Cryptochromes are proteins related to DNA photolyases and have been shown to function as blue-light photoreceptors and to play important roles in circadian rhythms in both plants and animals. The CPH1 gene from Chlamydomonas reinhardtii was originally predicted to encode a putative cryptochrome protein of 867 amino acids with a predicted molecular mass of 91 kD (Small et al., 1995). However, western blotting with antibodies specific to the CPH1 protein revealed the presence of two proteins that migrate at apparent molecular mass of approximately 126 and 143 kD. A reexamination of the assigned intron-exon boundaries has shown that the previously assigned intron 7 is in fact part of exon 7 which leads to a predicted protein of 1,007 amino acids corresponding to a size of 104.6 kD. The two forms of CPH1 that migrate slower on SDS-PAGE presumably result from unknown posttranslational modifications. In C. reinhardtii cells synchronized by light to dark cycles, the two slow migrating forms of CPH1 protein accumulate in the dark and disappear rapidly in the light. Both red and blue light are effective at inducing the degradation of the CPH1 proteins. Proteasomes are implicated because degradation is inhibited by MG132, a proteasome inhibitor. Studies with deletion mutants indicate that the C-terminal region is important for both the posttranslational modification and the protein’s stability under both light and dark conditions.

The discovery of the first blue light photoreceptor in Arabidopsis (Ahmad and Cashmore, 1993) has led to the subsequent discovery of similar proteins in several other species. Also known as cryptochromes, or CRYs, these include two each in mice (Kobayashi et al., 1998) and humans (Todo et al., 1996; van der Spek et al., 1996), one in Chlamydomonas reinhardtii (Small et al., 1995) and Drosophila melanogaster (Emery et al., 1998; Stanewsky et al., 1998), and five in fern (Adiantum capillus-veneris; Kanegae and Wada, 1998; Imaizumi et al., 2000). Cryptochromes show significant amino acid similarity to DNA photolyases in their N termini although cryptochromes lack photolyase activity. In addition, cryptochromes contain a C-terminal extension that appears to be unique for each cryptochrome in size and amino acid sequence (Lin and Shalitin, 2003). Recently, a second class of cryptochromes, DASH, has been identified, whose members share similarities to DNA photolyases in DNA binding and recognition (Brudler et al., 2003). These proteins have been hypothesized to have redox activity and act as transcriptional regulators.

Cryptochromes in Arabidopsis (AtCRY1 and AtCRY2) belong to the first class of cryptochromes, act as blue light photoreceptors, and have roles in the regulation of flowering time and plant development (Cashmore et al., 1999). The Arabidopsis CRY1 is known to regulate hypocotyl elongation and cotyledon growth in seedlings under high light intensities (Ahmad et al., 1995) while its close relative, AtCRY2, is involved in hypocotyl and cotyledon growth under conditions of low light (Lin et al., 1998). The AtCRY1 protein has been shown to interact with phytochrome A, a red light receptor (Ahmad et al., 1998a), while AtCRY2 and phytochrome B work antagonistically to regulate flowering time (Guo et al., 1998). Unlike AtCRY1, AtCRY2 is rapidly degraded in blue, green, or ultraviolet light (Lin et al., 1998). In addition, AtCRY1 and AtCRY2 have been shown to be involved in circadian signaling processes, although they are not required for circadian rhythmicity (Somers et al., 1998; Devlin and Kay, 2000).

Cryptochromes in D. melanogaster (dCRY1) and mice (mCRY1 and mCRY2) have been linked to circadian rhythm, although they appear to have vastly different functions within these organisms. While dCRY1 apparently acts as the photoreceptor for the circadian clock (Emery et al., 1998), mCRY1 and mCRY2 may be part of the clock mechanism itself and there is much debate regarding their function as photoreceptors (Kume et al., 1999; van der Horst et al., 1999; Van Gelder et al., 2002, 2003). In light to dark synchronized D. melanogaster, the degradation of the dCRY protein is...
also dependent on light, with a half-life of approximately 1 h in an overexpressing strain (Emery et al., 1998). The degradation is less rapid in wild-type flies; however, in neither strain is the protein degraded when flies are left in darkness at the end of the dark phase. This indicates that the protein degradation is initiated by exposure to light and is not due to regulation by the circadian clock.

During experiments designed to characterize the C. reinhardtii cryptochrome, Chlamydomonas photolyase homolog 1 (CPH1), we have found that two forms of CPH1 protein exist and each undergoes light-induced degradation. This relatively rapid degradation is dependent on light rather than the circadian clock and is inhibited by a proteasome inhibitor. During the initial phase of degradation, there is a decrease in mobility in SDS-PAGE that does not occur in the presence of a kinase inhibitor, suggesting that phosphorylation is involved. Interestingly, western blotting has revealed that both forms migrate at a higher molecular mass than predicted, possibly due to post-translational modifications.

RESULTS

The CPH1 Proteins Migrate as Two Bands at High Apparent Molecular Masses during SDS-PAGE

Using polyclonal antibodies directed against the CPH1 protein, western blots of total cell extracts from synchronized C. reinhardtii cells reveal two bands with apparent molecular mass of approximately 126 and 143 kD. These bands accumulate in the dark and disappear during the light phase (Fig. 1A). These molecular masses are much larger than the originally predicted 91 kD for the CPH1 protein. Preimmune sera antibodies do not react with these proteins (data not shown). The specificity of the antibody was confirmed by overexpressing the CPH1 gene in C. reinhardtii. For overexpression, the entire CPH1 gene was engineered to contain the FLAG epitope at the N terminus with expression driven by a heat shock promoter (pHSP-CPHg). Transformation of C. reinhardtii with pHSP-CPHg led to the successful overexpression of CPH1 in two transformants, HS88g and HS12g (Fig. 1B). In both strains the same 126- and 143-kD proteins are inducible by heat and are apparent using either antibodies against CPH1 (Fig. 1C) or antibodies against FLAG (Fig. 1D). For all other experiments where overexpression of the gene was employed, strain HS88g was used and for clarity is referred to as OxG (overexpression of gene).

A Reevaluation of the CPH1 Exon-Intron Assignments

Based largely on the results of reverse transcription (RT)-PCR, the CPH1 gene was originally predicted to contain eight exons and seven introns (Small et al., 1995). A reexamination of the assigned intron-exon boundaries revealed that deletions occurred during the reverse transcriptase step of the RT-PCR procedure (supplemental data available at www.plantphysiol.org). Northern blots together with ribonuclease protection assays have shown that the previously assigned intron 7 is in fact part of exon 7 which leads to a predicted protein of 1,007 amino acids corresponding to a size of 104.6 kD (supplemental data). Although 104.6 kD is much closer to the apparent molecular mass of the CPH1 proteins, there is still a discrepancy between the predicted and apparent molecular mass. Overexpression of the CPH1 cDNA as a polyhistidine tagged product in Escherichia coli results in a protein that migrates in SDS-PAGE at the predicted size (110 kD), as determined by Coomassie Brilliant Blue staining (Fig. 2A). When compared on western blots, overexpressed C. reinhardtii CPH1 migrates more
slowly than the protein expressed in *E. coli*, confirming the difference in size (Fig. 2B). We hypothesize that the species migrating according to apparent sizes of 126 and 143 kD are due to unknown posttranslational modifications.

The Levels of the CPH1 Proteins Are Decreased in a Light-Dependent Manner

Western blots of extracts from *C. reinhardtii* cells synchronized by a light to dark regimen show that the two high molecular mass forms of CPH1 accumulate in the dark and disappear quickly in the light (Fig. 1). A comparison of synchronized cells that were kept in the dark at the end of the dark phase or placed in the light demonstrates that it is light, rather than a circadian signal, that induces the down-regulation of the CPH1 proteins (Fig. 3A). If a circadian signal were responsible for the down-regulation of CPH1, similar levels would be expected in cells kept in the dark during the normal light phase as in cells exposed to the light. However, there is no significant decrease in CPH1 protein levels in cells remaining in the dark for 30 min (Fig. 3A) and up to 6 h after artificial dawn (data not shown). At a fluence rate of 23 μmol m⁻² s⁻¹, the steady state level of CPH1 decreased by one-half after approximately 15 to 20 min in white light as determined by western blotting and subsequent quantitation (Fig. 3A). Since the protein levels under these conditions represent a balance between rates of synthesis and degradation, a more accurate measure of the rate of degradation can be obtained if synthesis is inhibited with cycloheximide. When the rate of degradation was measured in the presence of cycloheximide, a value of 15 to 20 min was obtained (Fig. 3B).

It is possible that light causes a rapid degradation of CPH1 mRNA with a subsequent decrease in the protein due to constitutively active proteolysis. This is not the case as shown with northern-blot analysis of CPH1 mRNA levels taken before and after the beginning of the light phase (Fig. 3C). Quantification of three autoradiographs from separate northern-blot experiments shows a 10% (±3%) decrease in mRNA after 30 min in the light (data not shown). This decrease cannot account for the almost total disappearance of CPH1 proteins within that same time.

A second possibility is that light inhibits translation of the CPH1 mRNA with the reduction of protein due to the constitutively active proteolysis. If this were the case, then inhibiting protein synthesis should result in a similar rate of disappearance of the proteins. Addition of the protein synthesis inhibitor cycloheximide does not result in a decrease in the amount of the CPH1 proteins when the cells are kept for 30 min in the dark (Fig. 3D, 30 min dark, +CH). The results shown in Figure 3D, as well as Figure 3B, also show that protein synthesis is not required for the light-induced disappearance of the protein since addition of cycloheximide to the media just prior to the beginning of the light cycle has no effect (30 min light, +CH). These data suggest that light stimulated protein degradation, rather than a decrease in protein synthesis, accounts for the disappearance of the two forms of the CPH1 protein. Our data does not rule out the possibility that the CPH1 protein levels could also be influenced by the clock over the 24-h time scale.

Degradation of the CPH1 Proteins Is Induced by Blue or Red Light

Unlike Arabidopsis CRY2 (Lin et al., 1998) whose degradation is not triggered by red light, red light is just as effective as blue light in inducing the degradation of the CPH1 proteins (Fig. 4A). Exposure of cells to a low intensity (5 μmol m⁻² s⁻¹) of either blue or red light results in about the same level of degradation after 60 min. Either wavelength is effective at various intensities although at higher intensities the proteins disappear more rapidly (compare Fig. 4A, 5 μmol m⁻² s⁻¹ with Fig. 4B, 20 μmol m⁻² s⁻¹). In addition, constant light stimulation is not necessary for degradation. At 20 μmol m⁻² s⁻¹ a 5 min pulse of red light is sufficient to induce the breakdown of the CPH1 proteins (Fig. 4B). Similar results were obtained using blue light (data not shown).

The effects of red light do not appear to be the result of a signal from the photosynthetic machinery. The addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a known inhibitor of photosynthetic electron transport, has no effect on red-light-induced degradation (Fig. 4E). Photosynthetic electron transport produces oxygen as a byproduct; therefore, addition of DCMU to cells should also inhibit the production of oxygen. To ascertain that DCMU was effectively inhibiting photosynthesis, oxygen evolution was mea-
sured as described in “Materials and Methods” (data not shown).

CPH1 Proteins Are Degraded by the Proteasome

The degradation of the CPH1 proteins is inhibited by the addition of a proteasome inhibitor, MG-132, prior to the beginning of the light cycle (Fig. 4D). It is notable that there is an apparent increase in molecular mass during the initial phase of degradation and this form does not disappear in the presence of MG-132. This phenomenon is reproducible under white, blue, or red light and at various intensities of light. The addition of a general kinase inhibitor, 6-dimethylamino purine (6DMAP, 3 mM), prior to the beginning of the light cycle inhibits the shift and also results in the inhibition of degradation, suggesting a requirement of phosphorylation prior to degradation (Fig. 4E).

CPH1 Constructs with C-Terminal Tail Deletions Have Varying Degrees of Stability

The Arabidopsis CRY1 and CRY2 proteins differ the most in their C-terminal extensions. Since AtCRY1 is stable and AtCRY2 is labile in the light, a simple model would be that the C-terminal region of AtCRY2 has the required information to determine light lability. However, studies of chimeric Arabidopsis CRY1/CRY2 proteins have not resulted in the identification of a specific, discrete region of AtCRY2 that could account for the light-induced degradation of the protein (Ahmad et al., 1998b). Two deletion constructs of CPH1 were used to examine the role of the C-terminal region in the light-induced degradation of the CPH1 proteins in C. reinhardtii. One construct, pHSP-CPH1-D1, is predicted to encode a 91-kD protein of 867 amino acids of which the first 837 are identical to the CPH1 sequence. The second construct, pHSP-CPH1-D2, is predicted to encode a 78-kD protein of 729 amino acids, with the first 722 amino acids identical to CPH1. For convenience, cells overexpressing pHSP-CPH1-D1 or pHSP-CPH1-D2 will be referred to as OxΔ1 and OxΔ2, respectively. Please refer to the supplemental data online for maps and further descriptions of the constructs. The amino acid sequence of CPH1 and the C-terminal region of the two deletion mutants are shown in Figure 1 of the supplemental data.

When transformed separately into C. reinhardtii and overexpressed using heat shock, CPHΔ1 and CPHΔ2 migrate at the predicted molecular mass of 91 and 78 kD, respectively, as detected by antibodies against CPH1 (Fig. 5, A and B, arrows). This indicates that the presumed posttranslational modifications of the intact CPH1 protein require the region deleted in CPHΔ1 and CPHΔ2.
induced degradation. There is only 30% CPH1Δ2 present after 4 h in the light while almost no degradation has occurred when cells were kept for 4 h in the dark after heat shock. The rate of light-induced degradation of CPH1Δ2 is much slower than the endogenous CPH1 proteins, with a half-life of approximately 2 h for CPH1Δ2 versus 15 to 20 min for endogenous CPH1 under 23 μmol m⁻² s⁻¹ of white light. The features of the light-induced degradation of CPH1Δ2 are similar to the endogenous CPH1 proteins in that both blue and red light are effective and degradation is inhibited by proteasome inhibitors (data not shown).

Since light-induced phosphorylation may play a role in the degradation signal of CPH1 and CPH1Δ2, we analyzed the amino acid sequences for potential phosphorylation sites. CPH1 contains several potential casein kinase (CK2) phosphorylation sites including five in the extreme C-terminal region (Fig. 1, supplemental data). CPH1Δ2, which shows light-induced degradation, contains a single CK2 site at Ser-725 in its C-terminal region. Conversely, CPH1Δ1, which does not show light-induced degradation, is lacking any potential CK2 phosphorylation sites at its extreme C terminus. To test the possible role of this site in CPH1Δ2, we performed site specific mutagenesis converting Ser-725 to an Ala. However, this mutation did not eliminate the light-induced degradation of the protein (Fig. 6D).

DISCUSSION

Size Discrepancy

Western blots indicate two forms of the CPH1 protein migrating at apparent sizes of about 126 and
mutagenesis on CPH1

D refers to the amount of protein relative to zero time. Site-specific blotting were performed as described using antibodies against CPH1. Alternatively, synchronized OxΔ1 (B), OxΔ2 (C), or OxΔ2-S725A (D) cells were heat shocked for 30 min near the end of the dark cycle. Cycloheximide was added and cells were split into two flasks, one of which was covered in foil. Cells then remained in the dark for an additional 30 min until the lights normally turned on. Samples were taken at the indicated times after the lights turned on. Cells were quick frozen until sample buffer was added and SDS-PAGE and western blotting were performed as described using antibodies against CPH1. An average of two separate experiments is shown. “Percent” on y axis refers to the amount of protein relative to zero time. Site-specific mutagenesis on CPH1Δ2 converted Ser-725 to an Ala (S725A). Cells expressing this construct were labeled OxΔ2-S725A.

143 kD. At this time we have no evidence of differential splicing of the mRNA to account for two forms. Northern blots have consistently yielded a single band when hybridized with specific CPH1 probes (Small et al., 1995; Supplemental Fig. 4). The CPH1 mRNA was previously predicted to encode a protein with a molecular mass of 91 kD (Small et al., 1995). We have now shown that to be incorrect due to an artifact during the RT-PCR reaction and the corrected CPH1 mRNA is predicted to encode a protein with 1,007 amino acids and a molecular mass of 104,667 D (105 kD). Although 105 kD is much closer to the apparent molecular masses of the CPH1 proteins, there is still a discrepancy between the predicted and apparent masses. Overexpression of the CPH1 cDNA in E. coli results in a product of predicted size (Fig. 2). Hence there is nothing about the CPH1 sequence per se that dictates a retarded migration in SDS-PAGE.

There are several possible explanations for the size discrepancy, including posttranslational modifications such as glycosylation or phosphorylation. Whatever the posttranslational modification, it must occur very rapidly because we have never observed an increased accumulation of a 105-kD protein during the induction of the CPH1 gene by heat shock. Experiments have indicated that the presence of tunicamycin does not prevent the accumulation of CPH1 proteins after 8 h in the dark, suggesting that N-linked glycosylation is not involved (data not shown).

Light-Induced Degradation

Regardless of the reason for the high molecular masses, it is clear that both CPH1 proteins undergo rapid, light-induced degradation. During this process, there is a small upward shift in apparent molecular weight that is inhibited by the addition of a general kinase inhibitor. The proteins are not degraded under these conditions. We postulate that phosphorylation is required for light-induced degradation. Recently, the Arabidopsis CRY2 protein has been shown to undergo blue-light-dependent phosphorylation which results in a retardation of mobility in SDS-PAGE (Shalitin et al., 2002). The model proposed for the Arabidopsis CRY2 protein is that blue light absorption by the CRY2 protein results in a conformational change that exposes the protein to phosphorylation and leads to degradation. Such a scenario is conceivable for the CPH1 proteins. Interestingly, the CPH1 proteins are the first cryptochromes whose degradation is known to be induced under red light. In fact, there is no known cryptochrome, or related DNA photolyase protein, that absorbs light in the red region (>595 nm). Thus, to support the hypothesis that light induces a conformation change in the CPH1 protein it is necessary to postulate that C. reinhardtii CPH1 has a unique second chromophore that absorbs light in the red region of the spectrum. Absorption of either red or blue light would cause a conformational change in CPH1 proteins, leading to phosphorylation and degradation. Testing this hypothesis would require purifying the protein from C. reinhardtii cells under conditions that would retain the chromophores. An alternative hypothesis is that red and/or blue light activates a protein kinase that subsequently phosphorylates the CPH1 proteins. The identity of the red absorbing photoreceptor is an interesting question, especially considering that there is no known phytochrome in C. reinhardtii.

CPH1 Function

At this time, the function of the CPH1 proteins is unknown. Because the CPH1 proteins share much in common with other cryptochromes, they are presumed to have similar functions. For example, Arabidopsis CRY2 protein undergoes phosphorylation and degradation following absorption of blue light in a manner comparable to the CPH1 proteins. Overexpressing the C-terminal tail from either Arabidopsis CRY1 or CRY2 with a β-glucuronidase reporter results in a constitutive light response in dark-grown seedlings. It is thought that the AICRY1/2 C termini are held in an inactive state in the dark and are activated by
a light-driven redox reaction in the light (Yang et al., 2000). Upon activation, it is presumed that the cryptochromes bind to their respective, putative signaling partners. It is unclear if the mechanism is similar between the two Arabidopsis CRYs, especially considering that AtCRY2 is light-labile. Because CPH1 shares sequence homology with aCRY1/2 in the chromophore binding region, it is possible that CPH1 will undergo a similar redox-controlled activation.

It is also feasible that the CPH1 proteins function as transcriptional regulators, as do several other cryptochromes. There are numerous responses to blue light in C. reinhardtii, many of which involve gene transcription. Expression of a gene encoding a chlorophyll a/b binding protein (CABII-1) is inducible by blue light; levels peak early in the light period and diminish early in the dark period (Jacobshagen et al., 1996). Transcription of glutamate 1-semialdehyde aminotransferase, a protein involved in chlorophyll biosynthesis, is also induced by blue light (Matters and Beale, 1994). The action spectrum for resetting the circadian phototaxis rhythm in C. reinhardtii shows two peaks: one in the blue region (520 nm) and one in the red region (660 nm; Goto and Johnson, 1991; Kondo et al., 1991). As with the case of CPH1 proteins, inhibition of photosynthesis does not affect this response. C. reinhardtii has no known phytochrome and relatively few characterized responses to red light. In no case is the identity of the red light receptor known. As previously mentioned, cryptochromes have been shown to be involved in circadian rhythm in both plants and animals. It is intriguing to speculate that CPH1 plays a role in one or several of these processes in C. reinhardtii.

**CONCLUSION**

The CPH1 gene from C. reinhardtii encodes two proteins that migrate at apparent molecular masses of approximately 126 and 143 kD. Slow motion on SDS-PAGE is presumed to be due to unknown post-translational modifications. Both CPH1 proteins undergo light-induced degradation under white, red, and blue light, a process that is inhibited by a proteasome inhibitor. It is apparent that the C-terminal region is important for posttranslational modification and stability. Sequence homology and their lability in the light similar to AtCRY2 and D. melanogaster CRY suggest that the CPH1 proteins function as cryptochromes in C. reinhardtii.

**MATERIALS AND METHODS**

**Molecular Cloning**

Overexpression of CPH1Δ1 in E. coli and Antibody Production

CPH1Δ1 was previously called CPH1 and was the original construct used for sequencing purposes; however, as described in the text and supplemental data, CPH1Δ1 was found to contain a deletion when compared to the full-length cDNA CPH1. The cDNA for CPH1Δ1 was constructed by overlapping RT-PCR products as previously described (Small et al., 1995). The construct was moved to the vector pMAL-c2 (New England Biolabs, Beverly, MA) between the EcoRI and the HindIII sites resulting in a fusion protein with the maltose binding protein (MBP) but retaining the polyhistidine tag on the CPH1 protein. The fusion protein (MBP-CPH1) was purified on amylose affinity columns (New England Biolabs) according to the manufacturer’s specifications and polyclonal antibodies against the fusion protein were produced by BabCo (Richmond, CA).

**Construct for Overexpression of CPH1 in C. reinhardtii**

The CPH1 gene was engineered to contain the FLAG epitope at the N terminus of the CPH1 protein and expression was controlled by the heat shock promoter HSP70A (generously provided by C.F. Beck). HSP70A is contained in pCB745 and comprises a chimeric promoter made of 336 bp of HSP70A promoter and 215 bp of RBCS2 promoter (Schröda et al., 1999, 2000). The resulting construct is named pHSP-CPH1. Expression of C. reinhardtii was by the glass bead method (Kindle, 1990). Cells overexpressing this construct are referred to as OXG cells. For additional information, please refer to supplemental data online.

**Constructs for Overexpression of CPH1Δ1 and CPH1Δ2 in C. reinhardtii**

RT-PCR of C. reinhardtii RNA using primers PHR60 and PHR64 results in two predominate DNA bands at 1.1 and 1.4 kb. The 1.4-kb band was used to deduce the previously identified intron 7 (Small et al., 1995) and the 1.2-kb band suggested an alternatively spliced form. The construct pHSP-CPH1Δ1 was reengineered for the purpose of duplicating the sequences of the 1.1- and 1.4-kb RT-PCR products. Cells overexpressing these construct are referred to as OXΔ1 (1.4-kb product) and OXΔ2 (1.1-kb product) cells. For details see supplemental data online.

**Cell Culture**

C. reinhardtii CW15/Arg7 mating type (−), CW15 (+), 125 (+), or transformed cells were synchronized by inoculating cells to a starting density of 5 × 10⁴ in Tris-acetate phosphate medium (Harris, 1989) or Tris-acetate phosphate plus 75 μg/mL Arg media and incubating with constant shaking at 25°C during 12:12 h light to dark cycles. Cool-white fluorescent bulbs illuminated the cells at a fluence rate of 23 μmol m⁻² s⁻¹. Fluence rate was measured using a LI-COR (Lincoln, NE) light meter, model LI-250, attached to a LI-COR quantum probe. Cells were harvested in the mid-exponential phase at a density of approximately 4-6 × 10⁶ cells/mL. For cells grown in constant light, cool-white fluorescent bulbs illuminated the cells at an average fluence rate of 45 μmol m⁻² s⁻¹.

**Protein Assays**

**Western Blotting**

Approximately 6 × 10⁶ (1.5 mL at 4 × 10⁶/mL) synchronized cells were centrifuged and 1 × Laemmli sample buffer was added to the pellet to lyse cells. Samples were boiled for 3 min and proteins were separated using SDS-PAGE and were subsequently transferred to a polyvinylidene difluoride membrane. Bio-Rad (Hercules, CA) Precision Plus dual color standards were used as molecular mass markers.

Membranes were blocked in Tris-buffered saline (TBS) plus 0.05% (v/v) Tween 20 (TBS) buffer with 5% (w/v) nonfat dry milk (NFDM) for 1 h, washed briefly in TBS, and primary antibodies were added. Before use, sera containing antibodies against CPH1 and preimmune sera were partially purified using an AVID-AL column (Bioprobe, Tustin, CA) according to the manufacturer’s protocol. Primary antibodies against CPH1 were diluted to 1:1,000 in 1% (v/v) bovine serum albumin in TBS and incubated with the membrane for 1 h. M2 monoclonal antibodies against FLAG (Invitrogen, Carlsbad, CA) were diluted 1:1,000 in 1% bovine serum albumin, 2.5% NFDM in TBS, and incubated with the membrane for 1 h. Regardless of the primary antibody used, membranes were subsequently washed for 30 min in TBS or TBS plus 0.3% (v/v) Triton X-100. Secondary antibodies (goat anti-rabbit or
Chlamydomonas Cryptochrome Undergoes Light-Induced Proteolysis

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number L07561.

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LITERATURE CITED


got anti-mouse, Santa Cruz Biotechnology, Santa Cruz, CA) were diluted to 1:10,000 in TBST with 5% (v/v) NFDM and incubated with the membrane for 45 min. Membranes were again washed in TBST for 30 min. Chemilumininescent reagents (Amersham, Piscataway, NJ) were added according to manufacturer’s protocol and the membranes were exposed to film. Films were quantified using a ChemilImage 4000 with AlphaEase 3.3e software (Alpha Innotech, San Leandro, CA) which produces densitometry data.

Cell Treatment

Cycloheximide was added 15 min before the end of the dark phase to a final concentration of 10 μg/ml. This concentration of cycloheximide was found to effectively inhibit protein synthesis in Chlamydomonas (Kawazoe et al., 2000). In addition, this concentration of cycloheximide was sufficient to inhibit CPH1 synthesis in dark-grown cells (data not shown). DCMU, dissolved in ethanol, was added 5 min before the end of the dark phase to a final concentration of 10 μM. For control purposes, the same volume of ethanol was added to control cells. Dimethylaminopropine (dMDAP), genistein, or staurosporine were added 10 min before the end of the dark phase to a final concentration of 5 μM, 300 μM, or 150 nM respectively. These concentrations of kinase inhibitors (dMDAP, genistein, and staurosporine) were found to effectively inhibit gametogenesis in Chlamydomonas cells (Pan et al., 1996).

Heat Shock

Synchronized cells were wrapped in foil and placed into a rotating, darkened 40°C water bath 1 to 2 h into the light cycle. Aliquots were taken at 0, 30, and 60 min, microfuged, and the pellets quick frozen. Alternatively, light-grown cells were used and the same protocol was followed.

Blue Light Versus Red Light (Wavelength Studies)

Synchronized cells were placed in clear glass flasks at various distances from a Kodak Ektagraphic slide projector (Eastman Kodak, Rochester, NY) with a Phillips’ ELH 300W lamp. Fluence rate was measured using a LI-COR Light Meter, Model LI-250 attached to a LI-COR quantum probe. The light passed through colored filters before reaching the cells. For red light, Corning filter no. 2418 (Corning, NY) was used, which only transmits light above 595 nm. For blue light, Corning filter no. 4455 was used, which has a peak transmission at 495 nm. For far red light, Kodak filters numbers 22 and 47 were used, which together only transmit light above 720 nm. Transmission of colored filters was verified using a Beckman quartz spectrophotometer, Model DU (National Technical Laboratories, CA).

Northern Blotting

RNA was isolated from approximately 1.5 × 108 synchronized CW15 ARG7 (*) or transformed cells according to the manufacturer’s protocol using TRI reagent (Sigma-Aldrich, St. Louis). Poly(A+) RNA was subsequently isolated using the PolyATtract mRNA Isolation System (Promega, Madison, WI) according to the instructions and was denatured with formaldehyde and electrophoresed on a 1% (w/v) agarose/17% (v/v) formaldehyde gel, and was transferred onto a nylon membrane (Ambion, Austin, TX) via downward, passive flow for 2 h. Membranes were ultraviolet-crosslinked and were prehybridized in ULTRAhyb (Ambion). Hybridization with the appropriate probe occurred at 42°C and final washes were done in 0.2% sodium chloride/sodium phosphate/EDTA with 0.5% (w/v) SDS at 65°C. Blots were probed as previously described (Small et al., 1995). For a loading control, blots were also probed with the plasmid pCF8-13, which contains cDNA for the constitutively encoded genes in Chlamydomonas. Autoradiographs were analyzed using a Chemilager 4000 with AlphaEase 3.3e software (Alpha Innotech).

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