as a dimer with TIM enters the nucleus and represses its own transcription, was shown some years ago to undergo progressive phosphorylation (Lee et al., 1998). The clock protein DOUBLE-TIME (DBT), a (serine-threonine) casein kinase I homolog, was identified as responsible (Kloss et al., 1998), and mutations in DBT caused a lengthening of the behavioral period and greatly altered transcript and protein profiles of both *per* and *tim* (Suri et al., 2000). This action of DBT has been attributed to the regulation of PER feedback by delaying the time of PER translocation to the nucleus and altering the level of nuclear PER during the declining phase of the cycle (Bao et al., 2001). In mammals, PERIOD proteins undergo robust changes in phosphorylation, which affect the formation of multimeric complexes and their DNA-binding activity during negative transcriptional feedback (Akashi et al., 2002; Lee et al., 2001).

Compared to the numerous reports concerned with the role of protein kinases in the circadian mechanism, relatively few studies have been concerned with the enzymes that catalyze dephosphorylation. We have previously demonstrated that 3 phosphatase inhibitors specific for the PPP family, okadaic acid (OKA), calyculin A (CAL), and cantharidin (CAN), significantly affect the circadian system of the dinoflagellate *Lingulodinium polyedrum* (formerly *Gonyaulax polyedra*). These compounds each altered the period and/or phase of the bioluminescence rhythm and changed the circadian phase-shifting response to light pulses (Comolli et al., 1996). Based on these results, our model is that the inhibition of an *L. polyedrum* PP1- or PP2A-like enzyme prevents the dephosphorylation of a protein important in its circadian system, for instance, an analog of PER, FRQ, or CCA-1 (Dunlap, 1999; Sugano et al., 1999; Young and Kay, 2001). In support of this, the 3 inhibitors were shown to increase the phosphorylation state of *L. polyedrum* proteins in vivo and to block dephosphorylation of a PP1/PP2A-specific substrate in vitro at concentrations similar to those affecting the circadian system (Comolli et al., 1996).

In the present study, we identify PP1 in *L. polyedrum* cell-free extracts and isolate a cDNA with an open reading frame encoding a protein with over 70% amino acid identity to PP1c from other organisms. This gene is the 1st encoding a phosphatase to be isolated from a dinoflagellate; the properties of the isolated enzyme are consistent with it being the target of drugs that affect the circadian rhythm in vivo.

## MATERIALS AND METHOD

### Cell Culture and Preparation of Cell Extracts

*L. polyedrum* cells (strain 70) were grown in supplemented seawater medium (f/2) at 19 °C under a light-dark (LD) cycle of 12 h light/12 h darkness. For experiments involving constant conditions, cells were transferred to dim constant white light (30 μE $m^{-2}$ $sec^{-2}$) at the dark to light transition and collected 24 to 48 h later. Cultures (~$10^4$ cells/ml) were harvested by vacuum filtration onto Whatman #54 filter paper at the LD or circadian times indicated, then lysed in extraction buffer consisting of 50 mM Tris-Cl pH 7.5, 2 mM EDTA, 2 mM EGTA, 1 mM DTT, 100 mM NaCl, 500 μM PMSF, 10 μM E-64, and 100 μM benzamidine using a Mini-Beadbeater (Biospec Products, Bartlesville, OK). The resulting cell lysate was centrifuged for 20 min at 27,000 g at 4 °C, and the supernatant (referred to as cell extract) was used in phosphatase assays.

The same protocol was used for purifying the endogenous inhibitor, followed by heating for 10 min at 100 °C and centrifugation at 30,000 xg for 15 min. The supernatant was then used directly in assays or first dialyzed against several changes of 10 mM Tris-Cl pH 7.5, 2 mM β-mercaptoethanol, and 1 mM EDTA overnight at 4 °C using Spectra/Por #1 membrane (cutoff 6 to 8 kDa; Spectrum Medical Industries, Houston, TX).

Partial purification of phosphatase activity by ethanol treatment (Cohen et al., 1988) was performed as follows: 150 μl of 27,000 xg supernatant was mixed with 750 μl cold ethanol plus 1 mM PMSF and centrifuged at 4,200 xg for 5 min at 4 °C. The pellet was resuspended in 40 μl phosphatase assay buffer (see below) and centrifuged again, and the supernatant used for phosphatase assays or Western blotting.

### In Vitro Phosphatase Assays

Radiolabeled phosphorylase a phosphatase was prepared using phosphorylase kinase (Gibco-BRL protein phosphatase assay system). Assays measuring the release of $^{32}P$ from the substrate were performed as described using a 1:200 dilution of cell extract (Comolli et al., 1996). Assay times were typically 10 min, which is within the linear range of the dephosphorylation assay (data not shown). The concentration of protein in cell extracts was determined by Bradford assay or Pierce BCA protein assay.

Recombinant mammalian inhibitor-2 was obtained from New England Biolabs (Beverly, MA). Assays were regularly performed in triplicate and had a resulting standard deviation between trials of less than 10%.

### Cloning and Sequencing of *Lingulodinium polyedrum* PP1

Degenerate primers were designed from regions with 100% amino acid identity 1n a number of PP1s from other organisms (see Fig. 1) and adapted to *L. polyedrum* codon bias (Li et al., 1997). The sequences of the primers are 5′-TAYGGHTTYTAYGAYGAGTGYAAG-3′ and 5′-GGRTCVGACCASAGSAGRTCRC-3′ for the forward and reverse, respectively. These were used to amplify a 233 bp fragment of a putative PP1 from a λZAP cDNA library (provided by D. Morse). The fragment was generated from approximately $3 \times 10^6$ pfu of phage using 35 cycles of 95 °C, 1 min; 60 °C, 2 min; then 72 °C, 3 min. The product was then purified by agarose gel electrophoresis and ligated into pGEM-T vector (Promega) for sequencing. Comparison of the deduced amino acid sequence to known phosphatase sequences in the database, using the BLAST algorithm, was used to identify portions of a putative PP1 gene. A larger 714 bp fragment was amplified and cloned as described above using a nondegenerate primer (5′-TTATGATGAGTGTAAGCGGCG-3′) derived from the sequence of the 233 bp fragment and a vector primer. This fragment was labeled and used as a probe for screening $4 \times 10^6$ plaques from an *L. polyedrum* λZAP II cDNA library (Stratagene). Thirty-eight plaques potentially containing phosphatase cDNAs were identified. The longest of several isolated cDNAs (approximately 1.3 kb) corresponded to a putative PP1 cDNA clone, and the sequence of this cDNA was submitted to Genbank (accession number U52691). Comparison to *Brassica oleracea* PP1(X63558), human PP1α(X70848), and *Saccharomyces cerevisiae* GLC7 (M77175) (Fig. 2) was performed using ClustalW in the Lasergene software package (Dnastar, Inc., Madison, WI).

### Western Blotting

Cell extract or ethanol-precipitated fractions were heated in SDS-PAGE sample buffer, run on 10% SDS-polyacrylamide gels (35 μg protein per lane), and blotted onto nitrocellulose (Gelman Sciences, BioTrace NT, Ann Arbor, MI). The resulting blots were blocked with 5% nonfat dried milk, probed with rabbit polyclonal IgG raised against recombinant human PP1 (Santa Cruz Biotechnology FL-18) and goat anti-rabbit IgG secondary antibody labeled with alkaline phosphatase (BioRad). Antibody detection was performed by addition of NBT (p-nitro blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt).

## RESULTS

### Detection and Characterization of PP1c-Like Activity in Extracts

We had previously shown in *L. polyedrum* (Comolli et al., 1996) that okadaic acid, calyculin A, and cantharidin specifically inhibit PP1- and PP2A-type serine/threonine phosphatases at concentrations that were effective in altering circadian function in vivo (Ingebritsen and Cohen, 1983). Okadaic acid, the most selective of the 3 inhibitors, is roughly 100-fold more effective in inhibiting PP2A than PP1; the $IC_{50}$ for PP2A is approximately 0.2 nM, while that for PP1 is roughly 20 nM (Sheppeck et al., 1997). We took advantage of this property to determine the enzyme in *L. polyedrum* cell extracts that is responsible for dephosphorylating glycogen phosphorylase. While 3 nM okadaic acid had very little effect, 100 n3 resulted in approximately 89% inhibition (Fig. 3), while 1 μM blocked 98% of the activity, consistent with the predominance of PP1 and the lack of a PP2A-type activity in *L. polyedrum* extract. Also, this result suggests that in vivo effects of okadaic acid, which occurred in the micromolar range, were also due solely to the inhibition of PP1.

The presence of an inhibitor in extracts was suggested by the observation that the specific activity of the phosphatase in cell extracts increased dramatically upon dilution in assay buffer, with a 1:50 dilution having over 20-fold higher specific activity than the undiluted extract, and a 1:1000 dilution 40-fold higher (data not shown). In support of that suggestion, undiluted cell extract completely blocked phosphatase activity when added in a 1:1 ratio to 1:200 diluted extract, and was found to be heat-stable (100 °C, 5 min), like the PP1c-specific regulatory proteins, inhibitor-1, inhibitor-2 (I-2), or DARPP-32 (Cohen, 1989). The properties of this inhibitor closely resemble those of PP1c-specific regulatory proteins, which are 12 to 21 kDa in size and lack significant secondary structure and thus are heat-stable. This is the 1st evidence for

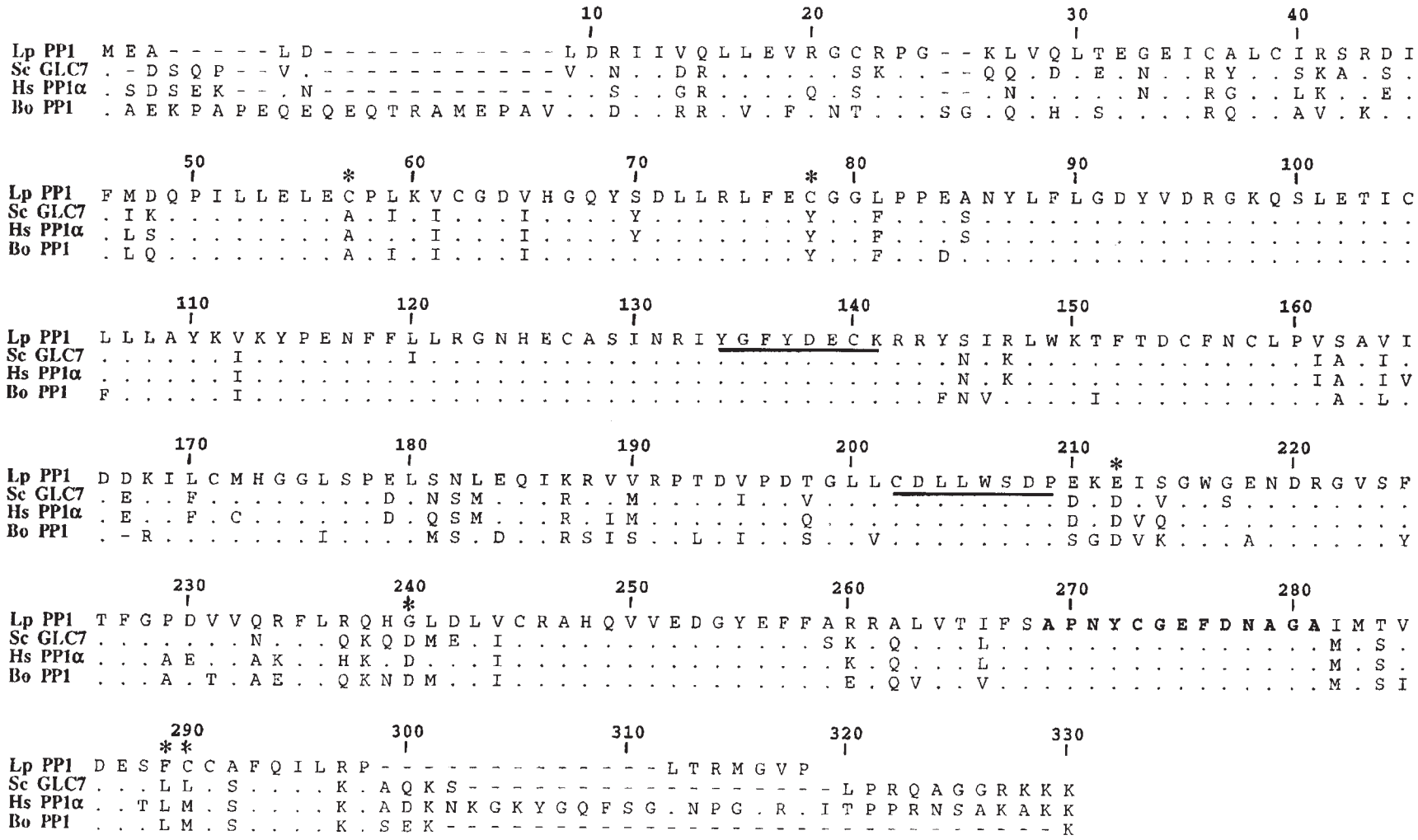


**Figure 1. Comparison of PP1c amino acid sequences. The deduced amino acid sequence of *L. polyedrum* PP1c was compared to that of *Saccharomyces cerevisiae* GLC7 (Genbank accession number M77175), human PP1a (X70848), and *Brassica oleracea* PP1 (X63558) using ClustalW. Dashes represent gaps in the protein sequence, and dots indicate where the amino acid is identical to that of *L. polyedrum* PP1. Also indicated are the conserved domains used in making degenerate primers (underlined). The β12-β13 loop is represented in bold, and residues that are conserved in all other PP1s but substituted in *L. polyedrum* PP1 are indicated by an asterisk (*).**

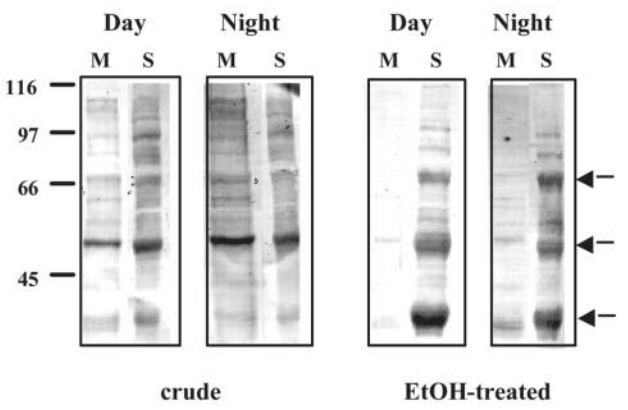


**Figure 2. Immunoblot of *L. polyedrum* cell extract and ethanol-treated fraction using anti-mammalian PP1 antisera. Cell extract was prepared from cells harvested in the light (day) or dark (night) of an LD 12:12 cycle. Each cell extract was then centrifuged to create membrane (M) and soluble (S) fractions. The migration of molecular weight standards (kDa) is indicated.**

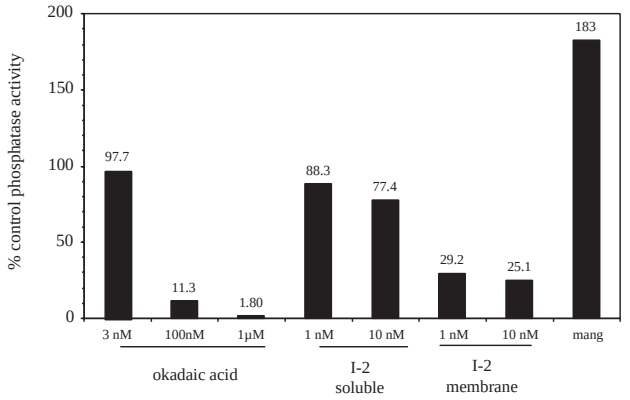


**Figure 3. Phosphorylase phosphatase activity of *L. polyedrum* extracts is indicative of PP1. Cell extracts diluted 1:200 were treated with okadaic acid (OKA), mammalian inhibitor-2 (I-2), and 5 mM $MnCl_2$ (mang) for 10 min prior to measuring the ability to dephosphorylate $^{32}$P-phosphorylase a. Activity is expressed as a percentage of a control assay receiving no treatment. In the case of I-2, both soluble and membrane-associated fractions were assayed.**

this class of regulatory proteins in dinoflagellates, and interestingly, there has been no evidence for the presence of these factors in other lower eukaryotes or plants (Shenolikar, 1994).

We also found that mammalian inhibitor I-2, which is available commercially, inhibits *L. polyedrum* phosphatase activity (Fig. 3). Cell fractionation studies had revealed that 47% of the total *L. polyedrum* phosphorylase phosphatase activity is soluble and the remainder membrane-associated. This is similar to the subcellular distribution of phosphorylase phosphatase activity in other organisms (e.g., MacKintosh and Cohen, 1989). Both fractions from *L. polyedrum* displayed similar sensitivities to okadaic acid, calyculin A, and cantharidin (data not shown), but different sensitivities to mammalian I-2. While 1 nM I-2 inhibited the soluble phosphatase activity by less than 13%, it blocked approximately 70% of the phosphatase activity associated with the membrane fraction (Fig. 3). Since I-2 is specific for PP1c, this result confirmed the existence of a PP1c homolog in *L. polyedrum*. The refractory nature of the soluble activity (inhibited only 23% by 10 nM I-2; Fig. 3) suggests that soluble phosphatase activity may be PP1c already associated with a regulatory protein that excludes I-2 binding. The membrane-associated phosphatase activity does not appear to be associated with a similar regulatory protein since 10 nM I-2 inhibits ~75% of this activity.

Although PP1 activity is not considered to be cation dependent, $Mn^{2+}$ has been shown to induce the conversion of mammalian PP1 complexed with I-2 from an inactive to an active form (Bollen and Stalmans, 1992). The phosphorylase phosphatase activity in *L. polyedrum* extract was similarly stimulated; 5 mM manganese chloride increased activity by almost 2-fold (Fig. 3), again indicating that the activity in *L. polyedrum* extract is PP1c-like. The treatment of cell extracts with denaturants such as ethanol has also been shown to result in the enrichment of the PP1c by removal of associated proteins (Cohen et al., 1988; Lee et al., 1999). Such a treatment of extracts resulted in a partial purification of PP1c and resulted in an over 25-fold increase in phosphatase specific activity (data not shown). This was yet another indication that the *L. polyedrum* phosphatase activity consists mainly of PP1.

### Isolation of an *L. polyedrum* PP1c cDNA

The detection of a serine/threonine phosphatase in *L. polyedrum* extracts with properties similar to PP1 led us to attempt to identify the gene for the catalytic subunit, PP1c. The primary structure of PP1c is highly conserved in all eukaryotic species, showing more than 55% amino acid sequence identity between such diverse phylogenetic groups as mammals, higher plants, algae, fungi, and protists (Lin et al., 1999). The central catalytic domains are the most conserved regions, while the more variable flanking regions are presumed to confer regulatory protein specificity. A

given organism may have up to 8 genes that encode PP1c isoforms, with higher plants at the high end of the scale and *Saccharomyces cerevisiae* at the other, with only a single gene (Lin et al., 1999). Many eukaryotes possess other serine/threonine phosphatases, such as PP2A and calcineurin/PP2B, which are related to PP1c and are thus considered part of the phosphoprotein phosphatase (PPP) family.

Degenerate primers corresponding to highly conserved segments of the central catalytic domain of PPP phosphatases (Fig. 1) were used to amplify putative phosphatase sequences from a cDNA library. The 233 bp fragment that was generated encoded a portion of a presumed phosphatase with significant similarity to PP1c, and a full-length cDNA was isolated using this fragment as a probe. The isolated cDNA contained an open reading frame (ORF) with a high GC content (64%) and a codon usage similar to other isolated *L. polyedrum* genes (Li et al., 1997). This ORF encoded a 302 amino acid protein having over 70% amino acid identity to PP1c from plants, yeast, and humans (Fig. 1). Comparing the putative PP1c *L. polyedrum* enzyme with those from other species, the central catalytic portion is most conserved, with the N- and C-termini being somewhat divergent, and it is somewhat smaller than most other PP1c isozymes, which range from 312 to 341 residues. The difference in length can be accounted for by a shorter C-terminus.

Although residues involved in catalysis, metal-binding, and phosphoserine/phosphothreonine binding are conserved, the putative *L. polyedrum* PP1c has some amino acid differences at positions that are invariant in PP1s from other species. The significance of most of these changes, specifically Ala57 to Cys, Tyr78 to Cys, Asp212 to Glu, Asp240 to Gly, Gln262 to Ala, and Lys298 to Arg (Fig. 1), is not evident. The Leu289 to Phe and Met290 to Cys substitutions may alter inhibitor binding since these amino acids are thought to be important in this interaction. However, the β12-β13 loop (residues 269-281), the region demonstrated to be involved in okadaic acid and I-2 inhibition of PP1c (Sheppeck et al., 1997; Watanabe et al., 2001), is conserved in *L. polyedrum* PP1c.

The 5′-UTR of the *L. polyedrum* putative mRNA has the unusual property of containing 2 short upstream open reading frames (uORFs). The 1st uORF begins 66 nucleotides upstream of the proposed PP1 start codon and contains 4 codons (including the stop codon). The 2nd uORF starts 41 nucleotides proximal to the putative PP1 start codon and contains 24 codons in an alternate reading frame and overlaps the PP1c coding

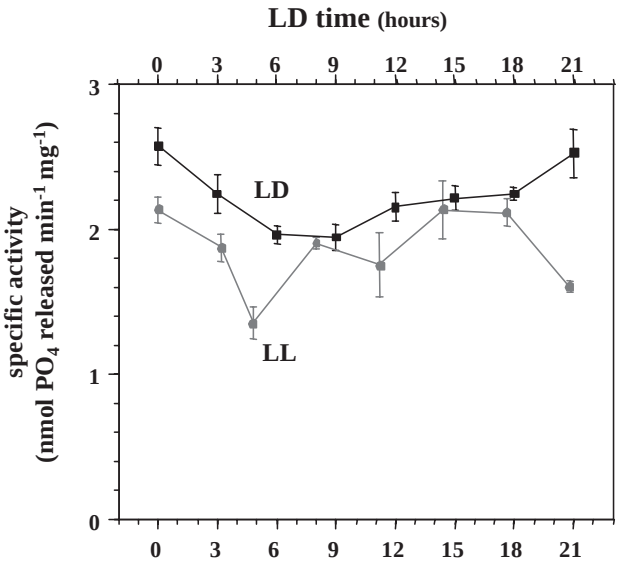


**Figure 4.** ***L. polyedrum*** **PP1 activity at different times. The phosphatase activities in extracts from cells maintained in a light-dark cycle (upper curve, LD) and in constant dim light (lower curve, LL) were measured at different times of day. Error bars show the standard deviation of triplicate assays. LD time zero represents dawn (lights on), and circadian time is calculated based on a 22.5-h free-running period.**

region by 27 nucleotides. Such uORFs are uncommon, and assuming that initiation must occur at the most proximal start codon on the mRNA, the uORFs may influence translational efficiency at a downstream start site (Kozak, 1987; Lohmer et al., 1993). Although a small uORF of 5 amino acids has been reported in the PP1 mRNA from *Brassica oleracea* (Rundle and Nasrallah, 1992), the translational control of PP1 production has not been studied in any organism. However, examination of PP1 messages present in GenBank revealed uORFs in the 5′-UTRs of maize, *Paramecium* and *Drosophila* mRNAs. This raises the possibility that the PP1 gene family has a high frequency of upstream start codons in the 5′-UTRs (Cavener and Cavener, 1993) and that this feature is used to regulate protein synthesis.

### Immunodetection of Phosphatases in *L. polyedrum*

To confirm that *L. polyedrum* expressed a PP1c with properties similar to those encoded by the isolated cDNA, cell extracts were tested for cross-reaction with polyclonal antisera capable of recognizing members of the PPP serine/threonine phosphatase family (Fig.

2). In the crude extract (left), the antisera strongly reacted with a 49 kDa protein, and weakly recognized several others; there appeared to be no significant difference in the amounts of the membrane-associated (M) and soluble-fraction (S) proteins. Ethanol treatment of either fraction (right) selectively enriched a 35 kDa protein, as well as a protein of approximately 69 kDa. The 49 kDa immunoreactive protein was less abundant after ethanol treatment, suggesting that it was not resistant to ethanol denaturation.

The ethanol-resistant proteins were by far more abundant in the soluble fraction, though a small amount was detectable in the membrane-associated fraction. The biochemical properties of the 35 kDa and 69 kDa immunoreactive proteins make them candidates for members of the *L. polyedrum* PPP gene family, possibly PP1c. The size of the smaller immunoreactive protein closely corresponded to the putative 34.3 kDa mass of the PP1c gene product. The identity of the higher molecular weight immunoreactive protein is not known, but due to the denaturing conditions present in the gel, it is unlikely that it is PP1c in association with a regulatory protein. It is more likely that this 69 kDa species is another PPP phosphatase, for example, the catalytic polypeptide of PP2B, which in other species ranges in mass from 53 to 62 kDa. Neither of these phosphatases is abundant in crude extract, but both are easily detectable after partial purification by ethanol treatment.

### Phosphatase Activity at Different Times of Day

PP1 activity measured in extracts from cells harvested at different times in either an LD cycle or in constant light showed no substantial circadian changes (Fig. 4). Also, the okadaic acid sensitivity of the phosphatase activity was similar when measured at different points in both LD and constant light (data not shown). Also, there were no significant differences in the amounts or the localization of the anti-phosphatase immunoreactive proteins in extracts prepared from cells during the day or night (Fig. 2). We also investigated if the inhibitory substance in *L. polyedrum* extract exhibited circadian changes in its activity; no evidence for this was found.

## DISCUSSION

Compared to the large number of eukaryotic enzymes that phosphorylate proteins, relatively few catalyze protein dephosphorylation. This is especially true for serine or threonine phosphorylation, where it has been estimated that there are roughly 20 times more kinases than phosphatases (Bollen, 2001; Cohen, 1989). As a consequence, many serine/threonine phosphatases are able to dephosphorylate a variety of different substrates (Aggen et al., 2000). For example, protein phosphatase 1, or PP1, is a major eukaryotic serine/threonine phosphatase that dephosphorylates phosphoproteins involved in a large number of cellular processes, including cell cycle regulation, protein synthesis, transcription, muscle contraction, T-cell activation, and carbohydrate metabolism (Bollen and Stalmans, 1992; Lee et al., 1999). Its catalytic subunit (PP1c) by itself has little apparent substrate specificity (Ingebritsen and Cohen, 1983) but can be modified to give specificity to a particular substrate or localize the enzyme to a subcellular region by interaction with regulatory proteins, more than 50 of which have been identified to date (Aggen et al., 2000; Bollen, 2001; Cohen, 2002). Other regulatory proteins, such as the heat-stable proteins DARPP-32, inhibitor-1, and inhibitor-2, modulate PP1c activity by blocking its activity toward all substrates (Bollen, 2001; Cohen, 2002; Sheppeck et al., 1997).

Although PP1c is present in most eukaryotes, the unusual properties of dinoflagellates, such as their atypical nuclear structure and their lack of signaling molecules such as a CDC2-like protein kinase (Salois and Morse, 1996), imply that intracellular regulatory factors such as serine/threonine phosphatases may be different. However, it has previously been demonstrated that the okadaic acid-producing dinoflagellate *Prorocentrum lima* possesses phosphatase activity with an inhibitor sensitivity and substrate specificity similar to that of rabbit PP1α (Boland et al., 1993). Our results definitively demonstrate that a marine dinoflagellate possesses a PP1c with an amino acid sequence and biochemical properties very similar to those of other eukaryotes. In addition, our data indicate the existence of at least one heat-stable regulatory protein with inhibitory capability similar to inhibitor-2. *L. polyedrum PP1* is the 1st phosphatase gene to be isolated from a dinoflagellate, and its conservation suggests that unicellular algae likely possess many intracellular regulatory networks similar to those of higher eukaryotes. *L. polyedrum* PP1 appeared to be expressed at a significant level since a protein of the same mass was detected by anti-PP1 immunoblots.

Our studies indicate that much if not all of the phosphorylase phosphatase activity present in *L.*

*polyedrum* extracts is due to PP1. Although PP1 and PP2A are homologous and share many biochemical properties, including the ability to dephosphorylate phosphorylase and the resistance to denaturation by ethanol, there are certain characteristics that are specific to PP1, many of which were displayed by the phosphatase in *L. polyedrum* extract. The activity was inhibited by okadaic acid in a manner consistent with the predominance of PP1 rather than PP2A activity (Comolli et al., 1996; Honkanen, 1993; Ishihara et al., 1989; Sheppeck et al., 1997). It was inhibited by endogenous heat-stable proteins such as I-2, characteristic of PP1c but not PP2A, and it occurs in both membrane and soluble fractions, as for PP1 from other species. Thus, it appears that *L. polyedrum* may be similar to *P. lima* in that they both lack PP2A-like activity. In support of this, our attempts to isolate a cDNA encoding PP2A from the *L. polyedrum* cDNA library were unsuccessful, as were attempts at chromatographic separation of PP2A-like activity from the phosphatase in *L. polyedrum* cell extracts (data not shown).

The core catalytic regions of all PP1c proteins are highly conserved, with almost one-third of the amino acids unchanged from those in diverse phylogenetic groups. This likely reflects structural and functional constraints in the evolution of the PP1c proteins. Phylogenetic analysis has revealed that although the *L. polyedrum* PP1c is closely related to that from the alga *Acetabularia cliftonii*, the 2 are on evolutionary branches separate from animal and plant PP1c enzymes (Lin et al., 1999). This likely does not mean that there is a difference in the catalytic properties of the enzyme but most likely reflects changes in the variable amino and carboxy terminal regions. These portions of the protein may have some influence on its substrate specificity or interaction with regulatory subunits. The isolation of a dinoflagellate ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO) that is nuclear-encoded and homologous to the proteobacterial form (form II) has suggested that dinoflagellate chloroplasts may have an evolutionary origin distinct from those of higher plants (Morse et al., 1995; Rowan et al., 1996). This may mean that the distant relationship between these photosynthetic organisms described in the PP1 phylogenetic tree may be an accurate representation of their evolutionary status.

The presence of an *L. polyedrum* cDNA encoding PP1c and the PP1-like activity in cell extracts strongly supports, but does not firmly establish, the conclusion that a PP1 is responsible, at least partially, for the effects of phosphatase inhibitors on the circadian system. PP1 knockout experiments would likely prove this beyond doubt, but such genetic manipulations have not been successful in dinoflagellates. Chemical genetics, therefore, is the best method presently available to confirm PP1's role in modulating the circadian clock. Our results, based on okadaic specificity, suggest that this drug can "knock out" PP1, but further work is needed to establish that the isolated clone actually encodes that activity detected in extracts, that the protein it encodes is responsible for the effects of inhibitors, and that this activity is only inhibited by relatively high concentrations of okadaic acid. If so, then this phosphatase may be considered to constitute a key component in the circadian mechanism of this organism.

In other circadian systems, the amount or activity of many factors that are critical to circadian function cycle (Allada et al., 2001; Dunlap, 1999; Harmer et al., 2001), but some do not (Young and Kay, 2001). For example, enzymes that affect the phosphorylation of core clock proteins in *Drosophila*, such as the protein kinases encoded by *dbt* (Kloss et al., 1998) and *shaggy* (Martinek et al., 2001), as well as mouse *casein kinase 1* (Ishida et al., 2001) are transcribed constitutively. Thus, although the in vitro phosphatase activity, the amount of immunoreactive protein, and the inhibitory capability in *L. polyedrum* extract were not found to fluctuate with a daily rhythm in either a light-dark cycle or constant light, this is not unexpected for a protein phosphatase associated with and playing an important role in the actual clock mechanism.

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