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Plk Is a Functional Homolog of *Saccharomyces cerevisiae* Cdc5, and Elevated Plk Activity Induces Multiple Septation Structures

KYUNG S. LEE* AND RAYMOND L. ERIKSON

Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138

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Plk is a mammalian serine/threonine protein kinase whose activity peaks at the onset of M phase. It is closely related to other mammalian kinases, Snk, Fnk, and Prk, as well as to *Xenopus laevis* Plx1, *Drosophila melanogaster* polo, *Schizosaccharomyces pombe* Plo1, and *Saccharomyces cerevisiae* Cdc5. The M phase of the cell cycle is a highly coordinated process which insures the equipartition of genetic and cellular materials during cell division. To enable understanding of the function of Plk during M phase progression, various Plk mutants were generated and expressed in Sf9 cells and budding yeast. In vitro kinase assays with Plk immunoprecipitates prepared from Sf9 cells indicate that Glu206 and Thr210 play equally important roles for Plk activity and that replacement of Thr210 with a negatively charged residue elevates Plk specific activity. Ectopic expression of wild-type Plk (Plk WT) complements the cell division defect associated with the *cdc5-1* mutation in *S. cerevisiae*. The degree of complementation correlates closely with the Plk activity measured in vitro, as it is enhanced by a mutationally activated Plk, T210D, but is not observed with the inactive forms K82M, D194N, and D194R. In a *CDC5* wild-type background, expression of Plk WT or T210D, but not of inactive forms, induced a sharp accumulation of cells in G_1. Consistent with elevated Plk activity, this phenomenon was enhanced by the C-terminally deleted forms WTΔC and T210DΔC. Expression of T210D also induced a class of cells with unusually elongated buds which developed multiple septal structures. This was not observed with the C-terminally deleted form T210DΔC, however. It appears that the C terminus of Plk is not required for the observed cell cycle influence but may be important for polarized cell growth and septal structure formation.

Reversible protein phosphorylation has emerged as a major regulatory mechanism, owing largely to the finding that protein kinases are involved in nearly all stages of cellular responses to a variety of extracellular signals, including the control of cell proliferation, differentiation, and organization of cellular structures. Protein kinases exert their regulatory effects by covalently phosphorylating other proteins and are also frequently regulated by phosphorylation.

Recently, a subfamily of protein kinases, which includes the products of the mammalian genes *PLK* (3, 10, 12, 15, 22), *SNK* (35), *FNK* (5), and *PRK* (24) and of *Xenopus laevis PLX1* (21), *Drosophila melanogaster polo* (25), *Schizosaccharomyces pombe plo1*$^+$ (28), and the *Saccharomyces cerevisiae* cell cycle gene *CDC5* (20), has been identified by molecular cloning and genetic analyses. Comparison of their deduced amino acid sequences reveals 50 to 65% identity in the N-terminal kinase domain. An extended conserved sequence exists through their C-terminal domains, with a strikingly conserved polo box (amino acids 410 to 439 in Plk) (3, 12).

Plk is an M-phase-specific protein kinase whose activity peaks at the onset of mitosis. Plk enzymatic activity gradually decreases as the M phase proceeds but persists longer than cyclin B-associated Cdc2 kinase activity (23). Plk is localized to the area surrounding the chromosomes in prometaphase, appears condensed as several discrete bands along the spindle axis at the interzone in anaphase, and finally concentrates at the midbody during telophase and cytokinesis (10, 23). In late M phase, a mitotic motor protein, CHO1/MKLP-1, colocalizes at the interzone and midbody with Plk. Furthermore, it interacts with Plk in vivo and is phosphorylated by Plk-associated kinase activity in vitro (23, 40). The expression, activation, and subcellular localization of Plk suggest that it plays an important role in normal M phase progression.

SNK (35), *FNK* (5), and *PRK* (24) are immediate-early genes inducible by serum. Snk becomes active during the early G_1 phase of the cell cycle (24a). Although Plk, Snk, Fnk, and Prk show high structural similarity throughout their entire amino acid sequences, the tissue distribution patterns of their mRNA expression are distinct, suggesting that these kinases have unique physiological roles in different cells or in a distinct phase of the cell cycle.

Mutations in *polo* cause abnormal mitotic and meiotic divisions due to abnormal spindle formation. This defect appears to lead to the production of polyploid cells. *polo* transcripts are abundant in tissues and at developmental stages in which there is extensive mitotic activity (25, 37). The *polo*-encoded product shows cyclical kinase activity, which peaks at late anaphase/telophase during the rapid cycles of mitosis in syncytial *Drosophila* embryos (7). *S. pombe plo1*$^+$ is an essential gene. Loss of Plo1 function leads to a mitotic arrest in which condensed chromosomes are associated with a monopolar spindle. It also results in the failure of F-actin ring formation and septal material deposition, both of which are required for septum formation and cell cleavage. Its overexpression, however, induces the formation of monopolar spindles and multiple septa without nuclear division (28). The *S. cerevisiae CDC5* is an essential gene for cell division. *CDC5* mRNA accumulates periodically at the G_2/M boundary. Cells depleted of Cdc5 result in a dumbbell-shaped terminal morphology with the nuclei almost divided but still connected by a thin bridge of chromatin (20), suggesting that its activity is required for the progression of M phase.

The M phase of the cell cycle is a carefully orchestrated

* Corresponding author. Mailing address: Dept. of Molecular and Cellular Biology, Harvard University, 16 Divinity Ave., Cambridge, MA 02138. Phone: (617) 495-9686. Fax: (617) 495-0681. E-mail: kslee@hubio2.harvard.edu.

process which requires a complex series of biochemical and motile events to ensure the equipartition of genetic and cellular materials. Although it is apparent from genetic and biochemical analyses in yeast and *Drosophila* that Plk may function in late M phase, a recent report (21) suggested that a Plk homolog (Plx1) from *Xenopus* phosphorylates and activates Cdc25 phosphatase, which, in turn, activates Cdc2. Thus, further characterization of Plk may provide important insights into the regulation of M phase.

Phosphorylation within the L_{12} loop, which connects protein kinase subdomains VII and VIII, is emerging as a regulatory mechanism common to many protein kinases. Activating phosphorylation sites for cyclic AMP-dependent protein kinase (cAPK), Cdc2, Cdk2, Erk1/2, Rsk, and Mek1 are present within this loop (for a review, see reference 26). In the case of Mek1, mutation of the two serine residues (S218 and S222) in this region to negatively charged Asp residues (Mek1S218D S222D) mimics the effect of phosphorylation and activates the capacity of Mek1 to phosphorylate mitogen-activated protein kinase several hundredfold (16). In all the members of the polo subfamily, Glu (E206 in Plk) and Thr (T210 in Plk) are found at these homologous sites. To aid in our understanding of the function of Plk, various Plk mutants were generated at these sites and expressed in *S. cerevisiae*. No other sequences closely related to Cdc5, Plk, or the polo box have been found in the Saccharomyces Genome Database (Stanford University, Stanford, Calif.), while at least three other closely related kinases, Snk, Fnk, and Prk, as well as distantly related ones, Sak-a and Sak-b (8), exist in mammalian cells. Thus, *S. cerevisiae* appears to be an ideal organism in which to study the function of various Plk mutants, permitting a simple interpretation of Plk expression.

In this communication, we demonstrate that ectopic expression of Plk complements the defect associated with the *cdc5-1* mutation in *S. cerevisiae*. In addition, expression of mutationally activated Plk mutants influences the cell cycle and induces the formation of multiple septal structures. The functional complementation between mammalian Plk and yeast Cdc5 indicates that Plk is another protein kinase that regulates cellular events conserved between budding yeast and mammals.

MATERIALS AND METHODS

Strains, growth conditions, and transformations. Yeast strains used in this study are 1788 (isogenic diploid of EG123, *MAT*α *leu2-3,112 ura3-52 trp1-1 his4 can1*^{r}) (34) and KKY921-2B (*MAT***a** *cdc5-1 leu2 trp1 ura1*) (20). Yeast cells were cultured in YEP (1% yeast extract–2% Bacto Peptone) supplemented with 2% glucose, 2% raffinose (Sigma, St. Louis, Mo.), or 2% galactose (J. T. Baker, Phillipsburg, N.J.) as required. Synthetic minimal medium (33) supplemented with the appropriate nutrients was employed to select for plasmid maintenance. Yeast transformation was carried out by the lithium acetate method (17).

Generation of Plk mutants and expression in Sf9 cells. Site-directed mutagenesis in a murine *PLK* cDNA was carried out with the Sculptor in vitro mutagenesis system (Amersham International plc, Little Chalfont, Buckinghamshire, England). Mutations at the indicated sites were generated in the pBluescript II KS(+)-PLK construct. A 1.4-kb *Sac*I fragment isolated from each partially digested mutant clone was ligated into pAC702-HA-PLK (23) digested with *Sac*I and dephosphorylated. All the pAC702-HA-PLK mutant clones were sequenced to confirm the introduced mutations in the *PLK* coding sequence. A C-terminally deleted wild-type Plk (Plk WT) (WTΔC) was constructed by digesting pAC702-HA-PLK with *Sma*I (an additional *Sma*I site exists in the polylinker at the 3′ end of the *PLK* cDNA) and self-ligating the resulting fragment. This deleted amino acid residues 356 to 604 from the *PLK* coding sequence. Various Plk constructs were transfected into Sf9 cells with BaculoGold (Pharmingen, San Diego, Calif.).

Expression of Plk mutants in yeast cells. The YCplac111-GAL1 vector was generated by inserting an 800-bp *Eco*RI-*Bam*HI fragment of the *GAL1* promoter sequence from the YEplac181-GAL1 vector (a 0.8-kb *GAL1* promoter sequence is inserted at the *Eco*RI and *Bam*HI sites of YEplac181 vector [9], a gift of M. Michelitch, Harvard University, Cambridge, Mass.) into the corresponding sites in the polylinker of the YCplac111 vector (9). A 2.4-kb *Dra*I fragment which contains the entire coding sequence and 3′ untranslated region of *HA-PLK* was isolated from each pAC702-HA-PLK mutant construct and cloned into YCplac111-GAL1 digested with *Xba*I, end filled, and dephosphorylated. To construct C-terminally deleted Plk mutants, YCplac111-GAL1-HA-PLK clones were digested with *Sma*I (an additional *Sma*I site exists at the 3′ end of *PLK* cDNA) and self-ligated, resulting in the loss of amino acid residues 356 to 604 from the *PLK* coding sequence.

To express various Plk mutant proteins in *S. cerevisiae*, yeast transformants harboring each construct were grown in YEP-raffinose to an optical density at 600 nm (OD_{600}) of 0.8 at 30°C. Cultures were then washed twice with water, resuspended in YEP-galactose at an OD_{600} of 0.05, and cultured continuously. All the *cdc5-1* transformants were cultured at 23°C. To examine the complementation of the *cdc5-1* defect by Plk expression, however, cells were cultured at 37°C.

A 2.82-kb fragment of *CDC5*, which contains the entire coding sequence, a 480-bp upstream sequence, and a 221-bp 3′ untranslated region, was amplified by PCR with 5′-TCCAAAATATAGAACGAATAAATATC-3′ and 5′-AAACGC TATATGAGAACTATTGAAAAGG-3′ as primers. Genomic DNA prepared from wild-type strain H4939-1b (a gift of L. Hartwell, University of Washington, Seattle, Wash.) was used as the template. A PCR product was cloned into YCplac111 vector digested with *Sma*I and dephosphorylated. Restriction and sequencing analyses confirmed that the cloned gene is *CDC5*. The introduction of single-copy *CDC5* fully complemented the defect associated with the *cdc5-1* mutation (data not shown). To generate a galactose-inducible *CDC5* construct, YCplac111-CDC5 was digested with *Dsa*I and *Eco*RI (an *Eco*RI site is present in the polylinker of the YCplac111 vector). A 2.4-kb fragment was isolated, end filled, and cloned into YCplac111-GAL1 digested with *Xba*I, end filled, and dephosphorylated. The resulting construct contains 41 bp of endogenous *CDC5* promoter sequence downstream of the *GAL1* promoter.

Immunoprecipitation and kinase assays. All the recombinant Plk proteins expressed in Sf9 cells and yeast cells are N-terminally tagged with the hemagglutinin (HA) epitope (HA-Plk) (23) and were immunoprecipitated by an affinity-purified anti-Plk antibody against 13 C-terminal amino acid residues (23) or by a monoclonal antibody, 12CA5, against the HA epitope peptide. Where indicated, anti-Plk antibody 8847 against the N-terminal sequence (13) (a gift of D. K. Ferris, National Cancer Institute, Frederick, Md.) was used to immunoprecipitate both HA-Plk and HA-PlkΔC proteins from yeast cell lysates. Yeast cells were lysed in TED buffer [40 mM Tris-Cl (pH 7.5), 0.25 mM EDTA, 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) (Pefabloc; Boehringer Mannheim, Indianapolis, Ind.), and 10 μg (each) of pepstatin A, leupeptin, and aprotinin/ml) with an equal volume of glass beads (Sigma). The obtained lysates were spun at 2,000 × *g* for 2 min to remove unbroken cells and beads. Supernatants were subjected to further centrifugation at 15,000 × *g* for 30 min to clarify heavy cellular materials. The resulting supernatants (S15) were diluted to 1 ml with TBSN buffer (20 mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EGTA, 1.5 mM EDTA, 0.5 mM Na_3VO_4, and 20 mM *p*-nitrophenyl phosphate [PNPP]) supplemented with protease inhibitors, then incubated with antibodies. Protein A-Sepharose 4B (Zymed, San Francisco, Calif.) was added, and the mixture was incubated for an additional hour to precipitate the antibodies.

For measuring the kinase activity of Plk, assays were carried out in a kinase cocktail (TBMD) (50 mM Tris-Cl [pH 7.5], 10 mM $MgCl_2$, 5 mM dithiothreitol, 2 mM EGTA, 0.5 mM Na_3VO_4, and 20 mM PNPP) supplemented with 3 μg of dephosphorylated casein (Sigma) and 25 μM ATP (5 μCi of [γ-^{32}P]ATP; 1 Ci = 37 GBq). Where indicated, purified tubulin (a gift of R. Kuriyama, University of Minnesota Medical School, Minneapolis, Minn.) was used as the substrate.

Western blot analyses. Western analyses were carried out as described previously (23). Affinity-purified Plk antibody or HA antibody was used at a concentration of 0.5 μg/ml. Proteins that interact with antibodies were detected by the enhanced chemiluminescence (ECL) Western detection system (Amersham, Arlington Heights, Ill.).

Flow cytometry analyses. To induce the expression of Plk, transformants were cultured in YEP-galactose as described above. At the indicated time points, cells were harvested. After being washed twice with H_2O, cells were fixed with 70% ethanol, then treated with RNase A (1 mg/ml) in phosphate-buffered saline (PBS) for 30 min at 37°C. After the cells were disrupted for 1 min with a sonicator (model W-375; Heat Systems-Ultrasonics Inc., Plainview, N.Y.), cells were stained with propidium iodide (50 μg/ml) in PBS. Flow cytometry analyses were performed with a Cellquest program (Becton Dickinson, San Jose, Calif.).

Cell staining and immunofluorescence microscopy. Yeast transformants were cultured in YEP-galactose to induce the expression of Plk proteins as described above. Formaldehyde was added directly to culture medium to a final concentration of 3.7%, mixed, and allowed to fix for 2 h. After the cells were washed with PBS twice, they were subjected to various stainings.

Indirect immunofluorescence was performed as described previously (30). For visualization of bud scars and other cell wall chitin patches, cells were stained with 0.01% Calcofluor (Fluorescent Brightener 28; Sigma) for 5 min. Fluorescence microscopy was performed with a Nikon Microphot SA microscope with a 63× Plan-apo objective. For visualization of the neck filament-associated septin ring structures, immunolocalization for Cdc10 (19, 36) was carried out. Briefly, cells obtained as described above were treated for 30 min with 200 μg of Zymolyase-20T (ICN Immunobiologicals, Irvine, Calif.)/ml in a buffer containing 1.2 M sorbitol, 40 mM potassium phosphate (pH 6.5), and 0.5 mM $MgCl_2$, then washed and resuspended in PBS supplemented with 0.1% bovine serum albumin

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          ***          218  222   *     ***
Mek1  CDFGVSGQL--IDSMANSFVGTRSYMSPE

                     206  210
Plk   GDFGLATKVEYEGERKKTLCGTPNYIAPE
Plx1  GDFGLATKVEYDGERKKTLCGTPNYIAPE
polo  GDFGLATRIEYEGERKKTLCGTANYIAPE
Plo1  GDFGLAALLMDDEERKMTICGTPNYIAPE
Cdc5  GDFGLAAVLANESERKYTICGTPNYIAPE
Sak   ADFGLATQLNMPHEKHYTLCGTPNYISPE
Snk   GDFGLAARLEPLEHRRRTICGTPNYLSPE
Fnk   GDFGLAARLEPPEQRKKTICGTPNYVAPE
Prk   GDFGLAARLEPPEQRKKTICGTPNYVAPE
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FIG. 1. Sequence alignment between Mek1 and polo subfamily members. In all the members of the polo subfamily, Glu (E206 in Plk) and Thr (T210 in Plk) exist at the corresponding sites of Ser218 and Ser222 in Mek1. Sequences of various cDNAs can be found in the following references: Mek1 (4), Plk (22), Plx1 (21), polo (25), Plo1 (28), Cdc5 (20), Sak (8), Snk (35), Fnk (5), and Prk (24). Two distantly related protein kinases, Sak-a/b, do not contain the polo box but also have the conserved Glu and Thr present in polo subfamily members. Asterisks indicate the conserved residues present in protein kinase subdomains VII and VIII.

(Sigma). Affinity-purified rabbit polyclonal antibody against Cdc10 (a gift of John Chant, Harvard University, Cambridge, Mass.) was used at a 1:250 dilution. Fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Jackson Immunoresearch Laboratories, West Grove, Pa.) was used at a 1:200 dilution. For visualization of DNA, either 40 μg of propidium iodide (Sigma)/ml or 2.5 μg of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Calbiochem, San Diego, Calif.)/ml was added before the secondary antibody was washed with PBS containing 0.1% bovine serum albumin. Stained cells were viewed under a Zeiss LSM410 confocal microscope equipped with a krypton/argon laser.

RESULTS

In vitro kinase activity of Plk mutants expressed in Sf9 cells. Phosphorylation within the L_{12} loop which resides between protein kinase subdomains VII and VIII has been shown to be important for activation of several protein kinases. In the case of Mek1, mutation of the two serine residues (S218 and S222) in this region to negatively charged Asp residues (Mek1S218D S222D) activates Mek1 several hundredfold (16). In all the members of the polo subfamily, Glu (E206 in Plk) and Thr (T210 in Plk) are found at these homologous sites (Fig. 1). In an attempt to understand the significance of these residues for Plk enzymatic activity, various mutations were introduced into these sites by using a murine *PLK* cDNA. Mutant proteins expressed in Sf9 cells were immunoprecipitated and subjected to immune complex kinase assays. Mutation of Thr210 to Asp (T210D) increased the in vitro kinase activity of Plk toward casein fourfold, whereas mutation of the same amino acid residue to Glu (T210E) increased it marginally. In contrast, mutation of Thr210 to Val (T210V) decreased Plk activity threefold (Fig. 2A). These data indicate that Thr210 plays an important role for Plk activity and that a negatively charged amino acid residue can substitute for its function. Since Mek1 requires phosphorylation at both Ser218 and Ser222 to achieve maximum activation (16), we examined whether the acidic residue immediately upstream, Glu206, present in the Plk wild-type sequence, is important for Plk activity. Mutation of Glu206 to Val (E206V), but not to Asp (E206D), decreased Plk activity threefold, suggesting the importance of a negatively charged residue at this position (data not shown). Moreover, a double mutant, E206V T210V, showed much less activity than either E206V or T210V (Fig. 2A and data not shown), indicating that Glu206 and Thr210 play equally important roles for the kinase activity of Plk.

A point mutation of the conserved Asp residue in the DFG sequence of kinase subdomain VII to Asn in *S. cerevisiae* Cdc28, mammalian Cdc2, and Cdks generates dominant-negative forms (27, 41). The mutation of the corresponding Asp to Arg, which is present in the *cdc5-6* mutant, results in an inactive enzyme (20). As expected, replacement of Asp194 with Asn or with Arg (D194N or D194R) abolished the kinase activity of Plk (Fig. 2A). Plk WT with the C-terminal domain, which includes the polo box, deleted (WTΔC) possessed threefold-increased kinase activity when expressed in Sf9 cells (Fig. 2B).

Plk complements the defects associated with *S. cerevisiae* *cdc5-1*. *CDC5* is apparently a unique gene in *S. cerevisiae*, since no other related sequences have been found in the Saccharomyces Genome Database. In contrast, several Plk-related kinases, such as Snk (35), Fnk (5), Prk (24), and Sak-a/b (8) exist in mammalian cells. We reasoned that yeast may be an ideal organism in which to study the function of Plk, since the likelihood of nonspecific effects by expression of Plk mutants may

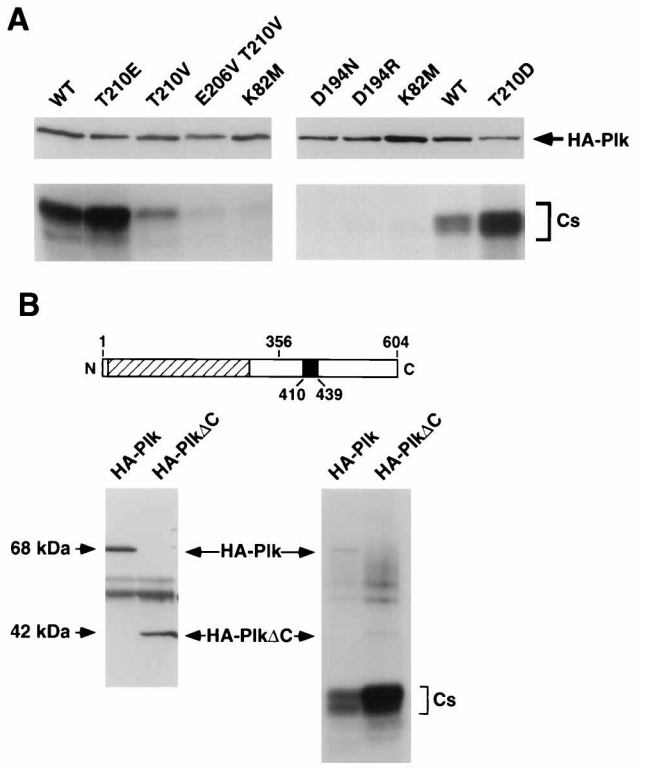

FIG. 2. In vitro kinase activity of Plk mutants expressed in Sf9 cells. HA-Plk mutant proteins were immunoprecipitated from Sf9 cells expressing various recombinant HA-Plk mutants, then subjected to kinase assays using casein as the substrate. (A) Casein phosphorylation activities of various Plk mutants. HA-Plk mutant proteins were immunoprecipitated by anti-Plk antibody. The kinase reaction mixtures were electrophoresed, and the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore). The two sets of gels are from two independent experiments. The blot was subjected to immunoblotting with anti-HA antibody to determine the amount of HA-Plk present in each immunoprecipitate (top panels) and was also exposed to detect casein (Cs) phosphorylation activities (bottom panels). (B) Casein phosphorylation activities by wild-type HA-Plk (WT) and its C-terminal deletion mutant, HA-PlkΔC. (WTΔC). The diagram at the top shows the region of C-terminal deletion. A hatched box denotes the kinase domain, while a solid box (amino acid residues 410 to 439) denotes the polo box. PlkΔC has lost amino acid residues 356 to 604, an area which includes the highly conserved polo box. The gels at the bottom show the results of immunoblotting with anti-HA antibody to determine the amount of HA-Plk and HA-PlkΔC immunoprecipitated (left panel) and detection of casein phosphorylation activities with the same blot (right panel).

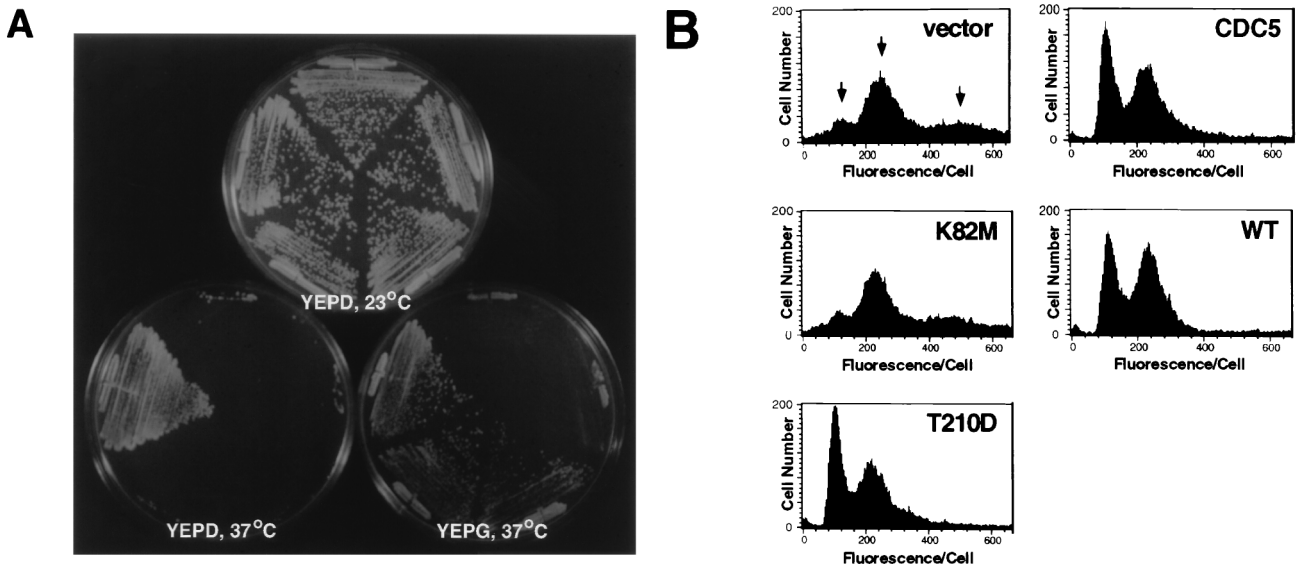

FIG. 3. Complementation of the *cdc5-1* defects by ectopic expression of Plk. (A) A *cdc5-1* mutant strain (KKY921-2B) transformed with various YCplac111-GAL1-HA-PLK mutants or YCplac111-CDC5 was selected on synthetic minimal plates lacking leucine. Transformants were streaked onto either YEP-glucose (YEPD) or YEP-galactose (YEPG) plates and incubated for 3 days at the indicated temperatures. Plasmids transformed are (clockwise from top on each plate) YCplac111-GAL1 vector, YCplac111-GAL1-HA-PlkK82M, YCplac111-GAL1-HA-Plk, YCplac111-GAL1-HA-PlkT210D, and YCplac111-CDC5. Cdc5 protein is expressed under its endogenous promoter and fully complements the *cdc5-1* defect (data not shown). Thus, it serves as a positive control. (B) Flow cytometric analyses of the *cdc5-1* cells expressing various forms of Plk mutants. Cells were cultured at 23°C in YEP-raffinose medium to an OD_{600} of 0.8. After the cultures were washed twice with water, cells were diluted in YEP-galactose to an OD_{600} of 0.05 and cultured continuously at 37°C for an additional 10 h. Then the cells were harvested, fixed, and analyzed. The first peak (first arrow) in the vector panel represents G_1 cells (1N), while the second peak (second arrow) represents G_2/M cells (2N). Consistent with a previous report (20), cells with more than a 2N DNA content (a broad cell population with the third arrow) are also apparent. Expression of Plk WT or T210D restores the *cdc5-1* cell division defect to a similar extent as expression of endogenous CDC5. CDC5, YCplac111-CDC5; vector, YCplac111-GAL1 vector; K82M, YCplac111-GAL1-HA-PlkK82M; WT, YCplac111-GAL1-HA-Plk; T210D, YCplac111-GAL1-HA-PlkT210D.

be diminished in yeast. Thus, we expressed various Plk mutants under the control of the *GAL1* promoter and asked (i) whether Plk WT or mutationally activated Plk is able to complement the defect associated with the *cdc5-1* mutation and (ii) whether expression of Plk mutants influences the cell cycle, cytoskeleton structures, or morphology.

Plk WT and mutant proteins were conditionally expressed under the control of the *GAL1* promoter. Expressed Plk protein was detected in YEP-galactose medium but not in YEP-glucose medium (data not shown). To examine whether Plk complements the defect associated with the *cdc5-1* mutation, cells were transformed with various YCplac111-GAL1-HA-PLK mutant constructs and selected on synthetic minimal plates lacking leucine. Obtained transformants were streaked on YEP-glucose or YEP-galactose plates and incubated for 3 days. As a comparison, the *cdc5-1* mutant transformed with YCplac111-CDC5, which expresses Cdc5 under the endogenous promoter, was also cultured. Expression of Plk WT and T210D complemented the cell division defect of the *cdc5-1* mutant to a similar extent as endogenous Cdc5, whereas K82M (an inactive form of Plk due to the mutation of the Lys82 in the ATP binding motif to Met [23]), D194N, and D194R did not (Fig. 3A and data not shown). However, WTΔC and the C-terminally deleted T210D (T210DΔC) complemented the *cdc5-1* defect to a lesser extent than the full-length forms (data not shown).

To quantitatively determine the complementation capability of Plk toward the *cdc5-1* defect, we examined cell doubling time, cell cycle profile, and cell volume changes upon expression of various Plk mutants at 37°C in YEP-galactose medium. Under these conditions, the *cdc5-1* mutant grew marginally, while the *cdc5-1* mutant transformed with YCplac111-CDC5 had a doubling time of 3.5 h. Expression of either Plk WT or T210D completely restored cell doubling time to that of cells expressing endogenous Cdc5, whereas expression of K82M, D194N, or D194R did not (data not shown). This suggests that expression of Plk active forms (Plk WT and T210D) is fully capable of complementing the cell division defect associated with the *cdc5-1* mutation.

To examine further whether the cell cycle defect observed in the *cdc5-1* mutant was also restored by Plk expression, we carried out flow cytometry analyses for various *cdc5-1* transformants cultured at 37°C in YEP-galactose medium. The majority of the *cdc5-1* mutant cells transformed with vector arrested when cells had achieved a 2N DNA content (after DNA replication). However, the introduction of YCplac111-CDC5 complemented the *cdc5-1* cell division defect, resulting in the regeneration of 1N DNA-containing populations (before DNA replication). Expression of Plk WT or T210D also restored this defect, whereas K82M, D194N, or D194R did not (Fig. 3B and data not shown). It is noteworthy that the complementation of the *cdc5-1* defect by T210D was marginally better than that by Plk WT. At the restricted temperature, the cdc5-1 mutant shows an enlarged and elongated cell morphology. This phenotype was also completely reversed by Plk WT or T210D, but not by K82M, D194N, or D194R, as judged by the profile of cell volume analyzed by flow cytometry analyses (data not shown).

Plk activity correlates with its capability to complement the *cdc5-1* defect. To examine whether the complementation of the *cdc5-1* cell division defect is dependent on Plk activity, the *cdc5-1* cells bearing various YCplac111-GAL1-HA-PLK mutant plasmids were cultured under inducing conditions for 10 h and harvested. After the cellular lysates were clarified to remove insoluble materials, we carried out Plk immune complex kinase assays using casein as the substrate. In agreement with

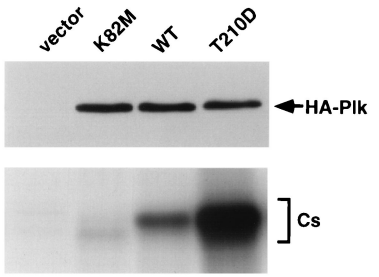

FIG. 4. Plk activity correlates closely with its capability to complement the *cdc5-1* defect. A *cdc5-1* mutant strain (KKY921-2B) transformed with various YCplac111-GAL1-HA-PLK mutants was cultured as described in the legend to Fig. 3B. The lysates were centrifuged at 15,000 × *g* for 30 min to clarify heavy cellular materials. From 500 μg of cellular proteins present in the S15 fraction, various HA-Plk mutant proteins were immunoprecipitated with anti-Plk antibody and subjected to in vitro kinase assays using casein (Cs) as the substrate. To determine the amount of HA-Plk proteins immunoprecipitated, immunoblotting was carried out with anti-HA antibody. Lane designations are as defined in the legend to Fig. 3B.

the results obtained from their expression in Sf9 cells (see Fig. 2A), casein phosphorylation activities were detected in Plk WT and T210D but not in the K82M, D194N, and D194R transformants (Fig. 4 and data not shown). The increased Plk activity of the T210D mutant was also consistent with the elevated activity of this mutant, previously observed in Sf9 cells (see Fig. 2A).

Ectopic expression of Plk in a *CDC5* wild-type background causes cells to accumulate in G_1. To study the phenotypes associated with Plk expression in a *CDC5* wild-type genetic background, various forms of Plk were expressed under *GAL1* control in a diploid wild-type strain, 1788. Expression of Plk WT partially inhibited cellular proliferation (Fig. 5A). This inhibition was enhanced by the expression of T210D but was not observed with kinase-inactive K82M. It was further enhanced by WTΔC or T210DΔC. In the case of the T210DΔC transformants, cell doubling time became lengthened gradually upon induction and never reached a saturation point, even after culturing for 41 h (Fig. 5A). Flow cytometry analyses revealed that expression of Plk active forms resulted in the accumulation of cells in G_1. This phenomenon was enhanced by the expression of WTΔC or T210DΔC (Fig. 5B) and was manifest 8 h after the cultures were shifted to YEP-galactose medium. Similar results were obtained with a haploid wild-type strain (data not shown), indicating that this is not ploidy specific. However, this phenotype was not observed in a *cdc5-1* background at the permissive temperature of 23°C. This may be due to crippled kinase activity of *cdc5-1* cells even under this condition. This speculation was supported by the observation that more than 70% of the *cdc5-1* cells still accumulate in a 2N DNA content (after DNA replication) when cultured at 23°C (data not shown). The expression of activated forms of Plk in a *CDC5* wild-type background results in inhibition of cellular proliferation, which correlates closely with accumulation of cells in G_1.

In *S. cerevisiae*, a cell coordinates growth and division at a single point, Start. Thus, the events that form the cell shape may be correlated to progression of the cell cycle. For example, DNA replication occurs at about the same time as bud emergence, and nuclear division occurs shortly before cytokinesis and septum formation. However, growth and division are not completely coupled processes inasmuch as growth can continue in cells blocked for division (18). To examine whether the influence of Plk on the cell cycle is coupled with cell growth, we

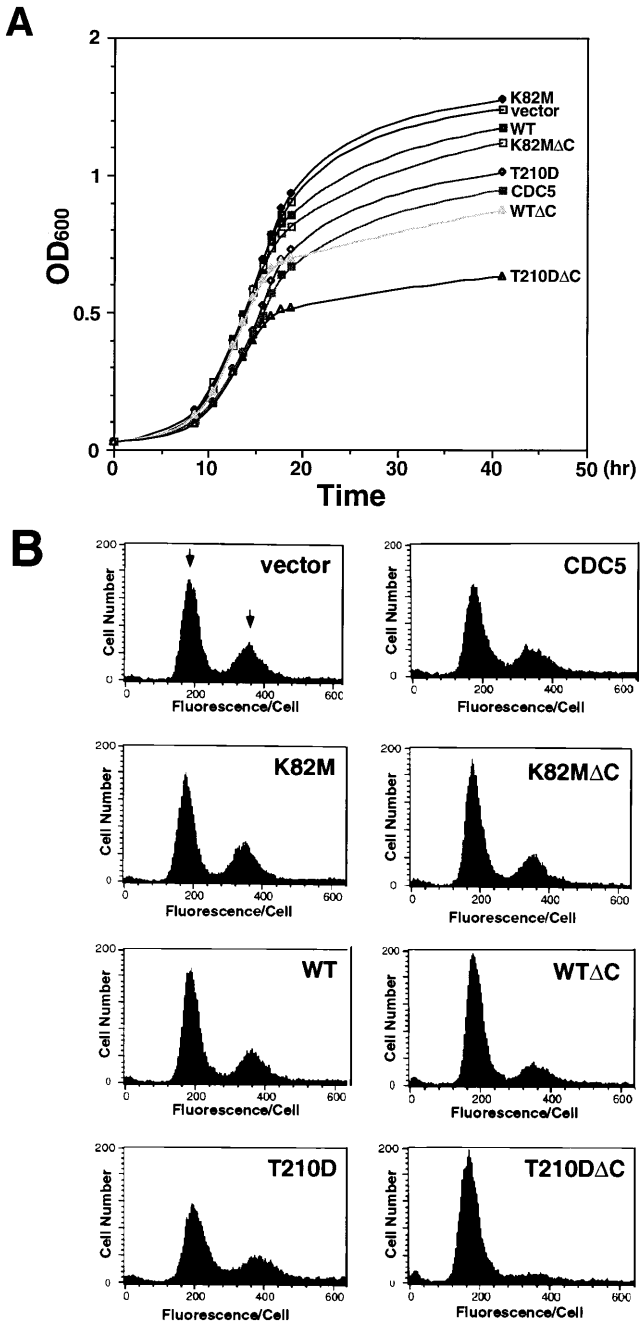

FIG. 5. (A) Expression of activated forms of Plk in a *CDC5* wild-type background inhibits cellular proliferation. A diploid wild-type strain, 1788, transformed with YCplac111-GAL1-CDC5 and various YCplac111-GAL1-HA-PLK mutants was cultured at 30°C in YEP-raffinose medium to an OD_{600} of 0.8. After the cultures were washed twice with water, cells were resuspended in YEP-galactose at an OD_{600} of 0.03 and cultured continuously. OD_{600} was measured at the indicated time points after the cultures were shifted to YEP-galactose. Like expression of Plk active forms, overexpression of Cdc5 inhibited cellular proliferation. The graphs were generated with the Cricket Graph program. (B) Expression of the activated forms of Plk results in the accumulation of cells in G_1. Cells cultured under inducing conditions for 8 h were harvested and subjected to flow cytometric analyses. We chose the cells induced for 8 h, since the inhibition of cellular proliferation was evident at this time point. The first peak (first arrow) in the vector panel represents G_1 cells (2N), while the second peak (second arrow) represents G_2/M cells (4N). vector, YCplac111-GAL1 vector; K82M (or K82MΔC), YCplac111-GAL1-HA-PlkK82M (or -HA-PlkK82MΔC); WT (or WTΔC), YCplac111-GAL1-HA-Plk (or -HA-PlkΔC); T210D (or T210DΔC), YCplac111-GAL1-HA-PlkT210D (or -HA-PlkT210DΔC); CDC5, YCplac111-GAL1-CDC5.

TABLE 1. Enrichment of unbudded cells by elevated Plk activity[a]

plasmid	(%) cells				
	⊙	◔	◑	◕	◉+
vector	58.0	25.8	4.3	11.9	0
K82M	61.2	25.0	4.1	9.7	0
WT	64.5	23.4	2.6	9.5	0
T210D	71.3	14.3	3.1	5.4	5.9
K82MΔC	60.9	24.1	3.9	11.1	0
WTΔC	74.7	16.4	3.7	5.2	0
T210DΔC	85.2	9.9	1.5	3.4	0

[a] A diploid wild-type strain, 1788, transformed with various Plk mutants was cultured in YEP-galactose at 30°C for 8 h. Cells were then fixed, stained with DAPI, and counted. More than 1,500 cells were counted for each type of transformant. The cells with elongated buds were present only in the T210D transformants.

counted cells in various budding stages. After induction for 8 h, 58 to 61% of wild-type cells transformed with vector, K82M, or K82MΔC were unbudded. An increased number of cells harboring Plk WT or T210D were unbudded, a result which was further enhanced with the C-terminal deletion mutants (Table 1). Thus, it appears that the accumulation of cells in G_1 by expression of various Plk mutants correlates closely with the enrichment of unbudded cells. One notable observation was the presence of cells with unusually elongated buds in the T210D transformants. About 21% of the budded cells and 41% of the large budded cells (5.9% of the total population) showed this unusual morphology, which was not observed in other Plk transformants.

The kinase activity of Plk correlates with its influence in the cell cycle. To examine whether the observed influence of the Plk mutants on the cell cycle is directly related to the increased kinase activity of Plk, the wild-type strain 1788 harboring various Plk mutants was cultured under inducing conditions for 8 h, harvested, and lysed. The obtained cellular lysates were subjected to centrifugation at 15,000 × *g* for 30 min to clarify heavy cellular materials. The levels of various Plk mutant proteins present in the total lysates varied, as it is apparent that kinase-inactive forms are more abundantly expressed than active forms (Fig. 6A). This observation implies that cells have a lower tolerance for kinase-active forms of Plk than for kinase-inactive forms, since their expression inhibits cellular proliferation (see Fig. 5A). Both the full-length and the C-terminally deleted forms of Plk were found in the pellet (P15) fraction from the 15,000 × *g* speed spin and were found at low levels in the supernatant (S15) fractions (data not shown). This indicates that Plk is present in large structures under these conditions of cell lysis. Attempts to solubilize Plk from P15 fractions with either 0.5 M NaCl plus 0.1% Brij 35 or 1% Nonidet P-40 plus 0.1% deoxycholate failed (data not shown). The nature of these complexes is under investigation.

To examine whether the influence of Plk on the cell cycle correlates with its kinase activity, Plk activity present in the S15 fraction was measured by in vitro immune complex kinase assays. Plk WT prepared from Sf9 cells phosphorylates purified tubulin 10-fold more efficiently than casein in vitro, while K82M does not (22a). We do not know the physiological significance of the efficient tubulin phosphorylation by Plk, however. From an equal quantity of the cellular proteins present in the S15 fractions, Plk was immunoprecipitated and subjected to kinase assays with tubulin as the substrate. In comparison to that of Plk WT, elevated Plk activity was found in the T210D transformants. Consistent with the observation made in Sf9 cells, a further increase in Plk activity was manifested by the C-terminally deleted T210DΔC mutant (Fig. 6B). The cell cycle influences observed with Plk expression correlate closely with an increase in the kinase activity. This suggests that expression of active forms of Plk is inhibitory for cellular proliferation and that the increased Plk activity is responsible for the enhancement of G_1 accumulation. It is notable that the amounts of various Plk mutants expressed appear to be inversely correlated with their specific activities (Fig. 6A and B).

Plk expression induces multiple septation structures. In an unbudded *S. cerevisiae* cell, a ring of chitin, which can be stained by the fluorescent dye Calcofluor, forms in the cell wall within which the bud emerges. A chitin-rich septal structure forms within the confines of the chitin ring as an early step in the separation of mother and daughter cells (31). After division, the chitin ring and septal structure remain on the mother cell as bud scars (30). Overexpression of Plo1 in *S. pombe* induces formation of multiple septa without nuclear division (28). To investigate whether there are any morphological phe-

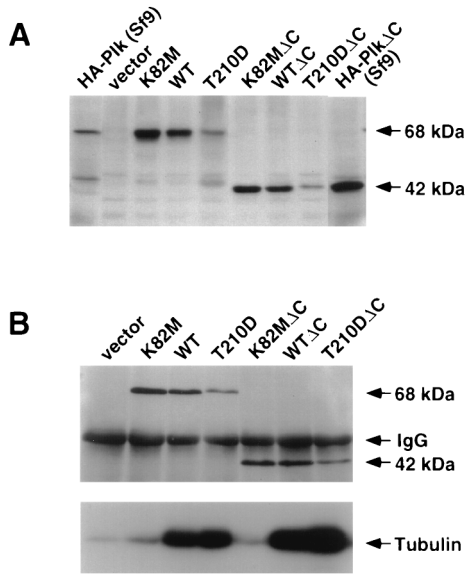

FIG. 6. (A) Expression of various Plk mutants in a wild-type *S. cerevisiae* strain. Yeast extracts from the 1788 strain harboring various HA-Plk mutants were prepared after the cells were cultured under inducing conditions for 8 h. An equal amount (100 μg) of total cellular proteins prepared from strain 1788 cells expressing various Plk mutants was loaded on each lane. For comparison, total cellular proteins prepared from Sf9 cells expressing recombinant HA-Plk or HA-PlkΔC were loaded as indicated. After the proteins were transferred onto a PVDF membrane, proteins interacting with anti-HA antibody were detected by immunoblotting. (B) Kinase activities of various Plk mutant proteins expressed in a wild-type *S. cerevisiae* strain. From 500 μg of the cellular proteins present in the S15 fractions, HA-Plk or HA-PlkΔC mutant proteins were immunoprecipitated with the anti-Plk antibody 8847 and subjected to in vitro kinase assays using purified tubulin as the substrate. Reaction mixtures were electrophoresed, and the proteins were transferred onto a PVDF membrane and exposed to detect tubulin phosphorylation activities. The same blot was subjected to immunoblotting with anti-HA antibody to determine the amount of HA-Plk or HA-PlkΔC protein present in each immunoprecipitate. Lane designations are as defined in the legend to Fig. 5.

notypes associated with G_1 accumulation and whether Plk expression induces multiple septal structures, wild-type 1788 cells transformed with various forms of Plk mutant were examined for the distribution of chitin and septin ring components. In dividing vector-transformed cells, strong Calcofluor staining was manifest at the bud neck. In the case of cells transformed with T210D, about 50% of cells with elongated buds had multiple chitin bands along the long bud projection, a configuration never seen in wild-type cells. Often multiple chitin patches were less distinct but broader (Fig. 7A). Notably, we failed to observe multiple chitin bands in the cells transformed with T210DΔC, even though its kinase activity is higher than that of T210D.

The apparent multiple bands of chitin observed in the T210D transformants led us to examine the distribution patterns of a septin component, Cdc10 (19, 36). In the control cells transformed with vector or K82M, only one septin ring structure was manifest at the bud neck; this becomes a pair of rings prior to and during cell cleavage (11). However, among the long-budded cells transformed with T210D, two or multiple septin ring structures were apparent. This phenotype was enhanced when cells were transformed with YEplac111-GAL1-HA-PlkT210D, which replicates in multiple copies. Among the cells with elongated buds, more than 50% showed multiple septal structures. Two distantly placed septin rings were manifest in a cell with one nucleus or two already-divided nuclei. The extra septin ring was placed either between the two divided nuclei or on the other side of a nucleus present in the long bud. Often, several septin ring structures along the elongated bud were evident in the dividing cells with two nuclei (Fig. 7B). However, in these cells, we failed to observe a pair of distinct septin rings which are present in the cells with normal cell cleavages. This suggests that cleavage planes are not fully developed in these cells. In the rest of the long-budded cells, additional septin rings were weakly stainable or less distinct and were sometimes detected as punctate signals along the elongated bud. In contrast, we failed to observe additional septin ring structures in the cells transformed with T210DΔC.

DISCUSSION

Plk is activated by mutation of Thr210 to Asp. Thus far, the activation mechanism of Plk is not clearly understood. We previously observed that in NIH3T3 cells Plk activity is increased 26-fold at the G_2/M boundary in comparison to that at the G_1/S boundary, whereas the amount of Plk protein increased a modest fourfold. About a fivefold increase in Plk specific activity was achieved within 1 h prior to the G_2/M boundary (23). During mitosis, Plk is phosphorylated on serine in vivo, and treatment of mitotic Plk immunoprecipitates with phosphatase 2A reduces the total amount of Plk activity to five- to 10-fold (13). Taken together, it appears that Plk is activated by a posttranslational modification mechanism prior to the G_2/M transition. However, it is not known whether this is due to phosphorylation by an upstream kinase or to autophosphorylation enhanced by a nonkinase activator.

Phosphoamino acid analysis with in vivo-labeled Plk revealed the presence of phosphoserines (13). However, this result does not completely exclude the possibility that Thr phosphorylation may be involved in Plk activation. The fourfold activation achieved by mutation of Thr210 of Plk to Asp is comparable to that observed at the G_2/M boundary of cycling cells. In addition, mutation of Thr210 to Val decreases Plk activity threefold, whereas mutation to Glu slightly increases it. The elevated kinase activity resulting from the Asp substitution at Thr210 increases the likelihood that Thr210 may be phosphorylated in vivo. Mutation of Thr216 to Asp, Glu, or Val completely abolished the kinase activity, however (data not shown). Further mutational analyses revealed that a conserved negatively charged residue, Glu206, is as important as Thr210 for Plk activity. Thus, it is apparent that both Glu206 and Thr210 play important roles for the kinase activity of Plk.

In an inactive *cdc5-6* allele, Asp222 in the conserved DFG motif in the kinase subdomain VII is replaced with Arg (20). The equivalent Asp residue in cyclic AMP-dependent kinase is known to be essential in the phosphotransfer reaction (39). An Asp-to-Asn point mutation at this position has been identified in one of the two dominant-negative mutant alleles of *CDC28* in yeast (27), and an analogous mutation was introduced into Cdc2, Cdk2, and other Cdks to create dominant-negative forms (41). Plk mutated at the corresponding Asp194 to Asn (D194N) or Arg (D194R) becomes inactive when expressed in Sf9 cells or in yeast (Fig. 2A and data not shown). In the *cdc5-1* mutant, expression of D194N or D194R inhibited cellular proliferation, whereas vector or K82M did not. This phenotype was not observed in a Cdc5 wild-type background, however (data not shown). These data suggest that both D194N and D194R function as weak dominant-negative proteins which are able to compete with the kinase activity of *cdc5-1* cells, but not with that of wild-type Cdc5.

Plk is a functional homolog of *S. cerevisiae* Cdc5. In various eucaryotic cells, several genes which possess a high sequence identity to *Drosophila polo* were identified. These include *PLK*, *SNK*, *FNK*, *PRK*, *PLX1*, *plo1*$^+$, and *CDC5*. The extended identity beyond the catalytic domain strongly suggests that they are closely related family members. However, whether these kinases have similar biological functions in different organisms and whether they are functionally interchangeable are not known.

Plk and Cdc5 have 51% sequence identity in the catalytic domain and 57% identity in the polo box, which is present in the noncatalytic domain. When Plk WT and various Plk mutants were expressed under the control of the *GAL1* promoter, the degree of complementation of the *cdc5-1* cell division defect correlated closely with the kinase activity of Plk measured in vitro. Conversely, two mutationally inactivated Plk mutants, D194N and D194R, function as dominant-negative forms, albeit weakly, in a *cdc5-1* mutant, but not in a Cdc5 wild-type background. These data indicate that Plk is a functional homolog of *S. cerevisiae* Cdc5. Since Plk and Cdc5 are from two organisms separated by a great phylogenetic distance, complementation of the *cdc5-1* defect by Plk indicates that yet another highly conserved mechanism is used for driving M-phase progression in eucaryotic cells.

Apparently, the multiple septation phenotype induced by expression of a Plk activated form, T210D, in *S. cerevisiae* is similar to that of Plo1 in *S. pombe*. Thus, the primary structural homology found between Cdc5 and Plo1 may suggest a common functional significance. Recently, while describing a potential role for Cdc5 in DNA replication, Hardy and Pautz reported that *S. pombe* Plo1 and *Drosophila* polo do not complement the *cdc5-1* temperature-sensitive defect (14). Failure to complement the *cdc5-1* defect by Plo1 or polo expression may be the result of inability of Plo1 or polo to substitute for other essential roles of Cdc5 in S phase, as proposed earlier (14).

Recently, a Plk homolog (Plx1) in *Xenopus* was implicated as an activator of Cdc25 phosphatase, which, in turn, activates Cdc2 (21). The high degree of functional conservation among the eucaryotic cell cycle regulators implies that the upstream components leading to the activation of Mih1 (an *S. cerevisiae* functional homolog of the *S. pombe* mitotic inducer Cdc25 and

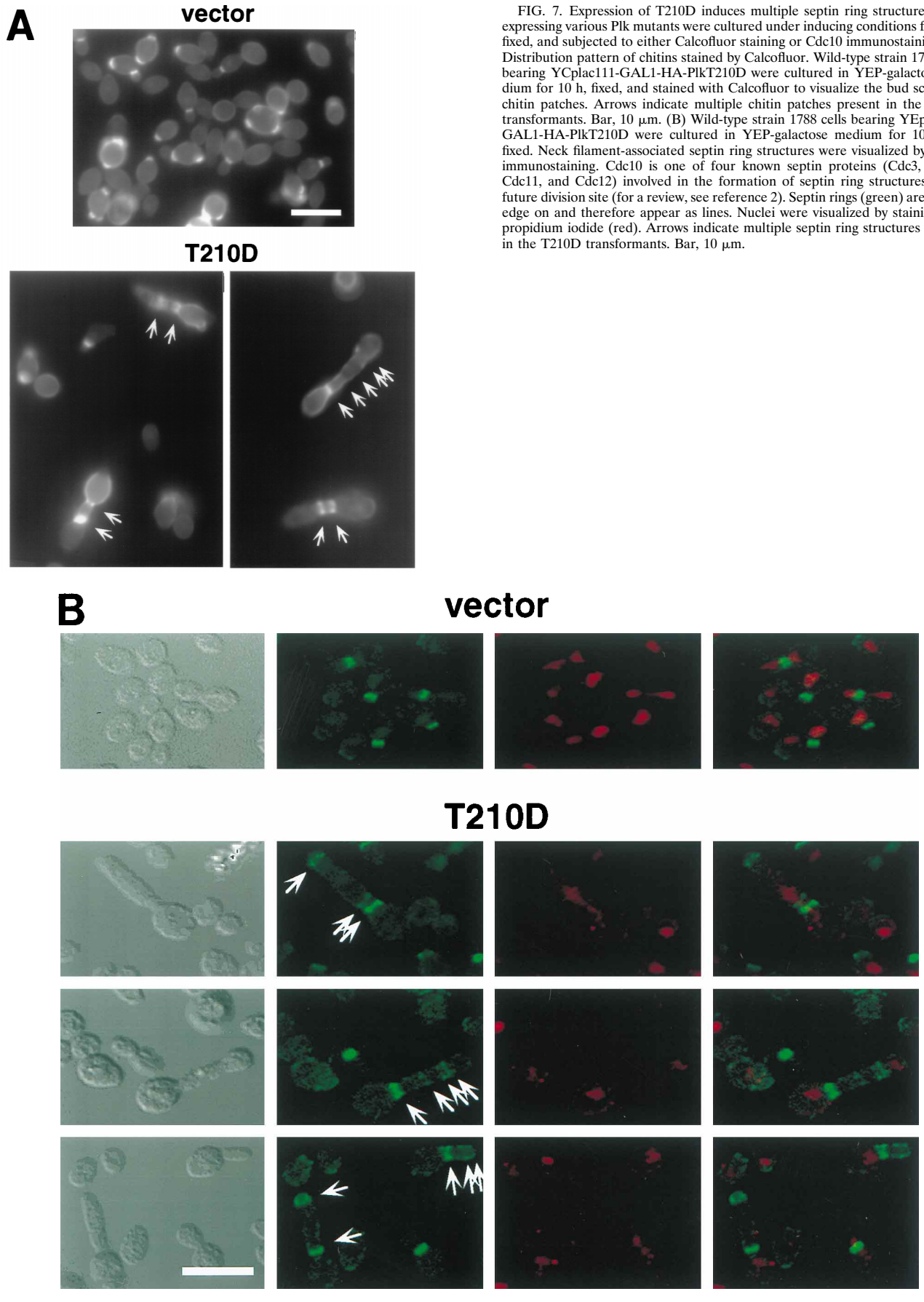

FIG. 7. Expression of T210D induces multiple septin ring structures. Cells expressing various Plk mutants were cultured under inducing conditions for 10 h, fixed, and subjected to either Calcofluor staining or Cdc10 immunostaining. (A) Distribution pattern of chitins stained by Calcofluor. Wild-type strain 1788 cells bearing YCplac111-GAL1-HA-PlkT210D were cultured in YEP-galactose medium for 10 h, fixed, and stained with Calcofluor to visualize the bud scars and chitin patches. Arrows indicate multiple chitin patches present in the T210D transformants. Bar, 10 μm. (B) Wild-type strain 1788 cells bearing YEplac111-GAL1-HA-PlkT210D were cultured in YEP-galactose medium for 10 h and fixed. Neck filament-associated septin ring structures were visualized by Cdc10 immunostaining. Cdc10 is one of four known septin proteins (Cdc3, Cdc10, Cdc11, and Cdc12) involved in the formation of septin ring structures at the future division site (for a review, see reference 2). Septin rings (green) are viewed edge on and therefore appear as lines. Nuclei were visualized by staining with propidium iodide (red). Arrows indicate multiple septin ring structures present in the T210D transformants. Bar, 10 μm.

the vertebrate Cdc25C protein phosphatase [32]) are likely to be conserved between yeast and vertebrates. Therefore, it will be interesting to examine whether an elevated Plk activity leads to the activation of Cdc28 (an *S. cerevisiae* functional homolog of the *S. pombe* and vertebrate Cdc2 protein kinase [1]) and suppresses the mitotic defect associated with a *cdc28-1N* mutation (29, 38).

Elevated Plk activity in a *CDC5* background induces accumulation of cells in G_1. The expression of Plk WT or T210D in a *CDC5* wild-type background results in the accumulation of the G_1 population. Consistent with an increase in kinase activity, these phenomena were further enhanced by the C-terminally deleted forms, indicating that the C terminus of Plk, which includes a highly conserved polo box, is dispensable for the observed G_1 accumulation effect (Fig. 5B). The observed G_1 accumulation does not appear to be the result of M phase acceleration, since no anuclear or unusually small cells which may be generated from a mitotic catastrophe were present in these populations. One possible explanation is that an unusually long G_1 phase may result from uncoordinated mitotic processes, which may be responsible for the increase in cell doubling time. Since both Cdc5 and Plk are regulated transcriptionally and are expressed in the late phases of the cell cycle, we cannot completely rule out the possibility that inhibition of cellular proliferation by elevated Plk activity may be partly due to the detrimental effect of unregulated Plk expression during all phases of the cell cycle.

Expression of T210D induces the elongated bud with multiple septin structures. *S. cerevisiae* displays pronounced cellular asymmetry during its normal growth and division. As a cell initiates the division cycle, polarized cell growth begins with an intrinsic spatial cue, established by cortical actin or septin cytoskeletal proteins. Polarization of actin directs secretion and cell surface expansion to produce bud growth (for a review, see reference 6). In a *CDC5* wild-type genetic background, expression of T210D induces cells with elongated buds. Strikingly, these cells were not present in the T210DΔC transformants. This finding led us to postulate that the C-terminal domain of Plk may be important for polarized cell growth by regulating cortical actin and septin cytoskeleton assembly. This hypothesis is supported by the observation that the formation of multiple septin ring structures is evident only in the T210D transformants, not in T210DΔC (Fig. 7B and data not shown).

The elongated bud phenotype associated with expression of T210D may be due to a mutationally acquired dominant-negative property caused by T210D, usurping common cellular proteins interacting with Cdc5. This is unlikely for two reasons. First, expression of T210D in the *cdc5-1* mutant does not cause any morphological or cell division cycle changes, but instead corrects the defect in M phase progression. Second, even Plk WT, when expressed in multiple copies under the control of the *GAL1* promoter (YEplac111-GAL1-HA-PLK), was also able to generate the elongated bud phenotype.

Once a yeast cell initiates a bud, a ring of septin proteins forms beneath the cell membrane at the future cleavage site. Four septin proteins (Cdc3, Cdc10, Cdc11, and Cdc12) are known and are components of the neck filaments encircling the mother bud neck. The septin ring defines the cleavage plane and functions as a scaffold for the recruitment of cytokinesis machinery to the mother bud neck (for a review, see reference 2). In *S. pombe*, loss of $plo1^+$ function leads to a defect in actin ring formation and septal material deposition. However, when Plo1 was overproduced, cells formed septa without nuclear division. Cell separation rarely followed, and cells with multiple septa accumulated (28). Similarly, in this study, expression of T210D induced the formation of multiple septin ring structures along the elongated bud. The presence of multiple septin rings implies that cells with elongated buds did not yet develop a fully functional cleavage plane for the completion of the septation processes. It is possible that the elevated Plk activity may result in the unbalanced progression of M phase, preventing cells from executing a normal cell cleavage process. Since cell growth and division are uncoupled processes after Start, failure of cell cleavage and continued cell growth may lead to further elongation of the bud. Moreover, unregulated Plk activity may have induced multiple cortical cues which initiate premature formation of septal precursor structures. Whether Plk directly regulates the components in the cortical cues through phosphorylation events, or whether the polo box in the C-terminal region of Plk plays a crucial role in interacting with these proteins, requires further investigation.

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REFERENCES

1. **Beach, D., B. Durkacz, and P. Nurse.** 1982. Functionally homologous cell cycle control genes in budding and fission yeasts. Nature (London) **300:**706–709.
2. **Chant, J.** 1996. Septin scaffolds and cleavage planes in Saccharomyces. Cell **84:**187–190.
3. **Clay, F. J., S. J. McEwen, I. Bertoncello, A. F. Wilks, and A. R. Dunn.** 1993. Identification and cloning of a protein kinase-encoding mouse gene, *Plk*, related to the *polo* gene of *Drosophila*. Proc. Natl. Acad. Sci. USA **90:**4882–4886.
4. **Crews, C. M., A. Allessandrini, and R. L. Erikson.** 1992. The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. Science **258:**478–480.
5. **Donohue, P. J., G. F. Alberts, Y. Guo, and J. A. Winkles.** 1995. Identification by targeted differential display of an immediate early gene encoding a putative serine/threonine kinase. J. Biol. Chem. **270:**10351–10357.
6. **Drubin, D. G., and W. J. Nelson.** 1996. Origins of cell polarity. Cell **84:**335–344.
7. **Fenton, B., and D. M. Glover.** 1993. A conserved mitotic kinase active at late anaphase-telophase in syncytial *Drosophila* embryos. Nature (London) **363:**637–640.
8. **Fode, C., B. Motro, S. Yousefi, M. Heffernan, and J. W. Dennis.** 1994. Sak, a murine protein-serine/threonine kinase that is related to the *Drosophila* polo kinase and involved in cell proliferation. Proc. Natl. Acad. Sci. USA **91:**6388–6392.
9. **Gietz, R. D., and A. Sugino.** 1988. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene **74:**527–534.
10. **Golsteyn, R. M., S. J. Schultz, J. Bartek, A. Ziemiecki, T. Ried, and E. A. Nigg.** 1994. Cell cycle analysis and chromosomal localization of human Plk1, a putative homologue of the mitotic kinases *Drosophila* polo and *Saccharomyces cerevisiae* Cdc5. J. Cell Sci. **107:**1509–1517.
11. **Haarer, B. K., and J. R. Pringle.** 1987. Immunofluorescence localization of the *Saccharomyces cerevisiae CDC12* gene product to the vicinity of the 10-nm filaments in the mother-bud neck. Mol. Cell. Biol. **7:**3678–3687.
12. **Hamanaka, R., S. Maloid, M. R. Smith, C. D. O'Connell, D. L. Longo, and D. K. Ferris.** 1994. Cloning and characterization of human and murine homologues of the *Drosophila polo* serine-threonine kinase. Cell Growth Differ. **5:**249–257.
13. **Hamanaka, R., M. R. Smith, P. M. O'Connor, S. Maloid, K. Mihalic, J. L. Spivak, D. L. Longo, and D. K. Ferris.** 1995. Polo-like kinase is a cell cycle-regulated kinase activated during mitosis. J. Biol. Chem. **270:**21086–21091.
14. **Hardy, C. F. J., and A. Pautz.** 1996. A novel role for Cdc5p in DNA replication. Mol. Cell. Biol. **16:**6775–6782.
15. **Holtrich, U., G. Wolf, A. Bräuninger, T. Karn, B. Böhme, H. Rübsamen-**

Waigmann, and K. Strebhardt. 1994. Induction and down-regulation of PLK, a human serine/threonine kinase expressed in proliferating cells and tumors. Proc. Natl. Acad. Sci. USA **91:**1736–1740.
16. **Huang, W., and R. L. Erikson.** 1994. Constitutive activation of Mek1 by mutation of serine phosphorylation sites. Proc. Natl. Acad. Sci. USA **91:** 8960–8963.
17. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
18. **Johnston, G. C., J. R. Pringle, and L. H. Hartwell.** 1977. Coordination of growth with cell division in the yeast *Saccharomyces cerevisiae*. Exp. Cell Res. **105:**79–91.
19. **Kim, H. B., B. K. Haarer, and J. R. Pringle.** 1991. Cellular morphogenesis in the *Saccharomyces cerevisiae* cell cycle: localization of the *CDC3* gene product and the timing of events at the budding site. J. Cell Biol. **112:**535–544.
20. **Kitada, K., A. L. Johnson, L. H. Johnston, and A. Sugino.** 1993. A multicopy suppressor gene of the *Saccharomyces cerevisiae* G_1 cell cycle mutant gene *dbf4* encodes a protein kinase and is identified as *CDC5*. Mol. Cell. Biol. **13:**4445–4457.
21. **Kumagai, A., and W. G. Dunphy.** 1996. Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from *Xenopus* egg extracts. Science **273:** 1377–1380.
22. **Lake, R. J., and W. R. Jelinek.** 1993. Cell cycle- and terminal differentiation-associated regulation of the mouse mRNA encoding a conserved mitotic protein kinase. Mol. Cell. Biol. **13:**7793–7801.
22a. **Lee, K. S., and R. L. Erikson.** Unpublished data.
23. **Lee, K. S., Y.-L. O. Yuan, R. Kuriyama, and R. L. Erikson.** 1995. Plk is an M-phase-specific protein kinase and interacts with a kinesin-like protein, CHO1/MKLP-1. Mol. Cell. Biol. **15:**7143–7151.
24. **Li, B., B. Ouyang, H. Pan, P. T. Reissman, D. J. Slamon, R. Arceci, L. Lu, and W. Dai.** 1996. *prk*, a cytokine-inducible human protein serine/threonine kinase whose expression appears to be down-regulated in lung carcinomas. J. Biol. Chem. **271:**19402–19408.
24a. **Liu, M.-A., and R. L. Erikson.** Unpublished data.
25. **Llamazares, S., A. Moreira, A. Tavares, C. Girdham, B. A. Spruce, C. Gonzalez, R. E. Karess, D. M. Glover, and C. E. Sunkel.** 1991. *polo* encodes a protein kinase homolog required for mitosis in *Drosophila*. Genes Dev. **5:**2153–2165.
26. **Marshall, C. J.** 1994. Hot lips and phosphorylation protein kinases. Nature (London) **367:**686.
27. **Mendenhall, M. D., H. E. Richardson, and S. I. Reed.** 1988. Dominant negative protein kinase mutations that confer a G1 arrest phenotype. Proc. Natl. Acad. Sci. USA **85:**4426–4430.
28. **Ohkura, H., I. M. Hagan, and D. M. Glover.** 1995. The conserved *Schizosaccharomyces pombe* kinase plo1, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G1 and G2 cells. Genes Dev. **9:**1059–1073.
29. **Piggott, J. R., R. Rai, and B. L. A. Carter.** 1982. A bifunctional gene product involved in two phases of the yeast cell cycle. Nature (London) **298:**391–393.
30. **Pringle, J. R.** 1991. Staining of bud scars and other cell chitin with calcofluor. Methods Enzymol. **194:**732–735.
31. **Roberts, R. L., B. Bowers, M. L. Slater, and E. Cabib.** 1983. Chitin synthesis and localization in cell division cycle mutants of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **3:**922–930.
32. **Russell, P., S. Moreno, and S. I. Reed.** 1989. Conservation of mitotic controls in fission and budding yeasts. Cell **57:**295–303.
33. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Siliciano, P. G., and K. Tatchell.** 1984. Transcription and regulatory signals at the mating type locus in yeast. Cell **37:**969–978.
35. **Simmons, D. L., B. G. Neel, R. Stevens, G. Evett, and R. L. Erikson.** 1992. Identification of an early-growth-response gene encoding a novel putative protein kinase. Mol. Cell. Biol. **12:**4164–4169.
36. **Slater, M. L., B. Bowers, and E. Cabib.** 1985. Formation of septum-like structures at locations remote from the building sites in cytokinesis-defective mutants of *Saccharomyces cerevisiae*. J. Bacteriol. **162:**763–767.
37. **Sunkel, C. L., and D. M. Glover.** 1988. *polo*, a mitotic mutant of *Drosophila* displaying abnormal spindle poles. J. Cell Sci. **89:**25–38.
38. **Surana, U., H. Robitsch, C. Price, T. Schuster, I. Fitch, A. B. Futcher, and K. Nasmyth.** 1991. The role of CDC28 and cyclins during mitosis in the budding yeast S. cerevisiae. Cell **65:**145–161.
39. **Taylor, S. S., D. R. Knighton, J. Zheng, J. M. Sowadski, C. S. Gibbs, and M. J. Zoller.** 1993. A template for the protein kinase family. Trends Biochem. Sci. **18:**84–89.
40. **Todokoro, K. (RIKEN, Ibaraki, Japan).** Personal communication.
41. **van den Heuvel, S., and E. Harlow.** 1993. Distinct roles for cyclin-dependent kinases in cell cycle control. Science **262:**2050–2054.