This model does not preclude a role for other cell cycle regulators such as Wee1 in the damage response (*14*). Furthermore, the fact that hChk1 phosphorylated hCdc25A and hCdc25B and that Ser^{216} is conserved among these Cdc25 proteins (*19*) suggests that hChk1 may regulate other DNA damage checkpoints, such as those controlling the G_1-to-S phase transition, through a similar mechanism.

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Mitotic and G_2 Checkpoint Control: Regulation of 14-3-3 Protein Binding by Phosphorylation of Cdc25C on Serine-216

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Human Cdc25C is a dual-specificity protein phosphatase that controls entry into mitosis by dephosphorylating the protein kinase Cdc2. Throughout interphase, but not in mitosis, Cdc25C was phosphorylated on serine-216 and bound to members of the highly conserved and ubiquitously expressed family of 14-3-3 proteins. A mutation preventing phosphorylation of serine-216 abrogated 14-3-3 binding. Conditional overexpression of this mutant perturbed mitotic timing and allowed cells to escape the G_2 checkpoint arrest induced by either unreplicated DNA or radiation-induced damage. Chk1, a fission yeast kinase involved in the DNA damage checkpoint response, phosphorylated Cdc25C in vitro on serine-216. These results indicate that serine-216 phosphorylation and 14-3-3 binding negatively regulate Cdc25C and identify Cdc25C as a potential target of checkpoint control in human cells.

A key step in regulating the entry of eukaryotic cells into mitosis is the activation of the protein kinase Cdc2 by the protein phosphatase Cdc25C. A complete understanding of mitotic control requires elucidation of the mechanisms that regulate the interactions between Cdc2 and Cdc25C throughout the cell cycle. Furthermore, although tremendous progress has been made in recent years in identifying proteins that participate in checkpoint control, it is unclear how these proteins interface with core cell cycle regulators to inhibit cell cycle transitions (*1*).

The Ser^{216} residue is the primary site of phosphorylation of Cdc25C in asynchronously growing cells (*2*). To determine if phosphorylation of Ser^{216} regulates Cdc25C function, we generated HeLa cell lines that allow conditional expression of either wild-type Cdc25C or a mutant of Cdc25C containing alanine at position 216 (S216A). In these cells, expression of Cdc25C and Cdc25(S216A) is under the control of a

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hybrid protein consisting of a bacterial tetracycline repressor and VP16 activator protein (3). Expression can be induced upon removal of tetracycline from the medium (Fig. 1A). Two electrophoretic forms were evident in the case of induced Cdc25C, a major form (species b) and a minor form (species a). A single electrophoretic form migrating in the position of species a was evident for induced Cdc25(S216A). Phosphatase treatment converted the electrophoretic mobility of species b to that of species a, demonstrating that phosphorylation of Ser^{216} is responsible for the shift in mobility of species b (4). The electrophoretic mobility of Cdc25C in SDS gels could therefore be used to monitor amounts of Ser^{216} phosphorylation in vivo. Phosphopeptide mapping experiments (5) revealed one major and several minor phosphopeptides for induced Cdc25C (Fig. 1B). The major phosphopeptide contains Ser^{216} (2) and was absent in maps of induced Cdc25(S216A).

To determine whether phosphorylation of Cdc25C at Ser^{216} was regulated by the cell cycle, we elutriated Jurkat cells and analyzed fractions for endogenous Cdc25C mobility by immunoblotting (6). Most Jurkat cell Cdc25C was phosphorylated on Ser^{216} in asynchronously growing cells and throughout the G_1 and S phases of the cell cycle (Fig. 2A). The G_2-M population of Jurkat cells contained Cdc25C phosphorylated on Ser^{216} but also contained the mitotic form of Cdc25C. We used a double-thymidine block and release protocol to monitor Ser^{216} phosphorylation in synchronized populations of HeLa cells (7). Amounts of Cdc25C phosphorylated at Ser^{216} remained constant throughout the first 9 hours after release, corresponding to passage through the S and G_2 phases of the cell cycle (Fig. 2B). Mitotic cells collected between 10 and 12 hours after release were enriched in the mitotic form of Cdc25C and had lower amounts of the Ser^{216}-phosphorylated form of Cdc25C. Phosphopeptide maps of Cdc25C and Cdc25(S216A) were generated from induced HeLa cells incubated with ^{32}P during mitosis (Fig. 2C) (5). Cdc25C and Cdc25(S216A) yielded identical phosphotryptic maps, and the Ser^{216}-containing phosphopeptide was not detected (this peptide migrates between phosphopeptides 7 and 8). Thus, Cdc25C is phosphorylated on Ser^{216} throughout interphase but not during mitosis.

We used chromosome spreading to assess the role of Ser^{216} phosphorylation in regulating mitotic entry (8, 9). Normal mitotic cells display intact chromosomes upon spreading, whereas chromosomes from cells that enter mitosis from S phase fragment upon spreading. Less than 0.1% of the mitotic nuclei derived from cells induced to express Cdc25C showed abnormal chromosome spreads, whereas 4.4 ± 0.7% of the mitotic nuclei derived from cells induced to

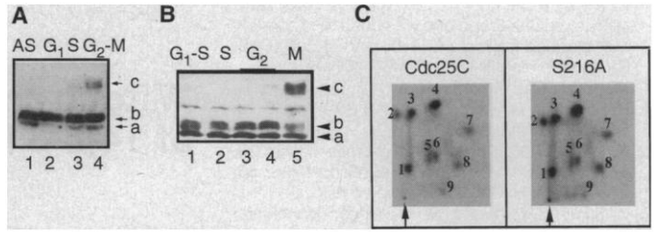

Fig. 2. Cell cycle regulation of Ser^{216} phosphorylation. (**A**) Asynchronous and elutriated populations of Jurkat cells were lysed, resolved by SDS-PAGE, and immunoblotted for Cdc25C. Lane 1, asynchronous population of Jurkat cells; lanes 2, 3, and 4, Jurkat cells enriched in G_1, S, or G_2-M, respectively. Species b is Cdc25C phosphorylated on Ser^{216} and species c is the mitotic form of Cdc25C. (**B**) HeLa cells were synchronized at G_1-S by a double-thymidine block (time 0, lane 1) and then allowed to proceed through S (4 hours, lane 2), G_2 (8 and 9 hours, lanes 3 and 4), and into mitosis. Mitotic cells were collected by shake-off between 10 and 12 hours after release (lane 5). Synchronized cells were lysed, resolved by SDS-PAGE, and immunoblotted for Cdc25C. (**C**) Induced cells were arrested in mitosis with nocodazole and incubated with ^{32}P-labeled inorganic phosphate. Mitotic forms of Cdc25C (left panel) and Cdc25(S216A) (right panel) were isolated by immunoprecipitation and subjected to two-dimensional tryptic phosphopeptide mapping. Arrows depict the origin.

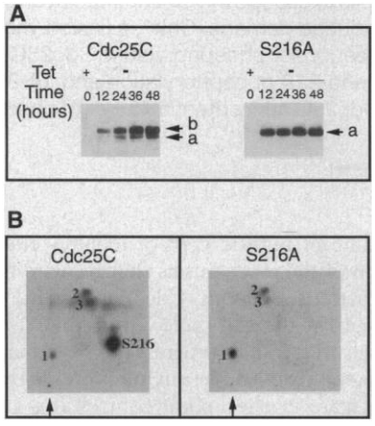

Fig. 1. Phosphorylation of Cdc25C on Ser^{216} in asynchronously growing HeLa cells. (**A**) Expression of Cdc25C and Cdc25(S216A) was induced by removal of tetracycline (Tet) from the media (3). At the indicated times (hours), cells were collected, and Cdc25C was visualized by immunoblotting. Species a and b refer to different electrophoretic forms of Cdc25C. (**B**) Induced cells were incubated with ^{32}P-labeled inorganic phosphate. Radiolabeled Cdc25C (left panel) and Cdc25(S216A) (right panel) were isolated by immunoprecipitation and subjected to two-dimensional tryptic phosphopeptide mapping. Arrows depict the origin.

Table 1. Effects of Cdc25(S216A) overexpression on mitotic and replication checkpoint control. Uninduced cells or cells induced to express either Cdc25C or Cdc25(S216A) were incubated for 8 hours in the presence of nocodazole (for examination of mitotic control) or were incubated for 8 hours in the presence of hydroxyurea followed by an additional 8 hours in the presence of hydroxyurea and nocodazole (for examination of replication checkpoint control). Cells were processed for chromosome spreading as described (8). Mitotic nuclei were identified from a population of 2000 nuclei each in three independent experiments. Data are presented as mean ± SEM.

Cells	Total mitotic nuclei (out of 2000 nuclei)	Percent mitotic nuclei	Normal mitotic nuclei	Abnormal mitotic nuclei
		Mitotic control		
Uninduced				
Cdc25C	594 ± 7	29.7% ± 0.4	594 ± 7	0 ± 0
Cdc25(S216A)	584 ± 8	29.2% ± 0.4	584 ± 8	0 ± 0
Induced				
Cdc25C	593 ± 5	29.6% ± 0.3	592 ± 5	0.3 ± 0.3 (0.06% ± 0.06)†
Cdc25(S216A)	617 ± 2	30.8% ± 0.1	589 ± 5	27.0 ± 4.0 (4.4% ± 0.7)†
		DNA replication checkpoint control		
Uninduced				
Cdc25C	11.0 ± 1.0	0.5% ± 0.07	11.0 ± 1	0 ± 0
Cdc25(S216A)	10.3 ± 0.9	0.5% ± 0.04	10.3 ± 0.9	0 ± 0
Induced				
Cdc25C	15.3 ± 1.8	0.8% ± 0.09	10.0 ± 1.5	5.3 ± 1.8
Cdc25(S216A)	76.0 ± 12	3.8% ± 0.60*	10.7 ± 0.9	65 ± 11

*The percent of Cdc25(S216A) mitotic nuclei are significantly increased compared with Cdc25C ($P < 0.01$, Student's t test). †Percent of abnormal mitotic nuclei. The percent of Cdc25(S216A) abnormal mitotic nuclei are significantly increased compared with Cdc25C ($P < 0.005$, Student's t test). The fact that only 4.38% of the Cdc25(S216A) mitotic nuclei displayed abnormal chromosomes indicates that Ser^{216} phosphorylation of Cdc25C is only one mechanism regulating entry into mitosis (28).

express Cdc25(S216A) showed abnormalities (Table 1).

Cells induced to express Cdc25C and Cdc25(S216A) were also analyzed for DNA replication and DNA damage checkpoint responses (9). A DNA replication checkpoint response was induced by incubation of the cells in the presence of hydroxyurea. Most cells induced to express Cdc25C were arrested in S phase as indicated by the low numbers of mitotic nuclei (Table 1). In contrast, five times more mitotic nuclei were counted in cells induced to express Cdc25(S216A) (Table 1). Although the number of normal nuclei were similar for uninduced and induced cells, cells induced to express Cdc25(S216A) had significantly more abnormal nuclei (Table 1). In addition, Cdc25(S216A) cells had detectable amounts of the mitotic form of Cdc25C and larger amounts of cyclin B1–associated histone H1 kinase activity (4). Finally, to generate a DNA damage checkpoint response, cells were gamma irradiated and monitored for their ability to delay in G_2 (9). In the absence of irradiation, the S phase cells from uninduced and Cdc25(S216A)-induced populations took between 8 and 12 hours to reach G_1 (Fig. 3, A and B). At 24 hours after irradiation, only 9% of the S phase cells from uninduced populations had cycled to G_1, indicating a 12- to 16-hour radiation-induced delay (Fig. 3C). Induced expression of Cdc25C resulted in a partial loss in the G_2 delay, which was exaggerated in the case of Cdc25(S216A) expression (Fig. 3, D, G, and H). By 12 hours, 9% of the Cdc25(S216A)-expressing cells were already in G_1 and 16% were in G_1 by 16 hours. After 24 hours, the G_1-S fractions represented 60% of the population. Cycling and arrest of BrdU-positive cells demonstrated that the observed results are due to a G_2 delay rather than to cells not releasing from G_1 (Fig. 3, A, B, C, and D).

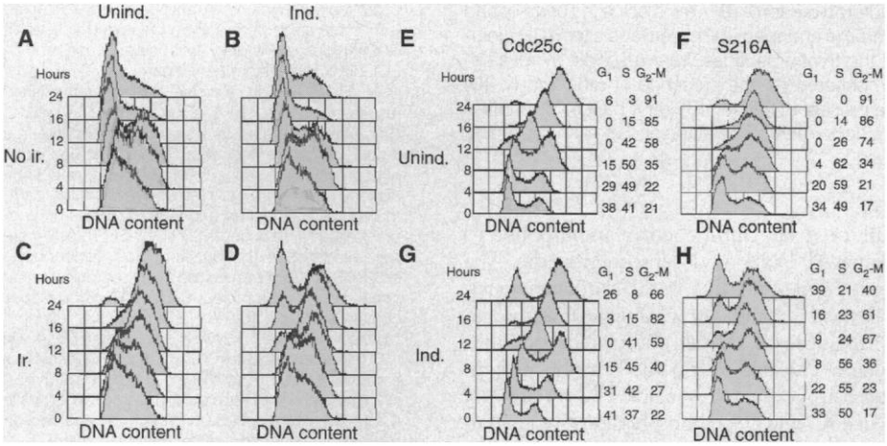

Fig. 3. Disruption of DNA damage checkpoint in Cdc25(S216A) cells. Uninduced cells or cells induced to express Cdc25C or Cdc25(S216A) were labeled with BrdU and then treated with 0 or 6 Gy of gamma irradiation. Cells were harvested at the indicated times and stained with PI and for BrdU as described (9). DNA content profiles for BrdU-positive cells are shown for uninduced and Cdc25(S216A) induced cells in (**A**) to (**D**). Total DNA content profiles and quantitation of G_1, S, and G_2-M irradiated cells are shown in (**E**) to (**H**).

Mutation of Ser^{216} abrogated the binding of 14-3-3 to Cdc25C, demonstrating that Ser^{216} is essential for the interaction. To determine whether phosphorylation of Ser^{216} was important for the interaction, we performed competition experiments using peptides consisting of amino acids 210 to 225 of Cdc25C that were either phosphorylated on Ser^{216} or unphosphorylated (13). Complexes consisting of 14-3-3 and Cdc25C-GST fusion protein purified from insect cells were disrupted by incubation with excess peptide containing phosphorylated Ser^{216} but not by unphosphorylated peptide (Fig. 4C). These results demonstrate that the phosphorylation of Ser^{216} is

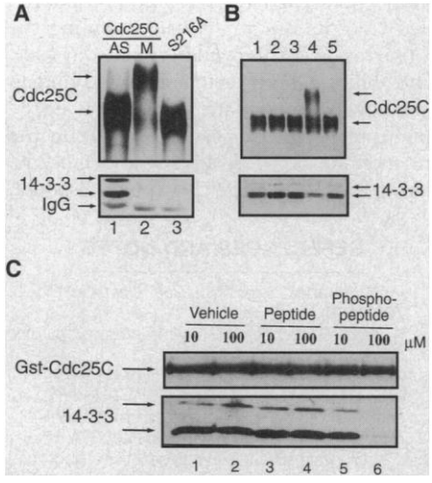

Fig. 4. Association of Cdc25C with 14-3-3 in a phosphorylation- and cell cycle–dependent manner. (**A**) Cells induced to express Cdc25C and Cdc25(S216A) were untreated (lanes 1 and 3) or were incubated in the presence of nocodazole (lane 2). Immunoprecipitates were prepared from 3 mg of total cellular protein by using 9E10 Myc-agarose and analyzed for Cdc25C (upper panel) and 14-3-3 (lower panel) by immunoblotting. AS, asynchronous cells; M, mitotic cells. (**B**) Cells induced to express Cdc25C were synchronized at G_1-S by a double-thymidine block (time 0, lane 1) and then allowed to proceed through S (4 hours, lane 2), G_2 (9 hours, lane 3), M (10 to 12 hours, lane 4), and G_1 (13 hours, lane 5). Cells were harvested at the indicated times, and immunoprecipitates of Cdc25C were prepared from 2 mg of total cellular protein. Cdc25C (upper panel) and 14-3-3 (lower panel) were visualized by immunoblotting. (**C**) Coprecipitates of GST-Cdc25C and 14-3-3 from overproducing insect cells were incubated in vehicle (lanes 1 and 2) or vehicle containing unphosphorylated (lanes 3 and 4) or Ser^{216}-phosphorylated (lanes 5 and 6) peptide at concentrations of 10 and 100 μM. Reactions were resolved by SDS-PAGE and immunoblotted for Cdc25C and 14-3-3.

The sequence bordering and inclusive of Ser^{216} in Cdc25C (RSPS^{216}MP) contains a potential recognition motif for binding of 14-3-3 proteins: RSXSXP where P is proline, R is arginine, X is any amino acid, and the underlined serine is phosphorylated (10). The 14-3-3 proteins belong to a highly conserved multigene family of small acidic proteins that associate with cell cycle and cell death regulators, oncogenes, and signaling molecules (11, 12). There are at least seven mammalian 14-3-3 isoforms ranging in size from 30 to 35 kD. Immunoblotting experiments were performed with a 14-3-3 antibody that recognizes several of the mammalian isoforms to determine whether a complex between Cdc25C and 14-3-3 proteins could be detected. We detected 14-3-3 proteins in immunoprecipitates of Cdc25C but not Cdc25(S216A) (Fig. 4A). Given that the mitotic form of Cdc25C was not detectably phosphorylated on Ser^{216} (Fig. 2C), we determined whether the binding of Cdc25C to 14-3-3 was lost during mitosis. HeLa cells induced to express Cdc25C were arrested in mitosis with nocodazole (3), and Cdc25C immunoprecipitates were monitored for the presence of 14-3-3 by immunoblotting. No 14-3-3 was immunoprecipitated with the mitotic form of Cdc25C (Fig. 4A). We used a double-thymidine block and release protocol to monitor 14-3-3 binding at other phases of the cell cycle (6). Binding of 14-3-3 was detected during the G_1, S, and G_2 phases of the cell cycle (Fig. 4B). Binding of 14-3-3 was reduced in fractions enriched for M phase cells.

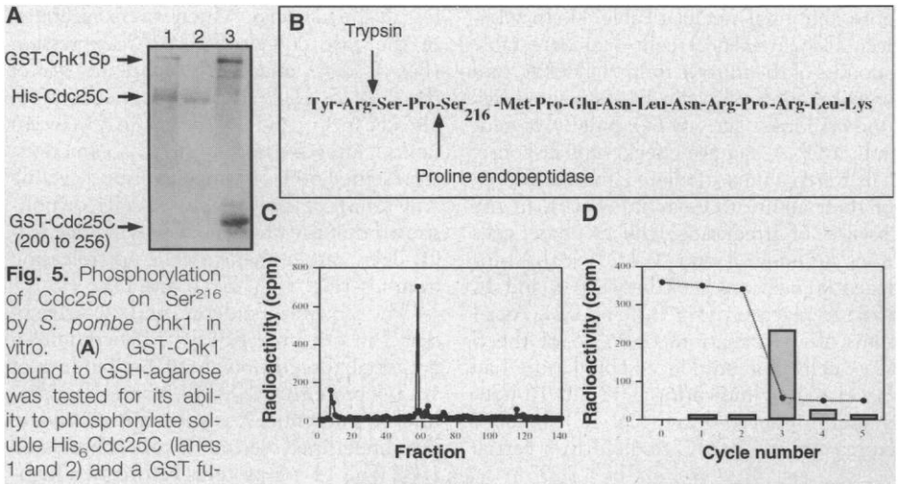

Fig. 5. Phosphorylation of Cdc25C on Ser^{216} by *S. pombe* Chk1 in vitro. (**A**) GST-Chk1 bound to GSH-agarose was tested for its ability to phosphorylate soluble His_6Cdc25C (lanes 1 and 2) and a GST fusion protein consisting of amino acids 200 to 256 of Cdc25C (lane 3). Reactions were separated into pelleted (lane 1) and supernatant (lane 2) fractions by centrifugation or were analyzed directly (lane 3). Proteins were resolved on a 7% gel and visualized by autoradiography. (**B**) Amino acids inclusive of and surrounding Ser^{216} showing NH_2-terminal trypsin and proline endopeptidase cleavage sites. (**C**) Radiolabeled His_6-Cdc25C was digested with trypsin, and the tryptic peptides were resolved by reverse-phase HPLC. Column fractions were collected and monitored for the presence of radioactivity. (**D**) Manual Edman degradation of tryptic phosphopeptide present in fraction 57 (right panel). The dotted lines indicate radioactivity remaining bound to the sequencing membrane at the end of each cycle, and bars represent radioactivity released from the membrane.

required for 14-3-3 binding to Cdc25C.

Mitotic hyperphosphorylation of Cdc25C on NH_2-terminal serine and threonine residues increases its intrinsic phosphatase activity (*1*). In contrast, phosphorylation of Cdc25C on Ser^{216} throughout interphase appears to negatively regulate Cdc25C. Our results suggest that the negative effects of Ser^{216} phosphorylation may be mediated by 14-3-3 binding. We have demonstrated that 14-3-3 is bound to Cdc25C during phases of the cell cycle when Cdc25C is phosphorylated on Ser^{216} and functionally inactive and is released in mitosis when Cdc25C is maximally active and not phosphorylated on Ser^{216}. We propose that Ser^{216} phosphorylation and 14-3-3 binding sequester Cdc25C from functionally interacting with Cdc2 in vivo, because the phosphatase activity of Cdc25C was not detectably altered in response to either Ser^{216} phosphorylation or 14-3-3 binding (*14*).

The fission yeast homologs of 14-3-3, Rad24 and Rad25, have been shown to play a role in mitotic and radiation checkpoint control (*15, 16*). Loss of either gene causes early entry into mitosis and partial loss of the radiation checkpoint, similar to the phenotype reported here for cells expressing the Cdc25C mutant (S216A). An inability to inhibit fission yeast Cdc25 activity could account for the observed *rad24*$^+$ and *rad25*$^+$ mutant phenotypes. The Chk1 protein kinase is another essential component of the DNA damage checkpoint in fission yeast (*17–19*). Cells that lack *chk1*$^+$ are viable but fail to delay mitotic entry in response to damaged DNA and subsequently die. We tested whether Chk1 from *Schizosaccharomyces pombe* could phosphorylate Cdc25C in vitro (*20*). Chk1 phosphorylated both full-length Cdc25C and a GST fusion protein consisting of amino acids 200 to 256 of Cdc25C (Fig. 5A). Phosphoamino acid analysis revealed phosphoserine (*21*), and trypsin digestion of Cdc25C followed by high-pressure liquid chromatography (HPLC) analysis gave rise to a single phosphopeptide that eluted in fraction 57 (Fig. 5C). Sequencing of this tryptic phosphopeptide before and after digestion with proline-specific endopeptidase identified Ser^{216} as the site of phosphorylation. Human Chk1 also phosphorylated Cdc25C on Ser^{216}, demonstrating the conservation of this regulatory pathway (*22*). The ability of both fission yeast and human Chk1 to phosphorylate Cdc25C on Ser^{216} implicates Chk1 as possibly regulating the interactions between 14-3-3 and Cdc25C during a DNA damage checkpoint response.

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3. Myc-epitope–tagged Cdc25C and Cdc25(S216A) were subcloned into pUHD10-3 (*25*). Plasmids were cotransfected with pBabe, a plasmid encoding a puromycin resistance gene, into the HeLa tTA cell line (*23*). Clones resistant to G418 (geneticin, Gibco) and puromycin were screened for inducible expression of Myc-tagged Cdc25C or Cdc25(S216A). Clones were expanded in Dulbecco's minimum essential medium (DMEM) containing G418 (400 μg/ml), puromycin (1 μg/ml), and tetracycline (2 μg/ml). Cells were trypsinized and washed four times with warm DMEM lacking tetracycline to induce protein expression. Upon replating, cells were grown in DMEM containing G418 and puromycin. Indirect immunofluorescence indicated that 85 to 90% of cells were induced to express Cdc25C and Cdc25(S216A) at levels 10- to 50-fold as high as endogenous Cdc25C (*4*). In some cases, cells were subjected to a double-thymidine block (*7*) or were incubated during the last 8 to 16 hours of induction with nocodazole (0.15 μg/ml) (Calbiochem) followed by mechanical agitation. Cells were lysed in mammalian cell lysis buffer [50 mM tris (pH 8.0), 2 mM dithiothreitol (DTT), 5 mM EDTA, 0.5% NP-40, 100 mM NaCl, 1 μM microcystin, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin (0.15 U/ml), 20 μM leupeptin, and 20 μM pepstatin]. Antibodies used for Cdc25C detection included a monoclonal antibody to the Myc epitope (9E10 myc-agarose, Santa Cruz Biotechnology), a monoclonal antibody generated to Cdc25C (174E10-3), and an affinity-purified rabbit polyclonal antibody to glutathione-S-transferase (GST). 14-3-3 proteins were detected with antibody to 14-3-3 β (K-19, Santa Cruz), which is broadly reactive with members of the 14-3-3 family of proteins. Bound primary antibodies were detected with horseradish peroxidase–conjugated anti-rabbit or anti-mouse secondary antibodies (Cappel) and an ECL detection system (Amersham).
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5. Cells were incubated for 4 hours in phosphate-free media supplemented with ^{32}P-labeled inorganic phosphate (4 mCi/ml), 2 mM glutamine, and 1.5% dialyzed calf serum. Cells were lysed in 1 ml of mammalian cell lysis buffer, and Cdc25C was immunoprecipitated with antibody 174E10-3. Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and visualized by autoradiography. The nitrocellulose containing radiolabeled protein was excised, blocked with 0.5% polyvinylpyrrolidone (PVP-40) in 100 mM acetic acid for 30 min at 37°C, washed six times with water, and digested with TPCK trypsin (Worthington) at a final concentration of 30 mg/ml in 0.1 M NH_4HCO_3 (pH 8.0). Proteolysis and two-dimensional phosphopeptide mapping were performed as described (*24*).
6. Jurkat cells (1×10^9) were suspended in 5 ml of cell dissociation solution (Sigma Chemical) and elutriated at 25°C with a Beckman elutriator rotor (model J2-MI) in RPMI medium containing 1% fetal bovine serum. Cells were processed for flow cytometric analysis as described (*25*) with a Becton-Dickinson FACScan, and data were analyzed with CELL QUEST software.
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9. Uninduced and induced cells were incubated for 8 hours in the presence of nocodazole (0.15 μg/ml) and then processed for chromosome spreading as described (*8*). For monitoring the DNA replication checkpoint, we incubated cells in the presence of 1 mM hydroxyurea (Sigma Chemical). After 8 hours, nocodazole was added to a final concentration of 0.15 μg/ml. After an additional 8 hours, cells were collected and analyzed for amounts of Cdc25C by immunoblotting, for cyclin B1–associated histone H1 kinase activity as described (*7*), and for chromosome integrity as described (*8*). For monitoring the DNA damage checkpoint, we labeled cells for 60 min with 20 μM bromodeoxyuridine (BrdU, Amersham) and then treated them with 0 or 6 Gy of gamma irradiation. Cells were harvested at 0, 4, 8, 12, 16, and 24 hours after irradiation, stained with propidium iodide (PI), stained with fluorescein isothiocyanate (FITC)–conjugated monoclonal antibodies to BrdU, and processed for flow cytometric analysis as described (*26*). Because induction of the Cdc25(S216A) mutant protein occurred more quickly than for wild-type Cdc25C, inductions of Cdc25C were initiated at least 16 hours before Cdc25(S216A) inductions. In

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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13. Stocks (1 mM) of phosphorylated (GLYRSPpSMPENLNRPR) (E, Glu; G, Gly; L, Leu; M, Met; N, Asn; Y, Tyr) and unphosphorylated peptides consisting of amino acids 210 to 225 of Cdc25C were prepared in 50 mM sodium phosphate, pH 7.0, and 0.5% sodium azide (vehicle). Lysates of Sf9 insect cells overproducing GST-Cdc25C were incubated with glutathione agarose beads. Beads containing Cdc25C:14-3-3 complexes were washed three times with buffer A [1× phosphate-buffered saline, 1% NP-40, 50 mM NaF, 5 mM EDTA, 2 mM DTT, 1 μM microcystin, 2 mM PMSF, 20 μM leupeptin, 20 μM pepstatin, and aprotinin (0.15 U/ml)], and peptides were added at the indicated concentrations in a final volume of 200 μl of buffer A. Incubations were carried out at 4°C for 1 hour followed by three washes with buffer A. Reactions were resolved by SDS-PAGE, and GST-Cdc25C and 14-3-3 were visualized by immunoblotting with GST and K-19 antibodies, respectively.
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20. A recombinant baculovirus encoding GST fused to *S. pombe* Chk1 was generated by inserting the fission yeast *chk1* gene into pGEX2TN followed by cloning of GST-*chk1* into pFASTBAC1. Virus was generated as described by the manufacturer (Gibco-BRL). Recombinant GST-Chk1 kinase was isolated from infected Sf9 insect cells on glutathione (GSH) agarose. Cdc25C was cloned into pET15b (Novagen) and purified as a soluble hexahistidine (His_6) fusion protein as outlined by the manufacturer. GST fused to amino acids 200 to 256 of Cdc25C [GST-Cdc25C(200–256)] was isolated as described (*2*). Kinase reactions contained GST-Chk1 bound to GSH agarose and either His_6-Cdc25C or GST-Cdc25C(200–256) in a buffer consisting of 50 mM tris (pH 7.4), 10 mM $MgCl_2$, 10 μM adenosine triphosphate (ATP), 1 mM DTT, and 10 μCi of [γ-^{32}P]ATP. Reactions were analyzed directly or were first centrifuged to isolate supernatant and pelleted fractions. Radiolabeled proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and visualized by autoradiography. The nitrocellulose membrane containing radiolabeled His_6-Cdc25C was excised and treated as described (*5*). Further digestion on selected HPLC fractions was performed with 2 units of proline-specific endopeptidase (ICN) in 0.1 M sodium phosphate, 5 mM EDTA (pH 7.4) at 37°C for 16 hours. Reactions were acidified in 1% trifluoroacetic acid (TFA) and loaded onto a Vydac C18 column (25 cm by 0.46 cm inner diameter). Reverse-phase HPLC was performed at 37°C. Reactions were loaded in 0.1% TFA (buffer A) and eluted with a gradient from 0 to 60% buffer B (90% acetonitrile, 0.095% TFA). Fractions were collected at 0.5-min intervals up to 90 min and counted for radioactivity. Selected fractions were immobilized on Sequenlon-AA membrane discs (Millipore) for NH_2-terminal sequencing. Manual Edman degradation was performed as described (*27*) with a coupling and cleavage temperature of 55°C.
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A Cyanobacterial Phytochrome Two-Component Light Sensory System

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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-

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