every experiment, lysates were prepared and monitored for amounts of Cdc25C and Cdc25(S216A) by immunoblotting, and in each case the Cdc25 (S216A) mutant accumulated to slightly smaller amounts than did wild-type Cdc25C.
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A Cyanobacterial Phytochrome Two-Component Light Sensory System

Kuo-Chen Yeh, Shu-Hsing Wu, John T. Murphy, J. Clark Lagarias*

The biliprotein phytochrome regulates plant growth and developmental responses to the ambient light environment through an unknown mechanism. Biochemical analyses demonstrate that phytochrome is an ancient molecule that evolved from a more compact light sensor in cyanobacteria. The cyanobacterial phytochrome Cph1 is a light-regulated histidine kinase that mediates red, far-red reversible phosphorylation of a small response regulator, Rcp1 (response regulator for cyanobacterial phytochrome), encoded by the adjacent gene, thus implicating protein phosphorylation-dephosphorylation in the initial step of light signal transduction by phytochrome.

The ability to cope with a continuously changing light environment is essential to the survival of all organisms that rely on sunlight for energy. Photosynthetic organisms, from bacteria to higher plants, possess numerous light-sensing molecules for perception and adaptation to fluctuations of intensity, direction, duration, polarization, and spectral quality of light (*1*). Most well known of these photoreceptors are the phytochromes, which sense ambient light conditions by their ability to photointerconvert between red (Pr) and far-red (Pfr) light-absorbing forms (*2*). The hypothesis that phytochrome is a light-regulated enzyme was proposed nearly 40 years ago (*3*). Despite evidence that purified plant phytochromes exhibit protein kinase activity (*4*) and possess a COOH-terminal domain similar to that of bacterial histidine kinases (*5*), the enzyme hypothesis remains controversial.

Identification of the *rcaE* gene from the cyanobacterium *Fremyella diplosiphon*, which encodes a protein that is structurally related to higher plant phytochromes and bacterial histidine kinases, has renewed interest in the possibility that phytochrome is a protein kinase (*6*). Other phytochrome-like open reading frames (ORFs) have been noted in the cyanobacterium *Synechocystis* sp. PCC6803 genome (*6*, *7*). One of these ORFs, locus slr0473, encodes a 748-residue polypeptide whose expression in *Escherichia coli* and incubation with phycocyanobilin (PCB), yielded an adduct with a red, far-red photoreversible phytochrome signature (*8*). Closer inspection of this phytochrome locus, which we have named *cph1* for cyanobacterial phytochrome 1, reveals another ORF only 10 base pairs (bp) downstream, locus slr0474, which we have named *rcp1* for response regulator for Cph1 based on this study (Fig. 1A). Because the COOH-terminal domain of Cph1 contains all conserved features of histidine kinase transmitter modules (Fig. 1B) and *rcp1* encodes a 147–amino acid protein related to the CheY superfamily of bacterial response regulators (Fig. 1C), which contain aspartate kinase receiver modules, we investigated whether these proteins represent a functional light-regulated transmitter-receiver pair (*9*).

Affinity-tagged versions of both proteins were cloned by polymerase chain reaction (PCR) and expressed in *E. coli* (*10*). That Cph1 is a functional phytochrome homolog was demonstrated by its ability to catalyze its own chromophore attachment to yield photoreversible adducts with the higher plant chromophore precursor phytochromobilin (PΦB) and its phycobilin analog PCB (Fig. 2A). Assembly with phycoerythrobilin (PEB), a phycobilin analog that lacks the C^{15} double bond found in PCB and PΦB, also produced a covalent adduct as visualized by zinc-blot analysis (Fig. 2B). The PEB adduct of Cph1 was photochem-

Section of Molecular and Cellular Biology, University of California, Davis, CA 95616, USA.

*To whom correspondence should be addressed. E-mail: jclagarias@ucdavis.edu.

ically inactive, thus demonstrating that photoisomerization of the C^{15} double bond is required for Cph1 photoactivity, as is the case for higher plant phytochromes (*11*). The Cph1 deletion mutant N514, which lacks the transmitter domain (*12*), also bound all three bilins covalently (Fig. 2B), yielding PΦB and PCB adducts with absorption difference spectra indistinguishable from the full-length photoreceptor (*13*). These data indicate that the NH_2-terminal region of Cph1 delimits a functional photosensory domain (Fig. 2C) consistent with the structure and photochemistry of eukaryotic phytochromes (*2*, *14*).

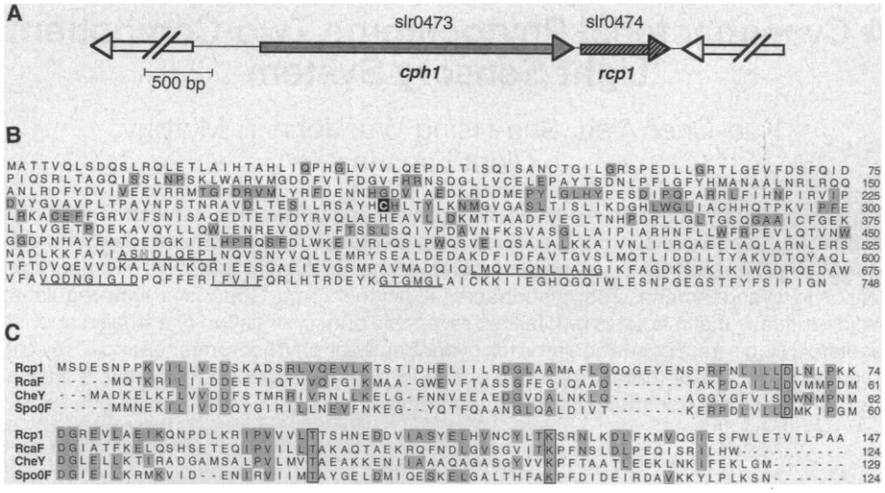

Fig. 1. Phytochrome operon of *Synechocystis* sp. PCC6803. (**A**) Genomic organization of the phytochrome-related gene *cph1* (locus slr0473) (GB:D64001, locus 1001165) and the adjacent small response regulator gene *rcp1* (locus slr0474) (GB:D64001, locus 1001166). (**B**) Deduced amino acid sequence of Cph1 (*26*). Highlighted residues are 100% conserved between Cph1 and 21 full-length eukaryotic phytochrome sequences in the nonredundant GenBank and EMBL databases (*13*). The conserved cysteine for bilin attachment for eukaryotic phytochromes is shown in a black box. Underlined protein sequences represent the five conserved motifs of transmitter modules (*9*). Outlined H represents the conserved histidine autophosphorylation site. (**C**) Multiple sequence alignment of Rcp1 and response regulators RcaF (*17*), CheY (*27*), and SpoOF (*18*). Invariant aspartate, threonine, and lysine residues of the CheY superfamily are boxed, and conserved residues are shaded.

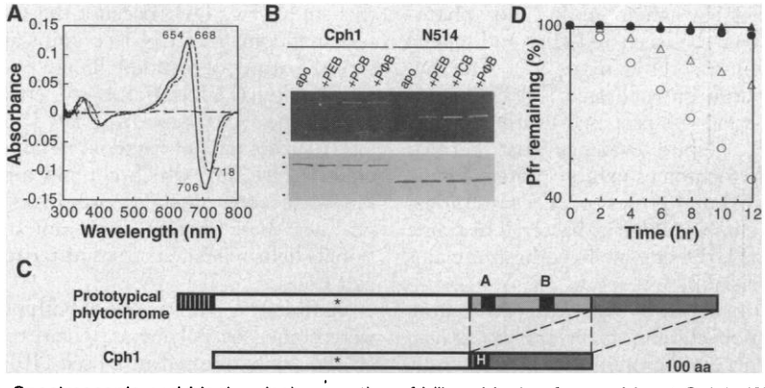

Fig. 2. Spectroscopic and biochemical properties of bilin adducts of recombinant Cph1. (**A**) Phytochrome difference spectra of 40% ammonium sulfate–fractionated, Cph1-containing protein extracts (*11*) after incubation with PΦB (short dashes), PCB (solid line), or PEB (long dashes). (**B**) Visualization of PΦB, PCB, and PEB adducts of Cph1 and N514 mutant on polyvinylidene difluoride (PVDF) membranes treated with zinc acetate (upper) (*11*) or alkaline phosphatase conjugated to streptavidin (lower) (*23*). Molecular mass markers at 119, 83, and 47 kD (top to bottom) are indicated with dots; apo, apoprotein. (**C**) Structural model for prototypical eukaryotic and *Synechocystis* apoprotein phytochromes. The phytochromes share a similarly sized photosensory domain (open rectangle) containing a conserved cysteine chromophore binding site (*) and a COOH-terminal transmitter-related module (dark-shaded rectangle). Prototypical phytochromes also contain a small NH_2-terminal extension and a second transmitter-related module (light-shaded rectangle) that contains the PAS A and B repeats (*13*, *28*). aa, amino acids. (**D**) Dark reversion of PCB and PΦB adducts of full-length Cph1 (● and ○, respectively) and N514 mutant (▲ and △, respectively) (*29*).

Cph1 is smaller than eukaryotic phytochromes; it lacks a 60– to 100–amino acid NH_2-terminal fragment found on the photosensory domains of prototypical phytochromes and approximately one-half of the COOH-terminal region (Fig. 2C). Removal of the NH_2-terminal portion of higher plant phytochromes blue-shifts its Pfr absorption maximum and attenuates its biological activity (*14*). Consistent with these observations, Pfr absorption maxima of Cph1-bilin adducts are blue-shifted relative to higher plant phytochrome bilin adducts whereas Pr absorption maxima are similar (Fig. 2A). The dark reversion properties of the two bilin adducts of Cph1 are particularly interesting (Fig. 2D). PCB adducts of Cph1 and the N514 deletion mutant display little dark reversion, whereas PΦB adducts show considerable dark reversion, with respective half-lives of 10 and 24 hours. In addition to demonstrating that Pfr stability depends on chromophore structure, these results indicate that the transmitter domain influences the conformational stability of the chromophore domain. In view of the potential role of dark reversion in the perception of photoperiod (*3*) and light direction (*15*), the identity of the natural Cph1 chromophore is of great interest.

To test whether Cph1 and Rcp1 represent functional transmitter and receiver molecules, we purified affinity-tagged versions of Cph1 and Rcp1 fusion protein (*16*) and analyzed the PCB adduct of Cph1 for protein kinase activity (Fig. 3A). Surprisingly, the Pr form of Cph1 exhibited adenosine triphosphate (ATP)–dependent autophosphorylation activity, whereas phosphorylation of the Pfr form was greatly reduced. Consistent with a histidine residue as the phosphorylation site, Cph1 autophosphorylation was base stable and acid labile. Similar experiments with the N514 mutant demonstrated that the transmitter domain was required for Cph1 autophosphorylation (Fig. 3A). That Rcp1 is a functional receiver substrate for Cph1 was established by phosphotransfer from Cph1 to Rcp1 (Fig. 3B). No phosphotransfer occurred with the Asp^{68} to Ala (D68A) mutant of Rcp1 (*16*), which lacks the conserved phosphate-accepting aspartate residue of receiver domains (*9*). The inability of the His^{538} to Lys (H538K) mutant of Cph1 to autophosphorylate and to support phosphotransfer to Rcp1 demonstrated that the conserved histidine at amino acid residue 538 in the Cph1 transmitter module is required for both activities (*13*). These data, taken together, demonstrate that Cph1 is a histidine kinase that mediates light-dependent phosphotransfer to Rcp1. Cph1 and Rcp1 thus represent a two-component regulatory sys-

tem in cyanobacteria that is modulated by red and far-red light.

The small amounts of Cph1 autophosphorylation and Rcp1 phosphotransferase activity exhibited by the Pfr sample (Fig. 3B) probably represent the presence of residual Pr and are consistent with the photoequilibrium mixture, containing 13% Pr, that results for higher plant phytochromes irradiated with saturating red light (*2*). To determine whether phosphorylated Pfr (Pfr*) was capable of phosphate transfer to Rcp1, we autophosphorylated Pr (Pr*), photoconverted it to Pfr*, and then incubated it with Rcp1 (Fig. 3C). By comparison with a control sample maintained in the Pr* form, phosphotransfer from Pfr* to Rcp1 was clearly prevented. Thus, the Pfr form of Cph1 lacks both autophosphorylation and Rcp1 phosphotransfer activities.

Organization of the cyanobacterial phytochrome operon is similar to the *F. diplosiphon rcaEF* operon, which encodes two elements of the complementary chromatic adaptation signal transduction pathway (*6*, *17*). This and our biochemical data suggest that the molecular mechanism of Cph1 action involves light-regulated protein phosphorylation-dephosphorylation as depicted in Fig. 4. In this model, Cph1 can exist as four species—Pr, Pr*, Pfr, and Pfr*—whose abundances are regulated both by light conditions and by Rcp1 phosphorylation status. By analogy to the multistep phospho-relay cascades proposed for complementary chromatic adaptation in *F. diplosiphon* (*17*), sporulation in *Bacillus subtilis* (*18*), and osmosensing in yeast (*19*), Rcp1 dephosphorylation could be mediated by phosphotransfer to another regulatory molecule. Alternatively, the two forms of the small receiver molecule—Rcp1 and phospho-Rcp1 (Rcp1*)—could have distinct regulatory activities like CheY (*9*).

In higher plants, Pfr is thought to be the active form of phytochrome (*2*). Our studies suggest that the light signal transduced by Cph1 involves regulation of Pr abundance rather than that of Pfr. However, Pfr (or Pfr*) could perform an as yet unidentified role in the signal transduction process, such as allosterically regulating the activity of a Rcp1 phosphatase or influencing phosphotransfer to another regulatory molecule. In view of the evidence presented here, the presence of a transmitter-like domain in higher plant phytochromes (*5*) and the observed protein kinase activity of purified higher plant phytochromes (*4*, *13*), we expect that the molecular mechanism of phytochrome function in plants will involve phosphorylation-dephosphorylation of transmitter- and receiver-containing signaling proteins like those prevalent in eubacteria and archaebacteria. It is intriguing that two-component regulatory family members have been identified in plants, including the putative plant hormone receptors for ethylene (*20*) and cytokinin (*21*). Given the physiological interplay between light and hormone responses in plants (*22*), we speculate that these receptors may be targets for integrated transduction of multiple signals.

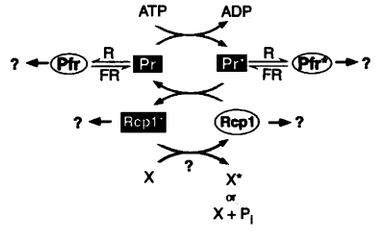

Fig. 4. Model for cyanobacterial phytochrome action. Cph1 can exist in four species—Pr, Pr*, Pfr, and Pfr*—whose relative levels are regulated by light, Pr autophosphorylation, and Rcp1 phosphotransferase activities. Species abundant in red (R) or far-red (FR) light are highlighted in white circles or black boxes, respectively. Phosphorylated Rcp1 (Rcp1*) is dephosphorylated by hypothetical molecule X by a multistep phospho-relay or protein phosphatase mechanism. See text for details. ADP, adenosine diphosphate; P_i, inorganic phosphate.

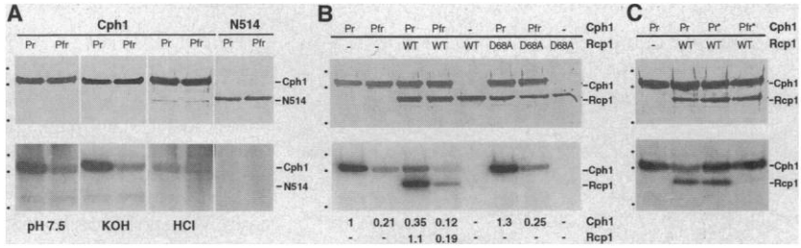

Fig. 3. Cph1 and Rcp1 represent a red, far-red light-regulated transmitter-receiver pair. (**A**) Autophosphorylation of purified PCB adducts of Cph1 and N514 mutant. Chemical stability of phospho-Cph1 on blots was assessed by treatment under neutral (50 mM tris HCl, pH 7.5), basic (3 M KOH), or acidic (1 M HCl) conditions at 25°C for 2.5 hours. (**B**) Phosphotransferase activities of purified Cph1 toward WT and D68A mutant of Rcp1. Normalized relative phosphorylation levels are indicated at the bottom of the figure. (**C**) Pfr* lacks Rcp1 phosphotransferase activity. Before addition of Rcp1, Pr* was prepared by Pr autophosphorylation for 30 min and either photoconverted to Pfr to produce Pfr* or kept as Pr*. Kinase assays were performed (*30*); proteins were resolved on SDS–10% polyacrylamide gels and transblotted to PVDF membranes for Coomassie blue staining (upper) or autoradiography (lower) (*11*). Molecular mass markers at 119, 83, and 47 kD (top to bottom) are indicated with dots.

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30. Protein kinase assays were performed in 25 μl of kinase buffer (*4*) with 80 mM KCl (Fig. 3B) or without KCl (Fig. 3, A and C) and containing 0.1 mM [γ-^{32}P]ATP (8000 cpm/pmol), 2.4 μg of Cph1-PCB adduct or 1 μg of N514-PCB adduct in Pr or Pfr form, and 2 μg of MBP-Rcp1 (WT and D68A). Reactions were initiated by adding ATP, mixtures were incubated 30 min at 30°C, and reactions were stopped by adding SDS sample buffer (*4*).
31. We are indebted to E. Campbell for providing us with purified *Synechocystis* DNA, K. C. McFarland for figure design, A. Skerra for core streptavidin, and D. Kehoe for critical review of the manuscript. Supported in part by National Science Foundation grant MCB-9604511 to J.C.L. and NIH National Center for Research Resources award 1 P41 RR06009 to the Pittsburgh Supercomputing Center.

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Differential Ligand Activation of Estrogen Receptors ERα and ERβ at AP1 Sites

Kolja Paech, Paul Webb, George G. J. M. Kuiper, Stefan Nilsson, Jan-Åke Gustafsson, Peter J. Kushner,* Thomas S. Scanlan*

The transactivation properties of the two estrogen receptors, ERα and ERβ, were examined with different ligands in the context of an estrogen response element and an AP1 element. ERα and ERβ were shown to signal in opposite ways when complexed with the natural hormone estradiol from an AP1 site: with ERα, 17β-estradiol activated transcription, whereas with ERβ, 17β-estradiol inhibited transcription. Moreover, the antiestrogens tamoxifen, raloxifene, and Imperial Chemical Industries 164384 were potent transcriptional activators with ERβ at an AP1 site. Thus, the two ERs signal in different ways depending on ligand and response element. This suggests that ERα and ERβ may play different roles in gene regulation.

Antiestrogens are therapeutic agents for the treatment and possible prevention of breast cancer. Tamoxifen (Fig. 1A) is an antiestrogen that is used in breast cancer chemotherapy and is believed to function as an antitumor agent by inhibiting the action of the estrogen receptor (ER) in breast tissue (*1*). Paradoxically, tamoxifen appears to function as an estrogen-like ligand in uterine tissue, and this tissue-specific estrogenic effect may explain the increased risk of uterine cancer that is observed with prolonged tamoxifen therapy (*2*). The related benzothiophene analog raloxifene (Fig. 1A) has been reported to retain the antiestrogen properties of tamoxifen in breast tissue and to show minimal estrogen effects in the uterus; in addition, it has potentially beneficial estrogen-like effects in nonreproductive tissue such as bone and cardiovascular tissue (*3–7*). One explanation for these tissue-specific actions of antiestrogens is that the ligand-bound ER may have different transactivation properties when bound to different types of DNA enhancer elements. The classical estrogen response element (ERE) is composed of two inverted hexanucleotide repeats, and ligand-bound ER binds to the ERE as a homodimer (Fig. 1B). The ER also mediates gene transcription from an AP1 enhancer element that requires ligand and the AP1 transcription factors Fos and Jun for transcriptional activation (Fig. 1B) (*8*). In transactivation experiments, tamoxifen inhibits the transcription of genes that are regulated by a classical ERE, but like the natural estrogen hormone 17β-estradiol [E_2 (Fig. 1A)], tamoxifen activates the transcription of genes that are under the control of an AP1 element (*9*).

At the end of 1995, a second ER (ERβ) was cloned from a rat prostate cDNA library (*10*), and, subsequently, the human (*11*) and mouse (*12*) homologs were cloned. The first identified ER has been renamed ERα (*10*). The existence of two ERs presents another potential source of tissue-specific estrogen regulation. Here we compared the transactivation properties of ERα and ERβ with a panel of five ER ligands with the use of a reporter gene under the control of either a classical ERE or an AP1 element (*13*). Our results show that ERα and ERβ respond differently to certain ligands at an AP1 element. These results suggest different regulatory functions for the two ER subtypes.

We examined the transactivation properties of ERα (*14*) and ERβ (*15*) at a classical ERE in response to the estrogens E_2 and diethylstilbestrol (DES) and the antiestrogens Imperial Chemical Industries (ICI) 164384, tamoxifen, and raloxifene (*16*). We conducted these experiments by transfecting HeLa cells with either an ERα or ERβ expression plasmid along with a reporter plasmid that contained a luciferase gene under the transcriptional control of an ERE (*17*). Both ERα (*18*) and ERβ (Fig. 2) showed the same transactivation profiles with the panel of ligands. E_2 and DES stimulated luciferase production 10-fold over ICI 164384, raloxifene, tamoxifen, and the control (no ligand added). The antiestrogens blocked E_2 stimulation in ligand competition experiments (*18*).

We next examined the ligand-induced transactivation behavior of ERα and ERβ at an AP1 site. With ERα, all five ligands stimulated luciferase transcription, including the antiestrogens ICI 164384, tamoxifen, and raloxifene (Fig. 3). This stimulation was dependent on transfected ER, as cells trans-

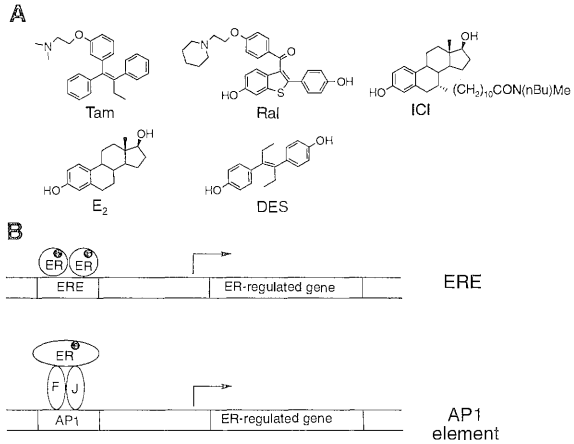

Fig. 1. (**A**) Structures of ER ligands. The estrogens E_2 and DES and the antiestrogens tamoxifen (Tam), raloxifene (Ral), and ICI 164384 (ICI) are shown. Bu, butyl; Me, methyl. (**B**) Models of ER action at a classical ERE and an ER-dependent AP1 response element. The filled circles represent the ligand bound to the ER. The AP1 proteins Jun and Fos are labeled J and F, respectively.

K. Paech and T. S. Scanlan, Departments of Pharmaceutical Chemistry and Cellular and Molecular Pharmacology, University of California, San Francisco, CA 94143–0446, USA.
P. Webb and P. J. Kushner, Metabolic Research Unit, University of California, San Francisco, CA 94143–0540, USA.
G. G. J. M. Kuiper, Center for Biotechnology, Karolinska Institute, Novum, S-14186 Huddinge, Sweden.
S. Nilsson, Karo Bio AB, Novum, S-14157 Huddinge, Sweden.
J.-Å. Gustafsson, Center for Biotechnology and Department of Medical Nutrition, Karolinska Institute, Novum, S-14186 Huddinge, Sweden.
*To whom correspondence should be addressed.