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CHARACTERIZATION OF A DINOFLAGELLATE CRYPTOCHROME BLUE-LIGHT RECEPTOR WITH A POSSIBLE ROLE IN CIRCADIAN CONTROL OF THE CELL CYCLE[1]

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Karenia brevis **(C. C. Davis) G. Hansen et Moestrup is a dinoflagellate responsible for red tides in the Gulf of Mexico. The signaling pathways regulating its cell cycle are of interest because they are the key to the formation of toxic blooms that cause mass marine animal die-offs and human illness.** ***Karenia brevis*** **displays phased cell division, in which cells enter S phase at precise times relative to the onset of light. Here, we demonstrate that a circadian rhythm underlies this behavior and that light quality affects the rate of cell-cycle progression: in blue light,** ***K. brevis*** **entered the S phase early relative to its behavior in white light of similar intensity, whereas in red light,** ***K. brevis*** **was not affected. A data base of 25,000** ***K. brevis*** **expressed sequence tags (ESTs) revealed several sequences with similarity to cryptochrome blue-light receptors, but none related to known red-light receptors. We characterized the** ***K. brevis*** **cryptochrome (Kb CRY) and modeled its three-dimensional protein structure. Phylogenetic analysis of the photolyase/CRY gene family showed that Kb CRY is a member of the cryptochrome DASH (CRY DASH) clade. Western blotting with an antibody designed to bind a conserved peptide within Kb CRY identified a single band at ~55 kDa. Immunolocalization showed that Kb CRY, like CRY DASH in** ***Arabidopsis*****, is localized to the chloroplast. This is the first blue-light receptor to be characterized in a dinoflagellate. As the Kb CRY appears to be the only blue-light receptor expressed, it is a likely candidate for circadian entrainment of the cell cycle.**

Key index words: **blue-light photoreceptor; cell cycle; circadian rhythm; cryptochrome; dinoflagellate;** ***K. brevis***

Abbreviations: **CPD, cyclobutane pyrimidine dimer; CRY, cryptochrome; EST, expressed sequence tag; GSP, gene-specific primer; Kb CRY,** ***Karenia brevis*** **cryptochrome; RACE, rapid amplification of cDNA ends**

Circadian rhythms are oscillations in biochemical, physiological, and behavioral functions of an organism with a periodicity of approximately 1 d (Sancar et al. 2000). The photoperiod provides the major input signal that serves to entrain circadian rhythms to a precise 24 h day (Roenneberg et al. 1998). Such rhythms are the key regulators of many facets of the physiology and behavior of dinoflagellates, including photosynthesis, vertical migration, cell division, and bioluminescence. However, the nature of the entraining light cues and light-dependent signaling pathways in dinoflagellates remains undefined. The current study was undertaken to gain insight into components of the light signaling pathways involved in entrainment of the circadian rhythm controlling the dinoflagellate cell cycle.

In all photosynthetic dinoflagellate species studied, photoperiodic entrainment of the cell cycle results in the synchronous progression of the population through G1, S, G2, and M phases. Populations dividing at $<1\ d^{-1}$ display ''phased'' cell

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division, with only a fraction of cells proceeding into the cell cycle on a given day. Those that do proceed into the cell cycle do so in a synchronous manner, while the others remain in G1. In the Florida red-tide dinoflagellate *Karenia brevis*, the dark–light transition serves to entrain the cell cycle by controlling the entry into S phase (Van Dolah and Leighfield 1999). In this study, we demonstrate that a circadian rhythm underlies the phased cell division observed in *K. brevis.* In order to begin characterizing the input signaling pathways responsible for entrainment of this circadian rhythm, we investigated the response of the cell cycle to red and blue light. Precedence for differential effects of red and blue light on the circadian cell-cycle rhythm is seen in the green alga *Chlamydomonas reinhardtii* P. A. Dang, in which the commitment point for cell division is shifted to a later time point and a larger cell size when grown in blue as compared with red light (Oldenhof et al. 2004). Both blue and red light have also been shown to alter the circadian rhythm of bioluminescence in dinoflagellates. In *Lingulodinium polyedrum* (F. Stein) J. D. Dodge, red and blue light both alter the circadian rhythm of bioluminescence, either similarly (inducing delay in early dark) or differentially, depending on the time of exposure (Roenneberg and Deng 1997). Further, the circadian period of *L. polyedrum* becomes shorter with increasing intensity of blue light but is lengthened in increasing red light (Roenneberg and Hastings 1988). These differential responses to red and blue light suggest the presence of distinct red- and blue-light receptors and accompanying signaling pathways in dinoflagellates.

Three families of photoreceptors have been identified in photosynthetic organisms: phytochromes, cryptochromes, and phototropins (Fankhauser and Staiger 2002). Phytochromes absorb in the red/far-red region of the visible spectrum (600–800 nm wavelength), while the cryptochromes and phototropins absorb in the UV-A/blue region (350–500 nm wavelength; Ahmad 1999). All three classes of photoreceptors have domain structures consisting of N-terminal chromophore-binding domains and C-terminal effector domains (Ahmad 1999).

Phototropins appear to be limited to higher plants (Christie and Briggs 2001). In contrast, both phytochromes and cryptochromes have been characterized in algae (Hegemann et al. 2001), although neither has been identified to date in dinoflagellates. We therefore screened a data base of *K. brevis* expressed sequences for cryptochrome- and phytochrome-like sequences. Among 25,000 sequences, we found seven expressed sequence tags (ESTs) with significant homology to cryptochromes, but found no sequences with similarity to phytochrome or other known red-light receptors. In this study, we characterize the cryptochrome blue-light receptor expressed in *K. brevis.*

Cryptochromes (CRYs), first characterized in *Arabidopsis thaliana* (Ahmad and Cashmore, 1993), have since been detected in numerous taxa, from bacteria to humans, making them the most widely distributed light sensors in nature (Hegemann et al. 2001). The CRYs evolved from photolyases that repair UV-induced DNA damage and belong to a gene family that includes (6-4) photolyases, cyclobutane pyrimidine dimer (CPD) photolyases, and plant and animal CRYs (Kanai et al. 1997). All CRYs bind light-capturing chromophores FAD and pterin (5,10 methenyltetrahydrofolate) in an N-terminal domain that has significant similarity to photolyases but lacks DNA repair activity. Unlike photolyases, plant and animal CRYs also possess a C-terminal extension thought to be involved in signaling (Ahmad 1999). A newly identified subgroup of the CRY gene family, CRY DASH (so-named for its presence in *Drosophila*, *Arabidopsis*, *Synechocystis*, and *Homo*), lacks the C-terminal extension found in the plant and animal CRYs; however, this subgroup does not exhibit photolyase activity.

Plant CRYs mediate photoentrainment as part of the input pathway for the circadian clock (Christie and Briggs 2001), whereas animal CRYs are essential components of the central oscillator in humans (Thompson and Sancar 2002), *Drosophila* (Emery et al. 1998), and mice (Vitaterna et al. 1999). The signaling pathways activated by CRYs are poorly defined, but in animals and plants, CRYs are localized to the nucleus (Lin 2000). In *Arabidopsis*, CRY DASH possesses a transit peptide and is localized to the chloroplast and mitochondria. Both *Arabidopsis* and *Synechocystis* CRY DASH proteins bind DNA and are proposed to act as transcriptional repressors (Brudler et al. 2003, Kleine et al. 2003), whereas among vertebrate CRY DASH proteins, no DNA-binding activity has been reported (Daiyasu et al. 2004). Thus, the function of CRY DASH subfamily members in vertebrates is not known to date (Daiyasu et al. 2004).

In this study, (i) the circadian rhythm of the cell cycle in *K. brevis* is described; (ii) evidence that the cell cycle is affected by red- and blue-light frequencies is presented; and (iii) the three-dimensional structure, phylogenetic relationship, and location of cellular expression of the putative blue-light receptor in *K. brevis* is characterized.

MATERIALS AND METHODS

Organism and standard culture conditions. *Karenia brevis* (Wilson isolate) was grown in filtered seawater (36‰) obtained from the seawater system at the Florida Institute of Technology field station, Vero Beach, Florida, enriched with f/2 media (Guillard 1973) and modified with the use of ferric sequestrene in place of EDTA · Na_2 and $FeCl_3 \cdot 6H_20$ and the addition of 0.01 μM selenous acid. Cultures were maintained in 25 × 150 mm flat-bottom glass culture tubes or 1 L glass bottles at 25°C ± 1°C with a 16:8 light:dark (L:D) cycle and cool-white light at 45–50 μmol photons · $m^{-2} \cdot s^{-1}$, determined using a LI-COR LI-250 Light Meter (LI-COR BioSciences, Lincoln, NE, USA). All experimental cultures were harvested during logarithmic phase growth.

Twenty-four-hour light experiment. Tube cultures of *K. brevis* (25 mL) were grown to mid-log phase. Individual cultures were harvested throughout the L:D photoperiod at circadian times (CT) 7, 10, 13, 17, and 21 h as a preexperimental control. On day 5, cultures were placed in continuous light, and individual cultures harvested throughout the diel cycle at the same time points for three consecutive days. Parallel cultures that remained on a 16:8 L:D photoperiod were also harvested at CT 7, 10, 13, 17, and 21 h for three consecutive days. Each sample was prepared for flow-cytometry analysis as described below.

Red and blue light experiment. Tube cultures of *K. brevis* were grown to mid-log phase and then placed in either red light, blue light, or white light, and maintained on a 16:8 L:D cycle. Roscolux® filters (Port Chester, NY, USA) of matched transmittance (approximately 20%) were used to obtain red (Roscolux #19) or blue (Roscolux #367) light. To achieve similar intensity for all treatments, the light intensity in the incubator was increased to 200 μmol photons $\cdot m^{-2} \cdot s^{-1}$, and neutral density filters were used for white-light controls, with all treatments thus receiving 35–40 $\mu E \cdot m^{-2} \cdot s^{-1}$. Triplicate tube cultures from each treatment were harvested on days 5 and 6 of experimental manipulation at CT 4, 7, 10, 13, 17, 19, 21, 23, and 1 h. Each sample was prepared for flow-cytometry analysis as described below.

Cell-cycle analysis. At each experimental time point, whole cultures were fixed with 2% gluteraldehyde and stored at 4°C for at least 12 h. Cells were then centrifuged 3 min at 700*g*. To extract cellular pigments, cells were resuspended in −20°C methanol for a minimum of 4 h. The cells were then centrifuged for 3 min at 700*g*, and their DNA stained by the addition of 10 $\mu g \cdot mL^{-1}$ propidium iodide (PI; Sigma, St. Louis, MO, USA) in PBS containing 10 $mg \cdot mL^{-1}$ RNase (Sigma) and 0.5% Tween 20. The DNA analysis was performed on an Epics MXL4 flow cytometer (Beckman Coulter, Miami, FL, USA) using a 5 W argon laser with a 488 nm excitation wavelength and 635 nm emission wavelength. Multicycle software (Phoenix Flow Systems, San Diego, CA, USA) was used to obtain the percentages of cells in each stage of the cell cycle, or cell-cycle distribution.

Transformation, plasmid purification, and sequencing. The cryptochrome ESTs expressed in pBluescript plasmids from the *K. brevis* cDNA library were transformed into One Shot® *E. coli* cells (Invitrogen, Carlsbad, CA, USA) for amplification. Positive clones were selected by blue/white screening and grown overnight in 3 mL LB ampicillin broth (LB Broth Base, Invitrogen; ampicillin, Sigma), and plasmid DNA was then extracted using Nucleospin Plasmid DNA Prep kit (Clontech, Mountain View, CA, USA). The DNA inserts were then sequenced from both ends of the EST inserts using T3 and T7 primers (Seqwright, Houston, TX, USA). Sequence traces were examined using Chromas software (Technelysium Pty. Ltd, Tewantin, Australia), and nucleotide sequences were edited using BioEdit (Tom Hall, Ibis Therapeutics, Carlsbad, CA, USA) and DNAStar (DNAStar Inc., Madison, WI, USA) software and then BLASTed (Altschul et al. 1997) against the National Center for Biotechnology Information (NCBI) nonredundant data base to identify similarity to known sequences.

5′ Rapid amplification of cDNA ends. *Karenia brevis* mRNA was extracted using the Dynabeads® mRNA Purification Kit (Dynal Biotech ASA, Oslo, Norway). Quantity was determined using a UV-vis spectrophotometer (Spectronic BioMate 3, Thermo Fisher Scientific, Waltham, MA, USA), and quality was determined using an Agilent® BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). The Clontech Marathon™ cDNA Amplification kit was used to perform 5′ rapid amplification of cDNA ends (RACE) to determine the amino-terminal nucleotides of the cryptochrome EST. Two gene-specific primers (GSPs) were designed for primary and secondary amplification of the transcript of interest, respectively: GSP1, 5′-ATA CCC TTC TCC CTT CAT GCC ATT GCG A-3′; and GSP2, 5′-CAC ATA AAT CGC GGA AGC ACT GGC ACA A-3′. The amplified DNA was then cloned into the Topo TA vector (Invitrogen) and transformed into OneShot® cells. Plasmid was extracted from 20 individual transformants and sequenced as described above using M13 forward and reverse primers. Sequence traces were examined using Chromas software, and nucleotide sequences were edited using DNAStar software. The 5′ RACE products were aligned with the contiguous sequence built from the ESTs from the cDNA library using BioEdit and DNAStar software.

Phylogenetic analysis. Alignment of the putative *K. brevis* cryptochrome (Kb CRY) amino acid sequence to a published alignment of the photolyase/blue-light receptor gene family (Daiyasu et al. 2004) was generated manually. The amino acid sequences, obtained from GenBank, included three class I CPD photolyases (from *Synechococcus*, *Halobacterium salinarum*, and *Thermus thremophilus*), 11 plant cryptochromes (including cryptochromes from *Arabidopsis* and *Chlamydomonas reinhardtii*), five animal cryptochromes (from *Drosophila melanogaster* and *Danio rerio*), and 23 known or putative CRY DASH proteins (including *Danio rubio, Synechocystis, Arabidopsis,* and *Neurospora crassa*), yielding a total of 42 aligned taxa and 191 characters. Thirty-two characters were constant, and 20 were parsimony-informative. We constructed phylogenies via distance and parsimony approaches using PAUP 4.0 b10 (Swofford 2002), using the class I CPD photolyases as an outgroup. A neighbor-joining (NJ) tree was constructed based on the number of amino acid differences, and 10,000 bootstrap replicates were used to assess statistical support for the nodes. We also implemented a Parsimony Ratchet analysis (Nixon 1999, Sikes and Lewis 2001). Briefly, we generated 10 independent searches of tree space, each consisting of 200 short heuristic search replicates using PAUP 4.0 b10 (Swofford 2002). A starting tree is generated with all characters, followed by branch swapping (tree-bisection-reconnection) using a randomly selected subset of characters (15% of total), and subsequently using all characters. The best tree is passed to the next replicate search, and the process is repeated 200 times. We calculated a majority-rule consensus tree across each of 10 independent searches to explore differences.

Protein model. A protein model of the amino acid sequence of *K. brevis* CRY DASH was generated using Swiss Model Deep View spdbv 3.7 Software (http://swissmodel.expasy.org/spdbv/). The amino acid sequence of *K. brevis* CRY DASH was BLASTed against the Swiss Model library of protein sequences derived from published X-ray structures. The *K. brevis* CRY DASH sequence had the highest homology to the *Synechocystis* CRY DASH protein (PDB code 1NP7; expectation value for the alignment: $1 \times e^{-84}$; 40% identical residues; alignment length 441 bp). Therefore, the known *Synechocystis* CRY DASH structure 1NP7A (Brudler et al. 2003) was used as a template to build this model. The two sequences were aligned, and the *K. brevis* CRY DASH sequence was threaded onto the structure of the *Synechocystis* CRY DASH. The threaded structure was submitted to the Swiss Model server for routine minimization, and the minimized result was returned for examination. The FAD cofactor is present in the 1NP7 template. Its relative position in the *K. brevis* CRY DASH structure is inferred. The amino acid sequence of *K. brevis* CRY DASH was also submitted to the SMART (Simple Modular Architecture Research Tool) server for domain assessment (http://smart.embl-heidelberg.de/).

Protein extraction and Western blotting. Cells were harvested from stationary phase cultures of *K. brevis* and centrifuged at 600*g* for 10 min. Supernatant was removed, and cells were centrifuged again to produce a pellet. Cells were then resuspended in Tri-Reagent® (Molecular Research Center Inc., Cincinnati, OH, USA), and protein was extracted according to the manufacturer's instructions. The Bradford Protein Assay (Bradford 1976) was used to quantify protein concentration

using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, USA), with a standard curve generated using a serial 1:2 dilution of BSA (200 $\mu g \cdot mL^{-1}$) for six standards. Linear regression of the absorbance of the standard curve (595 nm) was used to calculate the amount of protein in each sample.

For Western blotting, 15 μg of sample (protein extract or immunoprecipitated protein) were run on a gradient 8–12% SDS gel (Invitrogen) and then transferred to polyvinylidene fluoride (PVDF; Millipore, Billerica, MA, USA) at 4°C. The membrane was blocked in 5% milk in T-PBS (PBS with 0.5% Tween 20) for 1 h at room temperature. The membrane was then incubated in either primary antibody 1:1000 (affinity purified rabbit anti–Kb CRY, designed from conserved peptide [aa 77–101] in the translated sequence of Kb CRY), 1:1000 blocked primary antibody (preincubated with 100X peptide), or 1:1000 preimmune serum, overnight at 4°C. Following rinsing, horseradish peroxidase-linked donkey antirabbit Ig was then incubated with the PVDF membrane for 1 h at room temperature. After rinsing in T-PBS (3 times, 10 min) and PBS (1 time, 5 min), the Pierce ECL Western Blotting Detection Kit (Pierce, Rockford, IL, USA) was used to detect bands. The membranes were then exposed to film and developed for visualization.

Subcellular localization. An affinity-purified polyclonal peptide antibody was developed in rabbit against a synthesized peptide corresponding to the FAD-binding pocket of Kb CRY (Fig. 3, underlined sequence). When BLASTed on NCBI's GenBank, the only match against this peptide was CRY DASH. Immunolocalization was carried out using a modification of the protocol of Barbier et al. (2003). Cells were fixed in 4% paraformaldehyde in seawater for 10 min and centrifuged for 10 min at 250*g*. Cell pellets were then washed in PBS (150 mM NaCl, 10 mM Na_2HPO_4, 10 mM KH_2HPO_4, pH7.2) and resuspended in 2 mL, −20°C methanol and incubated at 4°C for 1 h. The pelleted cells were then incubated with normal goat serum at 4°C for 1 h and incubated overnight at 4°C with one of the following diluted in PBS with 0.1% Tween 20: anti–Kb CRY 1:50, anti–Kb CRY 1:50 blocked with 100X peptide (negative control), or monoclonal anti-α-tubulin 1:400 (positive control). Cells were also incubated in PBS with 0.1% Tween only as an additional negative control for nonspecific binding. The cells were then incubated with fluorescein isothiocyanate (FITC)–labeled goat antirabbit (FITC-labeled goat antimouse for tubulin) for 1 h at room temperature. After multiple washes in PBS, cells were counterstained with propidium iodide (PI; 100 $\mu g \cdot mL^{-1}$) for 5 min at room temperature to visualize nuclei. The cells were then mounted in Fluoromount-G (Southern Biotech, Birmingham, AL, USA). A Zeiss LSM 5 Pascal Confocal Microscope (Jena, Germany) was then used for imaging, with a lens magnification of ×630.

RESULTS

The Karenia brevis *cell cycle is under circadian control.* The cell-cycle distribution of *K. brevis* cultures was determined during log phase growth on a 16:8 L:D photoperiod and, after transfer to 24 h light, using flow cytometry (Fig. 1). In 16:8 L:D, cells were in G1 early in the light phase, S phase began between 10 and 13 h, and mitosis occurred late in the dark phase. When placed in continuous light, the cells continued to proceed through the cell cycle in a similarly phased manner as observed in 16:8 L:D. This indicates the presence of an underlying circadian rhythm. In fact, the percentage of cells proceeding through the cell cycle nearly doubled by day 3 in continuous light, while the temporal pattern of entry into various cell-cycle stages did not change. When *K. brevis* was placed in complete darkness, the cells did not enter the cell cycle at all, remaining in the G1 phase throughout the 24 h period (data not shown).

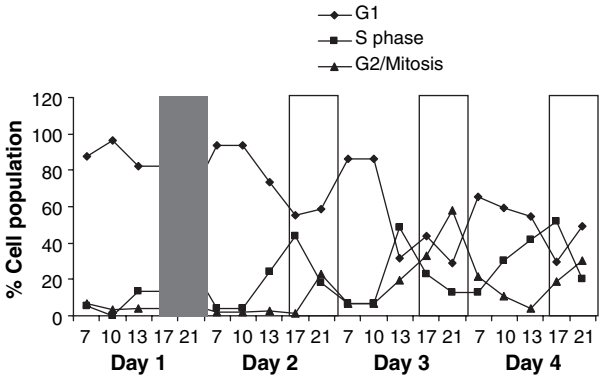

FIG. 1. Percentages of the cell population in each phase of the cell cycle in mid-log phase cultures on a 16:8 light:dark cycle (dark box indicates dark phase) and following their transfer to continuous light for 3 d (clear boxes indicate the absence of dark phase). G1: ♦; S phase: ■ G2/Mitosis: ▲.

Effects of light quality on cell-cycle progression. To assess the involvement of red- and blue-light signa-

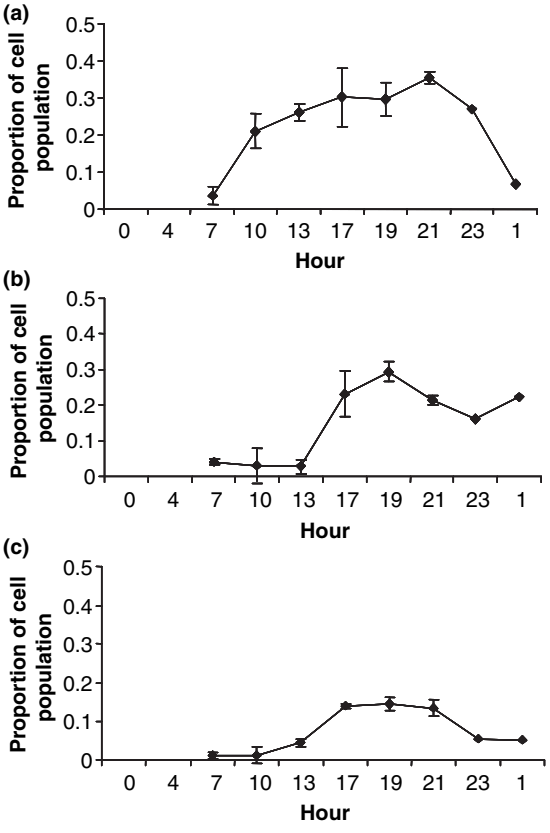

FIG. 2. Proportion of cell population in S phase when cells on a 16:8 light:dark cycle were transferred to blue light (a), red light (b), or white light (c) of similar intensity. Error bars are mean ± SEM of three independent experiments.

ling pathways in cell-cycle progression, cultures of *K. brevis* were grown to mid-log phase on a 16:8 L:D cycle and then transferred to red, blue, or white light of similar intensity. The percentage of S-phase cells was then determined by flow cytometry as a measure of cell-cycle entry. In the presence of blue light, the cells proceeded into S phase earlier, and a greater percentage of cells entered the cell cycle compared with the neutral density control (Fig. 2, a vs. c). This pattern was repeated in three independent experiments. Red light did not cause a consistent temporal change relative to white light, although more cells consistently entered S phase than under white light of similar intensity (Fig. 2b).

Characteristics of a blue-light receptor in Karenia brevis. Seven ESTs were identified in a *K. brevis* data base containing 25,000 5′ sequence reads with significant similarity (expectation value $< 1e^{-13}$) to blue-light receptors by BLASTx comparison with the GenBank nonredundant sequence data base. The *K. brevis* ESTs screened include 7001 ESTs described in Lidie et al. (2005), available at http://www.marinegenomics.org and GenBank dbEST data base. The remaining sequences are unpublished. All seven ESTs were found to represent overlapping regions of the same gene sequence. A contiguous nucleotide sequence generated from the overlapping ESTs has significant similarity to the *Synechocystis* CRY DASH with an e-value of $4e^{-84}$ (BLASTx; Fig. 3). A stop codon, TAA, a 50 bp 3′ untranslated region (UTR), and a poly-adenylated (poly-A) tail are identifiable in this sequence. A possible start codon, ATG, was identified 22 bp from the 5′ end. The translated amino acid sequence was used for subsequent alignments. The alignment of the Kb CRY with *Arabidopsis* CRY 1 (or HY4) showed 19% identity and 32% similarity. Notably, the C-terminal extension found in CRY 1 is not present in the *K. brevis* sequence. Alignment of the *K. brevis* sequence with CRY DASH proteins from *Arabidopsis* and *Synechocystis* shows higher identity and similarity of 32% and 46% versus 37% and 51%, respectively.

To determine if our EST contig represented the complete open reading frame (ORF), we carried out 5′ RACE using a GSP designed 216 bases from the 5′ end of the existing contig sequence (see Materials and Methods). The longest sequence obtained using this method added an additional 12 bp to the 5′ end

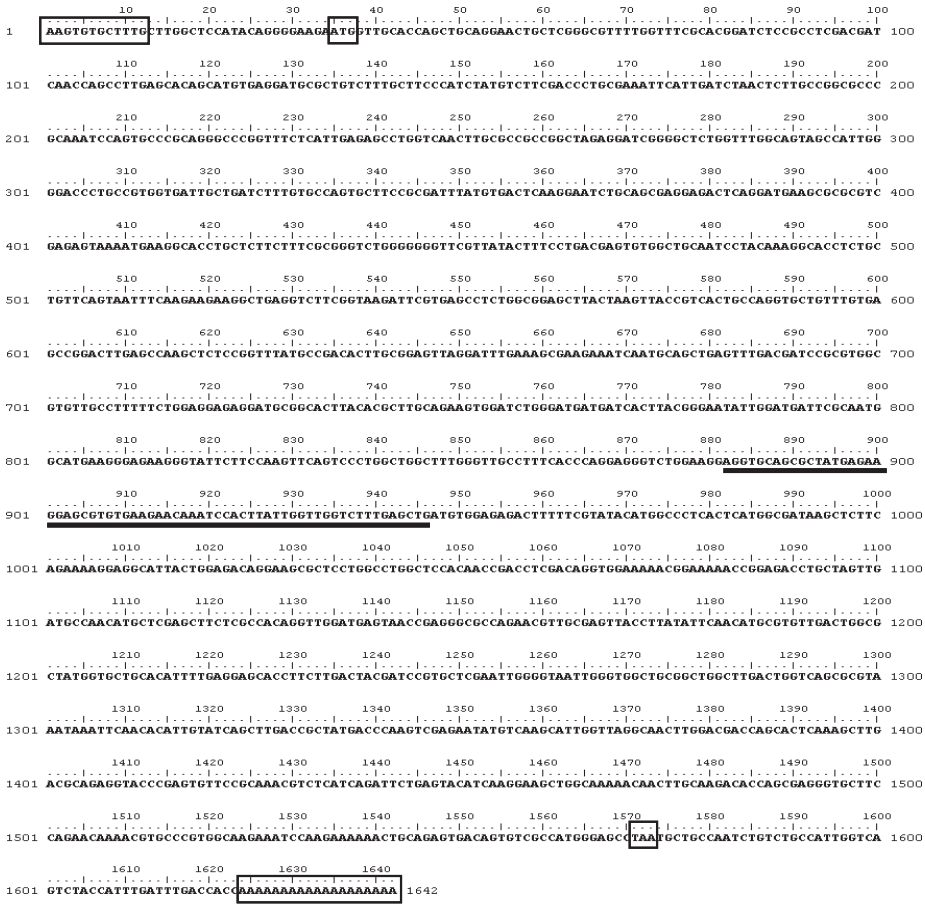

FIG. 3. The nucleotide sequence (1648 bp) of *Karenia brevis* cryptochrome (Kb CRY) contig generated from alignment of expressed sequence tags. The first boxed region denotes nucleotides identified by 5′ RACE. A start codon is shown in the second boxed region, with a 5′ UTR of 40 bp. A stop codon (TAA), 50 bp 3′ UTR, and poly-A tail are identifiable. Nucleotides underlined correspond to the peptide to which anti–Kb CRY was generated. RACE, rapid amplification of cDNA ends; UTR, untranslated region.

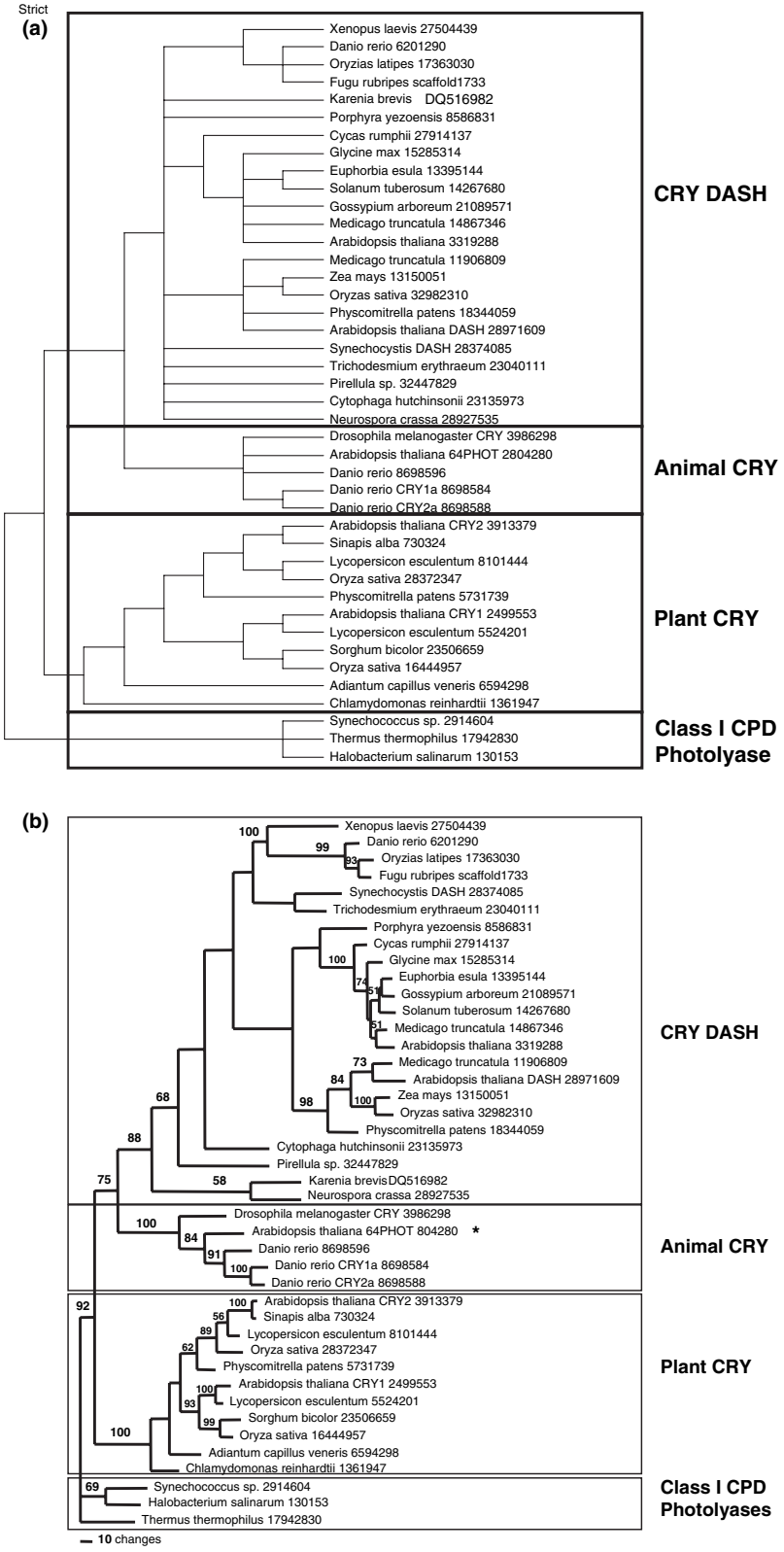

FIG. 4. (a) Parsimony-based and (b) neighbor-joining (NJ) phylogenies of the photolyase/cryptochrome gene family. The parsimony tree represents a strict (i.e., 100%) consensus of 200 replicate trees (see Materials and Methods). Numbers at the nodes of the NJ tree indicate bootstrap support from 10,000 replicate trees. GenBank Accession numbers are shown next to the species names.

of the contig (Fig. 3), resulting in a total sequence length of 1538 bp with a 3′ UTR of 50 bp (Fig. 3; GenBank Accession number DQ516982). Analysis of the 5′ end of this sequence by ChloroP did not provide evidence for a transit peptide. Therefore, it is unclear whether this sequence lacks the complete 5′ end of the ORF or whether this represents a 5′ UTR, with the ATG at position 40 representing the start codon for translation.

Phylogenetic analysis. Parsimony- and distance-based phylogenies of the members of the photolyase/blue-light receptor gene family indicate that the *K. brevis* sequence is embedded within a monophyletic and well-supported clade of CRY DASH alleles (Fig. 4; 100% consensus support using parsimony analysis and 88% bootstrap support using distance criterion). Within the CRY DASH clade, the plant sequences cluster separately from the animal and bacterial sequences. However, the phylogenetic relationships among CRY DASH proteins from other organisms are unclear. In the parsimony analysis (Fig. 4a), there is an unresolved polytomy among *Synechocystis, Trichodesmium, Karenia, Porphyra,* and *Pirellula.* In the distance-based analysis (Fig. 4b), the *K. brevis* allele clusters with the *Neurospora* CRY DASH allele, also a primitive eukaryote, but with low bootstrap support (i.e., 58%). The availability of CRY DASH sequences from additional protists may clarify this relationship.

Characterization of Karenia brevis *cryptochrome protein.* A protein model of *K. brevis* CRY DASH (Fig. 5) was generated by alignment of the *K. brevis* CRY DASH amino acid sequence with that of the *Synechocystis* CRY DASH structure derived from X-ray crystallography and present in the Swiss Model library. The photolyase domain (based on SMART analysis) is shown in yellow and is represented by amino acids 10–184. This photolyase-like region has an e-value of $6.90e^{-12}$. The FAD-binding domain (based on SMART analysis) is shown in green and is represented by amino acids 227–488, with an e-value of $3e^{-21}$.

We next developed an affinity-purified, polyclonal antibody to a 22-amino-acid peptide within a translated sequence of Kb CRY that has near identity with *Arabidopsis* CRY DASH and is located in its FAD-binding pocket (Fig. 3). BLAST analysis of this peptide against GenBank returned only CRY DASH sequences. Western blotting of a *K. brevis* protein extract revealed a major band of ~55 kDa (Fig. 6a). An average molecular weight of 55.04 kDa was predicted from the Kb CRY amino acid sequence (translated from the full ORF) using the ExPASy (Expert Protein Analysis System) Theoretical pI/Mw Tool (http://www.expasy.org/tools/pi_tool_html). When specific binding sites of anti–Kb CRY were blocked by incubation in the presence of peptide antigen, the major band at 55 kDa largely disappeared (Fig. 6c). Immunoprecipitation of Kb CRY with the antibody followed by Western blotting similarly produces the major band at ~55 kDa (Fig. 6b).

Localization of cryptochrome in Karenia brevis *cells.* Given the chloroplast localization of CRY DASH in *Arabidopsis*, the only other photosynthetic eukaryote in which CRY DASH has been studied, the absence of an apparent transit peptide prompted an immunocytochemical investigation to determine the

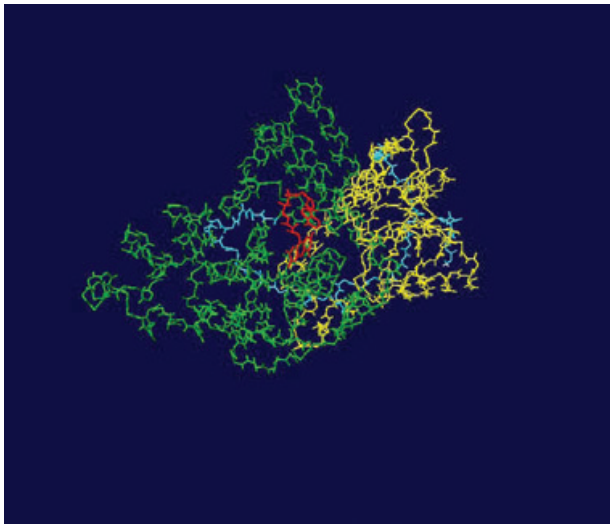

FIG. 5. Stick model showing regions of *Karenia brevis* CRY DASH that have significant homology to *Synechocystis* CRY DASH. The photolyase region is represented by yellow, and the FAD-binding site is represented by green. A bound FAD is shown in red. Blue regions are unique to *K. brevis.*

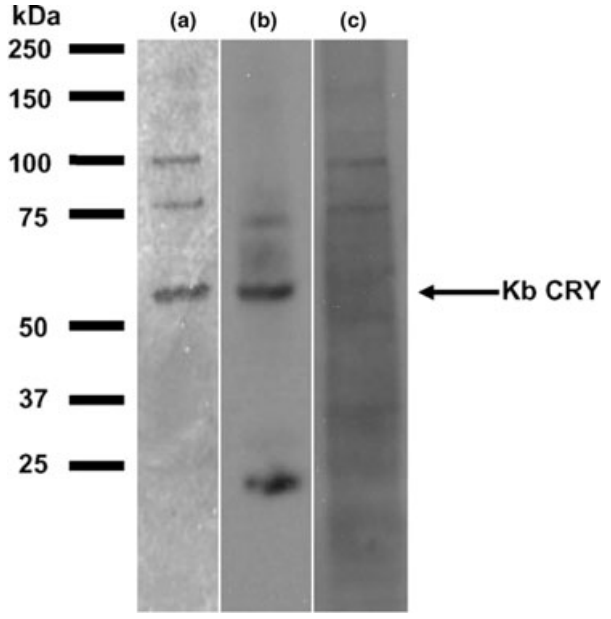

FIG. 6. Western blots with anti–*Karenia brevis* cryptochrome (Kb CRY). (a) Whole-cell protein extract probed with anti–Kb CRY, 1:1000. (b) Anti–Kb CRY immunoprecipitated protein, probed with anti–Kb CRY at 1:1000. (c) Whole-cell protein extract probed with anti–Kb CRY (1:1000) that was preincubated with 100x excess of CRY peptide to block specific binding, showing a loss of the major band at ~55 kDa.

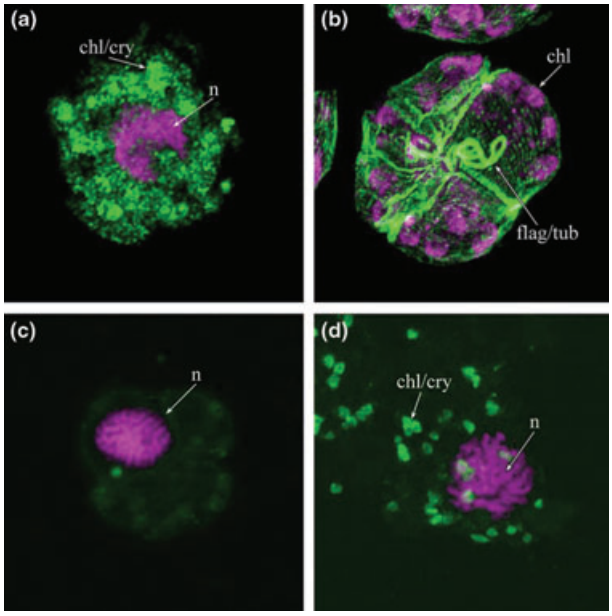

FIG. 7. Immunolocalization of *Karenia brevis* cryptochrome (Kb CRY). (a) Staining of whole cell with anti–Kb CRY (cry) shows chloroplast (chl) localization (green). The nucleus (n) stained with propidium iodide is magenta. (b) Staining of whole cell with anti-tubulin shows the flagella (flag/tub) and cortical cytoskeleton (green) and autofluorescence of the chloroplasts (magenta: chl). (c) Whole-cell staining with anti–Kb CRY blocked with 100x CRY peptide confirms specificity of binding in (a) and (d). (d) Ruptured cell that has released chloroplasts (chl), stained with anti–Kb CRY. chl, chloroplast; cry, anti-Kb CRY; flag, flagella; n, nucleus; tub, anti-tubulin.

cellular location of the mature protein. Immunolocalization carried out using anti–Kb CRY indicates that it is localized to the chloroplasts in this dinoflagellate (Fig. 7, a and d, green). The PI-stained nucleus is visible in magenta. To assist in structural interpretation of Figure 7a, tubulin staining (green) and chloroplast autofluorescence (magenta) are shown in Figure 7b. When the specific binding of anti–Kb CRY was blocked by incubation with an excess of CRY peptide, FITC staining was almost completely eliminated in the cell (Fig. 7c). The FITC staining was also absent in the cells stained with secondary antibody only (data not shown). Chloroplast localization is also convincingly seen in ruptured Kb CRY–stained cells releasing their chloroplasts (Fig. 7d, green).

DISCUSSION

The physiology of photosynthetic dinoflagellates is strongly influenced by the photoperiod, which serves to entrain underlying circadian rhythms, including those regulating photosynthesis, bioluminescence, vertical migration, and cell division. This study was undertaken to gain insight into the signaling mechanisms that entrain the cell cycle to the photoperiod. We first demonstrated that the cell cycle of *K. brevis* is under control of a circadian rhythm and is responsive to blue light. Through EST screening of a cDNA library, we next identified a blue-light receptor in *K. brevis* that is a member of the CRY DASH receptor family. Using an antibody developed from a conserved peptide in the translated Kb CRY sequence, we then characterized Kb CRY as a 55 kDa protein that is localized to the chloroplast. Whether the Kb CRY receptor mediates entrainment of the cell cycle remains to be demonstrated. However, these results identify the CRY receptor as a likely member of the blue-light signaling pathways involved.

Red and blue light have been shown to have varying effects on circadian rhythms in many organisms (Devlin and Kay 1999). In dinoflagellates, both phasic and constant light qualities contribute to the entrainment of circadian rhythms (Roenneberg 1995). Increasing intensities of constant blue light shorten the period of the bioluminescence rhythm in *L. polyedrum,* whereas increasing intensities of red lengthen it (Roenneberg and Hastings 1988). However, exposure to red plus blue light or to white light of increasing intensities does not alter period length; thus, the effects of red and blue light appear to be additive. Here we observed that *K. brevis* exposed to blue light entered the S phase earlier than cells exposed to white light of similar intensity, whereas red light had no consistent effect on the timing of S-phase entry. These results are consistent with the observations in *Lingulodinium* of differential responses to red and blue light. A number of studies on the *Lingulodinium* bioluminescence rhythm conclude that this system is primarily sensitive to blue light and responds to red light as the absence of blue light (Roenneberg 1995).

Although the bioluminescence rhythm appears to be responsive only to blue light, the circadian system in *Lingulodinium* is complex, consisting of two coupled oscillators controlling different rhythms that respond to light differentially. For example, Roenneberg and Morse (1993) demonstrated that the bioluminescence rhythm can be desynchronized from the aggregation rhythm, which appears to be sensitive to both red and blue light. Hastings and Sweeney (1960) determined that the action spectra associated with light-dependent phase shifts in *Lingulodinium* were similar to that of chl, with a large maximum in the blue range (475 nm) and a smaller one in red (650 nm). Later studies using the photosynthesis inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) confirmed that the inhibition of photosynthesis can alter the circadian phase (Johnson and Hastings 1989). Together, these results suggest that chl may play a role in the perception of red light by dinoflagellates. Furthermore, the effects of red light are not reversed by far-red light, indicating that the red-light effects are not mediated by phytochrome (Roenneberg and Hastings 1991).

Our *K. brevis* EST library screening further supports the conclusion that phytochrome is not

involved in dinoflagellate red-light reception. The 25,000 sequence *K. brevis* EST data base is to date the largest data base of expressed sequences available for a dinoflagellate. Among 25,000 sequences screened, no sequences were identified with similarity to a known red-light receptor. However, it remains possible that a red-light receptor is present in *K. brevis* but expressed at low level and thus missed in our screening, or that a red-light receptor exists among the approximately 70% of genes in the *K. brevis* library that remain unidentified.

Karenia brevis *expresses a cryptochrome DASH blue-light receptor.* Screening of the *K. brevis* EST data base identified a single CRY that was expressed at a relatively low level, with seven copies present among 25,000 ESTs. Although no other putative blue-light receptor was identified, it is possible that other photoreceptors are present but unknown. Phylogenetic analysis places the Kb CRY within the monophyletic CRY DASH clade of the cryptochrome/photolyase gene family, with substantial bootstrap support (88%). Results of our phylogenetic analysis of the CRY family are consistent with those of Daiyasu et al. (2004), wherein all CRY DASH proteins fall into one monophyletic group with the animal CRYs as the next closest neighbor (bootstrap probability of 75). Western blot analysis using an antibody to a conserved peptide in the Kb CRY translated sequence identified a protein of ~55 kDa, which is in good agreement with the expected size of this gene product and at the smaller end of the size range of cryptochrome family members (55–70 kDa), as would be expected since it lacks the 3′ extensions present in plant and animal CRYs (Sancar et al. 2000). Like *Arabidopsis* CRY DASH (Kleine et al. 2003), immunolocalization results demonstrate that this protein is localized to the chloroplasts of *K. brevis*. However, we did not identify a transit peptide in the nucleotide sequence obtained from library screening and 5′ RACE. This may indicate that 5′ RACE did not yield the full-length cDNA sequence, or it may simply be absent.

Signal sequences that are specific for membrane transport systems are not well conserved (Schatz and Dobberstein 1996), and it appears that many dinoflagellate plastid-targeted proteins are relatively divergent and possess odd characteristics—for example, a tandem fusion of translation elongation factor Ts (Waller et al. 2006). However, it is known that dinoflagellates possess a system that transports proteins to the plastid, similar to that of *Euglena,* whose plastids are also enclosed by three membranes (van Dooren et al. 2001). Nassoury et al. (2003) determined that in *Lingulodinium*, nuclear-encoded proteins transit through the Golgi apparatus from the endoplasmic reticulum (ER) on their way to the plastid, similar to *Euglena*. It has been suggested that *Euglena* chloroplast proteins are transported in vesicles through the ER and Golgi to the chloroplast as integral membrane proteins, with a large part of the protein located on the cytoplasmic side of the vesicle (van Dooren et al. 2001). Although it has not been directly shown, it has also been suggested that plastid-targeted proteins lacking a transit peptide may pass through this route (Nassoury et al. 2003). Information on *K. brevis* transit peptides from other gene sequences will provide additional insight into protein translocation systems in dinoflagellates.

The exact function of the CRY DASH subfamily is yet to be elucidated. The general domain structure of cryptochromes includes an amino-terminal chromophore-binding domain responsible for light perception and a carboxyl-terminal extension that is implicated in signaling (Ahmad et al. 1998). The CRY DASH proteins lack the C-terminal extensions present in plant and animal CRYs that are thought to confer their signaling activity. *Synechocystis* and *Arabidopsis* CRY DASH proteins bind DNA and are proposed to serve as transcriptional repressors (Brudler et al. 2003, Kleine et al. 2003). However, DNA-binding activity has not been demonstrated in animal CRY DASH proteins (Daiyasu et al. 2004). The protein model of Kb CRY using *Synechocystis* CRY DASH crystal structure as a template reveals a photolyase-like N-terminal region, present in all CRY family members, and an FAD chromophore-binding site. A surface protein model of the *K. brevis* CRY DASH (data not shown) also identifies a large area of this protein that is positively charged and thus may also bind DNA, as does CRY DASH in *Synechocystis*. As *Synechocystis* CRY DASH has been shown to inhibit transcription, it is possible that Kb CRY acts as a transcriptional repressor of chloroplast DNA, given its location in this organelle. However, similar models have not been published for animal CRYs that lack DNA-binding activity; therefore, the functional significance of this charge distribution is not yet clear.

Given that Kb CRY appears to be the only blue-light receptor present in *K. brevis,* and the blue-light effects observed on cell-cycle progression, Kb CRY is a likely candidate for a photoreceptor involved in the input signaling pathways of the circadian clock in this organism. Definitive analysis of its role will likely require gene-silencing strategies not yet developed in dinoflagellates.

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