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Plk Is an M-Phase-Specific Protein Kinase and Interacts with a Kinesin-Like Protein, CHO1/MKLP-1

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***PLK* (*STPK13*) encodes a murine protein kinase closely related to those encoded by the *Drosophila melanogaster polo* gene and the *Saccharomyces cerevisiae CDC5* gene, which are required for normal mitotic and meiotic divisions. Affinity-purified antibody generated against the C-terminal 13 amino acids of Plk specifically recognizes a single polypeptide of 66 kDa in MELC, NIH 3T3, and HeLa cellular extracts. The expression levels of both poly(A)$^+$ *PLK* mRNA and its encoded protein are most abundant about 17 h after serum stimulation of NIH 3T3 cells. Plk protein begins to accumulate at the S/G_2 boundary and reaches the maximum level at the G_2/M boundary in continuously cycling cells. Concurrent with cyclin B-associated cdc2 kinase activity, Plk kinase activity sharply peaks at the onset of mitosis. Plk enzymatic activity gradually decreases as M phase proceeds but persists longer than cyclin B-associated cdc2 kinase activity. 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. Plk and CHO1/mitotic kinesin-like protein 1 (MKLP-1), which induces microtubule bundling and antiparallel movement in vitro, are colocalized during late M phase. In addition, CHO1/MKLP-1 appears to interact with Plk in vivo and to be phosphorylated by Plk-associated kinase activity in vitro.**

Reversible protein phosphorylation is 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. The protein kinases that act in these processes are also frequently regulated by phosphorylation. Recently, a subfamily of protein kinases has been identified by molecular cloning and genetic analyses; this subfamily includes the mammalian genes *SNK* (42) and *PLK* (*STPK13*) (4, 10, 13, 14, 22), the *Drosophila melanogaster polo* gene (25), and *Saccharomyces cerevisiae* cell cycle gene *CDC5* (*MSD2*) (18). Comparison of their deduced amino acid sequences shows they are closely related, since they display 50 to 65% identity in the N-terminal kinase domain. A lower but significant degree of sequence identity extends through the C-terminal domains of these protein kinases, with one strikingly conserved region (amino acids 410 to 439 in Plk) (4).

SNK was first identified as a member of this protein kinase subfamily as an immediate-early gene inducible by serum and phorbol ester. Its mRNA is markedly elevated within 1 h after mitogen treatment and is synergistically increased by cycloheximide treatment of cells (42). *PLK* (*STPK13*) was identified by PCR cloning as a gene expressed in primitive murine hemopoietic progenitor cells (4) and shown to be regulated during the cell cycle in NIH 3T3 cells and during terminal erythrodifferentiation in murine erythroleukemia (MEL) cells (22). *PLK* mRNA is highly expressed in rapidly dividing cell populations found in fetal and newborn tissues and adult hemopoietic tissues and thus is strongly correlated with the mitotic activity of cells and tissues (22). Moreover, most human tumors of various origins expressed high levels of *PLK* mRNA, although its expression was undetectable in surrounding tissues, suggesting that its expression is associated with cell proliferation (14). Although both *SNK* and *PLK* show striking structural identity 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 response to different signals.

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 developmental stages in which there is extensive mitotic activity (25, 43). The *polo*-encoded product shows cyclical kinase activity, which peaks at late anaphase/telophase during the rapid cycles of mitosis in syncytial *Drosophila* embryos (8). *S. cerevisiae CDC5* is an essential gene for cell growth. Cells depleted of Cdc5 result in a dumbbell terminal morphology, with the nuclei almost divided but still connected by a thin bridge of chromatin. Moreover, *CDC5* mRNA is periodically accumulated at the G_2/M boundary, suggesting that it is important for the proper progression of M phase (18). Taken together, the structural and initial functional characteristics of Plk, Snk, polo, and Cdc5 suggest that these kinases have an important role in cell division and proliferation.

The M phase of the cell cycle is a highly coordinated process by which a eucaryotic cell ensures the equipartition of its chromosomes and cytosolic contents during the cell division (reviewed in reference 26). It comprises a complex set of motile events, many of which involve microtubule-dependent motor proteins. Protein phosphorylation has been suggested as an important regulatory mechanism for the elaborate processes of M-phase events, and many phosphoproteins are known to be associated with mitotic apparatuses such as microtubule-organizing centers, kinetochore, and midbody (5, 44, 45, 49). The cdc2-cyclin B complex has been shown to be localized to centrosomes and spindles, and its important roles during M-phase progression have been suggested (2, 3, 17, 33–36, 38). A growing body of evidence suggests that many other protein kinases also function at M phase (12, 15, 19, 27, 28, 41, 47, 50).

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Whether these other protein kinases can be directly or indirectly regulated by the cdc2 kinase is poorly understood.

A monoclonal antibody (CHO1) raised against mitotic spindles isolated from Chinese hamster ovary (CHO) cells resulted in identification of a novel 95/105-kDa spindle component (40), which has been shown to be required for mitotic progression (31). The gene which encodes this antigen (human mitotic kinesin-like protein 1 [MKLP-1]) was identified in a HeLa cDNA expression library, and its protein product was shown to cross-bridge antiparallel microtubules which slide over one another in vitro (30). Moreover, a cDNA corresponding to the mRNA for the CHO1 antigen (we shall refer to the protein product as CHO1 in this report) was identified in a CHO cell expression library; its sequence predicts a protein with 76% identity to MKLP-1 at the amino acid level. Baculovirus-expressed full-length CHO1 and an N-terminal motor domain polypeptide of this protein caused bundling of brain microtubules in vitro, whereas the C-terminal polypeptide did not (20).

In this report, we demonstrate that Plk is an M-phase-specific protein kinase which interacts with CHO1/MKLP-1 in vivo. The expression, activation, and subcellular localization of Plk suggest that it plays important roles for normal M-phase progression.

MATERIALS AND METHODS

Cell culture. NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum (HyClone Laboratories Inc., Logan, Utah) and penicillin G (100 U/ml) plus streptomycin (100 μg/ml) (Gibco-BRL Life Technologies, Grand Island, N.Y.). Mouse erythroleukemia (MEL) cells and HeLa cells were cultured in the same medium as NIH 3T3 cells except 10% fetal bovine serum (Intergen, Purchase, N.Y.) and 10% calf serum, respectively, were used. Sf9 cells were cultured as described previously (16).

To examine the expression patterns of the *PLK* transcript and its encoded product upon mitogen stimulation in NIH 3T3 cells, cells were cultured for 48 h under conditions of serum deprivation (DMEM containing 0.5% calf serum) to inhibit cell proliferation. After the culture dishes were washed twice with warm DMEM, cells were serum stimulated by the addition of warm DMEM containing 15% calf serum.

To obtain ^{35}S-labeled proteins, HeLa cells were cultured in methionine-deficient minimum essential medium Eagle (Sigma, St. Louis, Mo.) supplemented with 400 ng of nocodazole (Sigma) per ml for 1 h. After 30 min of incubation with 100 μCi of Tran^{35}S-label (ICN Biomedicals, Irvine, Calif.) per ml, 15% calf serum was added, and the cells were incubated for 4 h. Nonadherent mitotic cells were mechanically released. For some experiments, exponentially growing MEL cells were labeled as described above.

Cell cycle synchronization. NIH 3T3 or HeLa cells synchronized at the G_1/S boundary were obtained by doubly treating the cells with 2.5 mM thymidine (Sigma) for 16 h, with an 8 h-release interval. To examine the cell cycle dependency of Plk expression and its kinase activity after release from the G_1/S block, cells were washed twice and released into fresh warm DMEM containing 15% calf serum. To obtain mitotically synchronized NIH 3T3 or HeLa cells at prometaphase, double-thymidine-blocked cells were released into the medium containing 400 ng of nocodazole per ml and incubated for additional 12 h. Floating cells were washed off, and nonadherent mitotic cells were mechanically released from the culture dishes, washed three times with cold DMEM, and then released into fresh warm DMEM containing 15% calf serum. At the indicated time points, cells were collected for immunocomplex kinase assays and Western blot (immunoblot) analyses (see below). To obtain mitotic NIH 3T3 or HeLa cells, cells were directly treated with 400 ng of nocodazole per ml and incubated for additional 16 h. To examine the cell cycle stages, flow cytometry was carried out with a FACScan (Becton Dickinson, San Jose, Calif.), and data were analyzed by CellFIT-DNA software version 1.2.

Isolation of *PLK* cDNA. On the basis of sequence information (4), a pair of PCR primers (5′-CCGCCAAGCTTCCTATTACCTGCCTCACC-3′ and 5′-ATAGAATTCCCACTTGCTGACCCAGAA-3′; sequences not underlined are linker sequences) within the *PLK* coding sequence were synthesized to carry out reactions using a mouse thymus cDNA library as a template. An expected 300-bp PCR product was cloned in a pBluescript II KS (+) vector and was identified as a fragment containing *PLK* cDNA sequence. This fragment was subsequently used to screen an 8.5-day embryonic mouse cDNA library, and the isolated full-length *PLK* cDNA sequence was confirmed by partial sequencing analysis and diagnostic restriction enzyme digestions.

RNA isolation and Northern (RNA) blot analyses. Total RNA was isolated by lithium chloride precipitation (1). Poly(A)$^+$ RNA was isolated from total RNA by using the PolyATract mRNA isolation system (Promega, Madison, Wis.), and the transcript size was determined by reference to RNA size markers (Promega). Equal amounts of RNAs were loaded onto 1.0% agarose-formaldehyde gels and transferred to nylon membranes. Northern blot analyses were carried out as described previously (42).

Generation and affinity purification of a rabbit polyclonal antibody against Plk. The C-terminal 13-amino-acid segment of Plk protein, which is identical in both murine and human Plk sequences, was chosen to synthesize a peptide with an additional acetylated Cys at its N terminus (Ac-CLSSRSASNRLKAS-OH). The peptide was conjugated to diphtheria toxoid and injected into New Zealand White rabbits (carried out by Chiron-Mimotopes, Clayton, Victoria, Australia). The obtained immune sera were affinity purified with the epitope peptide. Cross-linking of the peptide to thiopropyl-Sepharose 6B (Pharmacia, Uppsala, Sweden) and affinity purification of the Plk antibody were carried out according to the manufacturer's protocol.

Western blot analyses. Affinity-purified Plk antibody and a monoclonal CHO1 antibody were used at 0.5 μg/ml and 1:10,000 dilution, respectively. Rabbit polyclonal Erk1/2 antibody 691 (Santa Cruz Biotechnology, Santa Cruz, Calif.), mouse monoclonal cyclin B1 antibody GNS1 (Santa Cruz Biotechnology), and rabbit polyclonal cdc2 C-terminal domain antibody cdc2-CT (Upstate Biotechnology Inc., Lake Placid, N.Y.) were all used at 1:2,000 dilution. Western analyses were done as reported previously (16), and proteins which interact with antibodies were detected by an enhanced chemiluminescence Western detection system (Amersham, Arlington Heights, Ill.).

Generation and expression of HA-Plk and HA-PlkK82M in Sf9 cells. An *Eco*RI site was created directly upstream of the start codon (ATG) of the *PLK* cDNA, and a 2.1-kb *Eco*RI fragment, containing the complete *PLK* coding and 3′ untranslated sequence, was prepared. After filling in of the sticky ends of both *Eco*RI sites, this fragment was ligated into pUC19-HA [a gift of Leslie Berg, Harvard University; a hemagglutinin (HA) tag (TYPYDVPDYAS) which is flanked by a *Stu*I-*Nru*I-*Sca*I polylinker at the 5′ end and *Nae*I-*Sma*I-*Hpa*I polylinker at the 3′ end was inserted at the *Sma*I site of pUC19], digested with *Nae*I, and dephosphorylated. A 2.2-kb *Sca*I-*Hpa*I fragment generated from pUC19-HA-PLK was cloned into pAC702 (Invitrogen Corp., San Diego, Calif.). The resulting pAC702-HA-PLK clone was sequenced to confirm the in-frame insertion of the HA tag at the N terminus of the *PLK* coding sequence.

To mutagenize the lysine of the ATP binding site of *PLK* in pBluescript II KS(+) to methionine, a non-sense-strand oligonucleotide with a change to a methionine codon, 5′-GACTTAGGCACGATCATGCCTGCGAACACC-3′, was synthesized. Site-directed mutagenesis was carried out with the Sculptor in vitro mutagenesis system (Amersham International plc, Amersham, Buckinghamshire, England). A 100-bp *Bgl*II fragment containing the mutation site was isolated and ligated to pAC702-HA-PLK digested with *Bgl*II and dephosphorylated. Both pAC702-HA-PLK and its ATP binding site mutant, pAC702-HA-PLKK82M, were transfected into Sf9 cells by using BaculoGold (Pharmingen, San Diego, Calif.). For some experiments, both HA-Plk and HA-PlkK82M were purified through the immunoaffinity column made with affinity-purified Plk antibody. Cross-linking of antibody to protein G plus agarose (Santa Cruz Biotechnology) was carried out with dimethyl pimelimidate (Sigma) as described previously (39), and bound Plk was eluted with the Plk epitope peptide.

Immunoprecipitation and kinase assays. Plk immunoprecipitation was carried out either with affinity-purified Plk antibody in the presence or absence of its epitope peptide or with monoclonal antibody 12CA5 for HA-Plk. Both affinity-purified Plk antibody and antibody 12CA5 were used at 4 μg/ml for immunoprecipitation. CHO1/MKLP-1 immunoprecipitation was carried out with a rabbit polyclonal CHO1 antibody. Cells were lysed in 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, 20 mM *p*-nitrophenyl phosphate, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) (Pefabloc; Boehringer Mannheim, Indianapolis, Ind.), 10 μg of pepstatin A per ml, 10 μg of leupeptin per ml, 5 μg of aprotinin per ml]. Supernatants obtained from ultracentrifugation at 150,000 × *g* for 35 min (S150 fractions) were incubated with antibodies for 1 h; then protein A-Sepharose 4B (Zymed, San Francisco, Calif.) was added, and each mixture was incubated for an additional 1 h to precipitate the antibodies.

For measuring Plk kinase activity, assays were carried out in a kinase cocktail (TBMD) containing 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 *p*-nitrophenyl phosphate and supplemented with 3 μg of dephosphorylated casein (Sigma) and 50 μM ATP (5 μCi of [γ-^{32}P]ATP; 1 Ci = 37 GBq) if not specified otherwise. All reactions were done at 30°C for 30 min and terminated by the addition of 5× Laemmli sodium dodecyl sulfate (SDS) sample buffer (21). Proteins were separated on SDS–10% polyacrylamide gels, and ^{32}P was detected by autoradiography. When necessary, appropriate bands were excised from the dried gels, and incorporated ^{32}P was measured by a liquid scintillation counter (Beckman model LS 6800).

To assay cdc2 kinase activity, the S150 fraction was incubated with 10 μl of yeast p13^{suc1} agarose conjugate (Upstate Biotechnology Inc.), and then the precipitates were washed and subjected to kinase reactions as described above, using 3 μg of histone H1 (Calbiochem, La Jolla, Calif.) as a substrate.

To detect CHO1/MKLP-1 phosphorylation by Plk-associated kinase activity, Plk immunoprecipitates, prepared as described above, were subjected to in vitro kinase reactions in TBMD kinase cocktail supplemented with 25 μCi of [γ-^{32}P]ATP. Reactions were terminated by the addition of SDS to a final concentration of 1% and boiled for 5 min to disrupt the interaction of associating

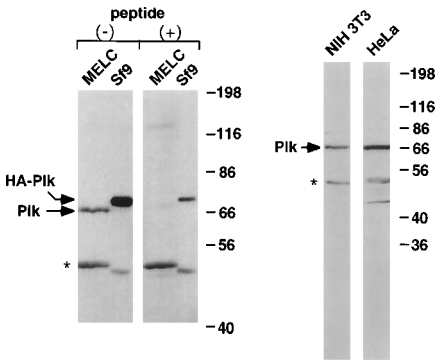

FIG. 1. Detection of Plk by affinity-purified antibody. Total cellular proteins prepared from Sf9 cells expressing HA-Plk as well as from growing MELC, NIH 3T3, and HeLa cells were subjected to immunoblotting with affinity-purified Plk antibody. In the indicated lanes [peptide (+)], Plk antibody was preincubated with its epitope peptide for 1 h to detect epitope-specific proteins. Asterisks denote a major cross-reacting band with the secondary antibody. Sizes are indicated in kilodaltons.

proteins. Samples were diluted to radioimmunoprecipitation assay (RIPA) buffer conditions (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 5 mM EGTA, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS) and centrifuged briefly to remove the beads. The resulting supernatant was then divided for subsequent CHO1/MKLP-1 and Plk immunoprecipitations.

Immunofluorescence microscopy. NIH 3T3 cells were grown on collagen (Vitrogen 100; Celtrix, Santa Clara, Calif.)-coated glass coverslips, washed briefly with phosphate-buffered saline (PBS), and lysed for 1 min in a microtubule-stabilizing (PMEG) buffer [100 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 5 mM $MgSO_4$, 5 mM EGTA, 0.5 mM EDTA, 0.9 M glycerol (pH 6.9)] containing 0.5% Triton X-100 and then fixed in methanol for 7 min and acetone for 2 min at −20°C. After four washes with PBST (PBS plus 0.1% Triton X-100), the coverslips were incubated for 45 min with affinity-purified Plk antibody at a concentration of 2 μg/ml in PBS containing 3% bovine serum albumin or with the CHO1 antibody diluted to 1:1,000. After being washed five times with PBST for 5 min each time, coverslips were further incubated for 45 min with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G (heavy plus light chain) secondary antibody (Gibco-BRL, Gaithersburg, Md.) diluted to 1:300 for Plk staining or with Texas red-labeled goat anti-mouse immunoglobulin M at a concentration of 5 μg/ml for CHO1/MKLP-1 staining. To stain chromosomes, cells were further treated with PBST containing propidium iodide (1 μg/ml; Sigma) and RNase I (500 μg/ml; Sigma) for 5 min. After five additional washes with PBST, cells were subjected to confocal microscopy. Double staining was performed by incubating the coverslips with both CHO1 and Plk antibodies in parallel with either the CHO1 antibody or the Plk antibody alone. Both the affinity-purified Plk antibody preincubated for 1 h with a threefold (in mass ratio) excess of its epitope peptide and the secondary antibodies alone were used as negative controls. To eliminate the possibility of any cross-reaction between one of the secondary antibodies and the other primary antibody, both secondary antibodies were switched and used as additional controls. Stained cells were viewed under a Bio-Rad MRC-600 confocal microscope equipped with an argon laser. Propidium iodide and Texas red were detected by A.1 filter blocks, while fluorescein fluorescence was detected by A.2 filter blocks. Each image is the Kalman-averaged product of approximately 10 scans generated by the Bio-Rad COMOS software.

RESULTS

Detection of p66^{PLK} protein in various mammalian cell lines. Using affinity-purified Plk antibody, we were able to detect a single polypeptide of 66 kDa from MELC, NIH 3T3, and HeLa cell lines and a 68-kDa protein from Sf9 cells expressing HA-Plk. When the Plk antibody was preincubated with its epitope peptide for 1 h before the Western analyses, recognition of a 66-kDa protein or a 68-kDa protein for HA-Plk was blocked or greatly diminished, respectively (Fig. 1). This antibody also immunoprecipitated from lysates of [^{35}S] methionine-labeled HeLa and MEL cells a 66-kDa protein which was recognized by the Plk antibody in the subsequent Western analyses (data not shown). After lysis in TBSN buffer (see Materials and Methods), about 80% of the total Plk was found in the S150 cellular fraction.

Both Plk protein and its transcript are expressed only at the late stage of the cell cycle. A 2.2-kb *PLK* transcript was detected in total RNAs prepared from various cell lines such as NIH 3T3, MEL, Jurkat, and 2B4 mouse T-cell hybridoma cells (data not shown) and was selected as poly(A)$^+$ RNA from total RNAs of both NIH 3T3 and MEL cells that we examined. Since *PLK* and *SNK* are structurally related and appear to have the potential for similar functions in mammalian cells, we investigated whether the Plk expression pattern is similar to that of Snk during the cell cycle. Since the *SNK* transcript is induced within 1 h after treatment of serum (42), we examined *PLK* expression from the very early stages of serum stimulation up to 32 h. Neither serum-starved cells nor serum-stimulated cells expressed detectable levels of 2.2-kb *PLK* transcripts at up to 14 h after serum stimulation (Fig. 2A), whereas at 30 min to 1 h, a *SNK* transcript is markedly elevated (data not shown and reference 42). A 2.2-kb *PLK* transcript appeared about 14 h after serum stimulation, confirming the previous report (22).

To determine whether the expression pattern of the 2.2-kb *PLK* transcript in serum-stimulated NIH 3T3 cells correlates with that of Plk protein, we prepared cells at the indicated time points after serum stimulation. Western analysis of total pro-

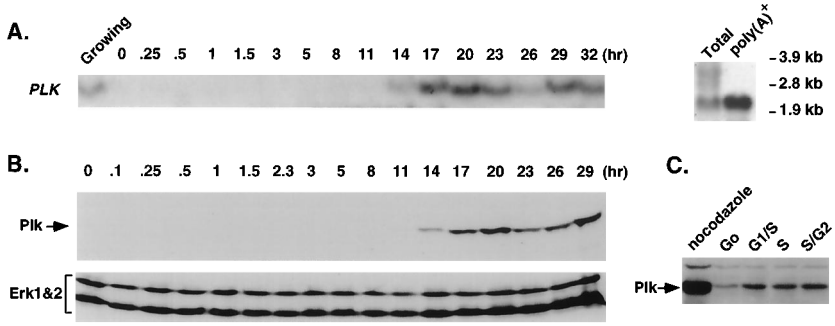

FIG. 2. Expression of the *PLK* transcript and its encoded product after serum stimulation. NIH 3T3 cells were serum starved for 48 h and stimulated with 15% calf serum. (A) Following stimulation, total RNA was prepared at the indicated time points. Fifty micrograms of total RNA was loaded in each lane. A *Sac*I 1-kb fragment within the *PLK* coding sequence was used as a probe. The size of the *PLK* transcript was determined with RNA markers (Promega). (B) Similarly, total cellular proteins were prepared at the indicated time points and subjected to immunoblotting with affinity-purified Plk antibody. The same blot was used for blotting with an Erk1/2 antibody to provide an internal control for equal loading. (C) Total cellular proteins were prepared from NIH 3T3 cells that had been serum starved (G_0), doubly thymidine blocked (G_1/S boundary), released from the double thymidine block for 3 h (S phase), released from the double thymidine block for 6 h (S/G_2 boundary), and nocodazole blocked (M phase) and subjected to Western analysis. Likely because of the continued synthesis of Plk during the blocks at both G_1/S and prometaphase, Plk amounts shown in G_1/S and nocodazole lanes are exaggerated. The basal level of Plk in Go could be detected only with prolonged exposure.

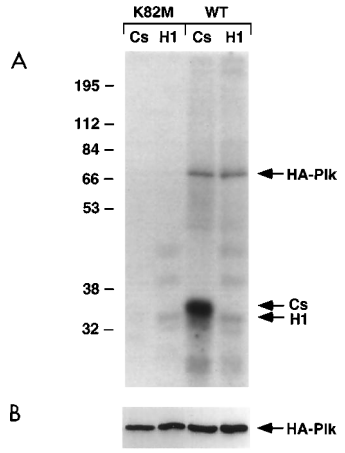

FIG. 3. Protein kinase activity of Plk in vitro. (A) Plk immunoprecipitates prepared from Sf9 cells expressing HA-Plk or kinase-inactive HA-PlkK82M were used to perform kinase assays using either casein or histone H1 as the substrate. HA-Plk indicates autophosphorylated signals. Cs, casein; H1, histone H1. (B) The same samples were subjected to immunoblotting with the Plk antibody to determine the amount of HA-Plk or HA-PlkK82M present in each immunoprecipitate. WT, wild type. Sizes are indicated in kilodaltons.

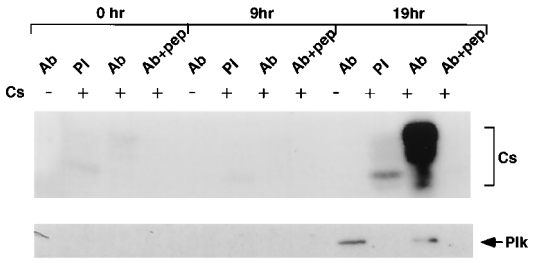

FIG. 4. Appearance of Plk kinase activity after serum stimulation. Immunoprecipitates were prepared from the cell lysates of serum-stimulated NIH 3T3 cells at the indicated time points. Kinase assays were performed with casein as an in vitro substrate. Immunoprecipitations carried out with preimmune serum or with the Plk antibody preincubated with its epitope peptide were used as controls. The same blot was used subsequently to detect the immunoprecipitated Plk. PI, preimmune serum; Ab, affinity-purified Plk antibody; pep, epitope peptide of the Plk antibody; Cs, casein.

teins showed that Plk began to accumulate at the 14-h time point and reached maximum level 20 h after serum stimulation (Fig. 2B). Flow cytometry analysis revealed that at the 14-h time point, about 4% of the cells had already reached G_2 phase, while 55% were in S and the rest were still in G_1. The G_2/M population gradually increased and reached 18% at the 20-h time point, while 37% were in S phase (data not shown). These data suggest that Plk protein becomes more abundant as more cells are in the G_2/M phase and that its expression is regulated at the transcriptional level. Since serum-stimulated cells from G_0 may differ from continuously cycling cells, total cellular proteins were prepared from cells doubly blocked with thymidine (cells at the G_1/S boundary), released from the double thymidine block for 3 h (cells in S phase) or 6 h (cells at the S/G_2 boundary), and mitotically arrested with nocodazole treatment (cells at M phase). Subsequent Western analysis showed that the Plk protein was maximally expressed at M phase, but a basal level of Plk was also detected in G_0, G_1, and S phases (Fig. 2C).

Plk autophosphorylates at Ser and Thr residues but not on Tyr residues. To investigate which amino acid residues are autophosphorylated, Plk was immunoprecipitated from MELC and Sf9 cells expressing HA-Plk and then subjected to an immunocomplex kinase reaction. Phosphoamino acid analyses of autophosphorylated Plk revealed primarily phosphoserine and a weak phosphothreonine signal. No phosphotyrosine was detected (data not shown). Both *Drosophila* polo and *S. cerevisiae* Cdc5 protein kinases utilize casein as their in vitro substrate (8, 18). We used baculovirus-expressed HA-Plk and the ATP binding site mutant, HA-PlkK82M, to determine their abilities to phosphorylate casein and histone H1. The results show that Plk prefers casein over histone H1 as a substrate (Fig. 3) and that casein phosphorylation occurs primarily on serine residues (data not shown).

Plk activity is maximum at the late stage of the cell cycle and correlates with its expression level. To examine whether Plk activity correlates with its expression level, NIH 3T3 cells arrested at G_0, stimulated for 9 h with serum, or stimulated for 19 h with serum were lysed, and their S150 fractions were subjected to Plk immunoprecipitation and subsequent kinase assays. Plk immunoprecipitates prepared from both G_0-arrested cells and cells serum stimulated for 9 h had no significant Plk activity, consistent with the low basal level of Plk protein. However, Plk immunoprecipitates prepared from 19-h serum-stimulated cells had a strong phosphorylation activity toward casein which was not observed in immunoprecipitates prepared with either the Plk antibody preincubated with its epitope peptide or the equivalent volume of preimmune serum (Fig. 4).

Plk activity appears concurrently with cdc2 kinase activity during the G_2 phase. To further investigate when Plk becomes active during the cell cycle, HeLa cells synchronized at the G_1/S boundary by a double thymidine block (all cells were in either G_1 or early S phase) were released, and at the indicated time points both Plk protein and enzymatic activity were analyzed. Protein levels reached a maximum level between 7 and 8 h after release from the G_1/S block. In addition, cyclin B1 appeared to become most abundant at these time points, whereas the levels of Erk1/2 and cdc2 remain constant (Fig. 5A). Flow cytometry analysis showed that about 50% of cells

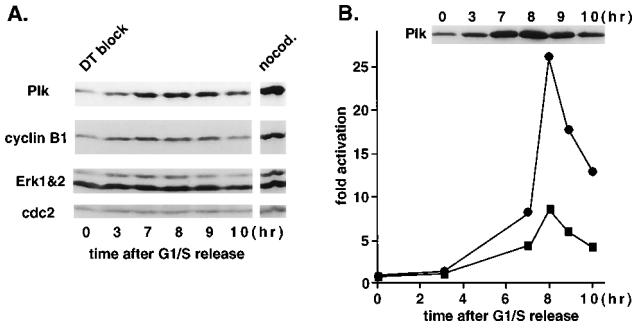

FIG. 5. Plk activity appears during the G_2 progression, concurrently with cdc2 kinase activity. (A) Protein levels of Plk and cyclin B1 after release from the double thymidine block. Both Plk and cyclin B1 become more abundant about 7 h after release. Mitotically arrested cells (nocod. lane) were prepared independently by treating the exponentially growing cells with nocodazole for 16 h. The levels of Erk1 and Erk2 were used as controls for equal amounts of loading. DT block, double-thymidine-blocked cells. (B) Concurrently with the H1 kinase activity of cdc2 (■), Plk activity (●) peaks at 8 h after release from the double thymidine block. Inset, the amount of immunoprecipitated Plk protein subjected to kinase assays at the indicated time points. Mitotically arrested cells (nocod. lane in panel A) showed 41-fold-increased activity for cdc2 and 44-fold-increased activity for Plk in comparison with the activities at their zero time points of G_1/S release (data not shown). The amount of Plk protein described in the text was determined by an analytical Western analysis comparing the quantity of purified Plk protein loaded on a gel with enhanced chemiluminescence signals.

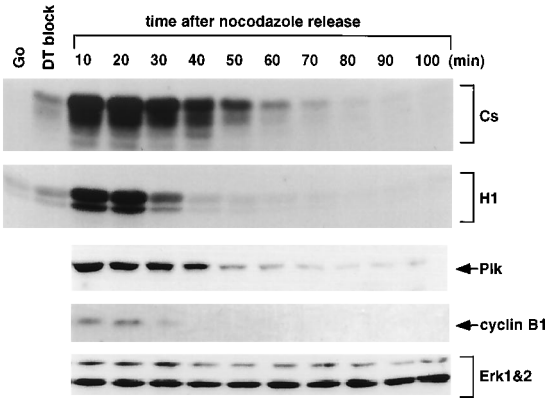

FIG. 6. Modulation of Plk activity during M-phase progression. Mitotically synchronized cells at prometaphase were prepared by double thymidine block and subsequent nocodazole treatment (see Materials and Methods). Cells were released into prewarmed medium and collected at the indicated time points. S150 fractions of cellular lysates were subjected to immunocomplex kinase assays to examine the kinase activity of Plk on casein. The same lysates were used to examine the kinase activity of cdc2 on histone H1. The same samples were subjected to immunoblotting with anti-Plk, anti-cyclin B1, and anti-Erk1/2 antibodies. DT, double-thymidine-blocked cells; Go, cells serum starved for 48 h; Cs, casein; H1, histone H1.

were in late S phase and the rest were in G_2 phase at the 7-h time point, and about 95% of cells were in G_2 phase at the 8-h time point (data not shown), indicating that most of the Plk proteins were synthesized in late S and G_2 phases. The protein kinase activity of Plk was compared with that of cdc2. Figure 5B shows that Plk activity peaks at 8 h after release from the G_1/S block, concurrent with cdc2 kinase activity. It should be noted that Plk activity increased about 26-fold during the 8 h after release from the G_1/S block, in contrast to the 8-fold increase observed for cdc2. During the same period, Plk protein increased about fourfold, as determined by a quantitative Western analysis. This finding implies that Plk activity is also regulated at the posttranslational level. Similar results were obtained with NIH 3T3 cells (data not shown).

Plk kinase activity peaks at the onset of mitosis and persists longer than cdc2 kinase activity. Plk is highly expressed in proliferating cells and tissues, and polo and Cdc5 protein kinases are known to play an important role in normal mitosis. polo is shown to be activated during the anaphase and telophase of the rapid cycles of mitosis in syncytial *Drosophila* embryos (8).

Since Plk is abundantly expressed and its activity peaks at the late stage of cell cycle, we examined the modulation of its expression and activity during the progression of M phase of the cell cycle in NIH 3T3 cells. Cells were released from a nocodazole block, collected at the indicated time points, and subjected to immunocomplex kinase assays and Western analyses as described in Materials and Methods. To monitor whether the cells were synchronously released from the mitotic block and progressing through M phase, cdc2 kinase activity and the level of cyclin B1 were examined as internal controls. While the protein level of cdc2 remained constant (data not shown), the cdc2 activities decreased sharply when cyclin B1 was degraded 30 min after nocodazole release. The protein levels of Erk1 and Erk2 remained constant, however. Plk activity decreased gradually but persisted longer than cdc2 kinase activity (Fig. 6). The close correlation between the protein level and the enzymatic activity of Plk late in M phase suggests that posttranslational modifications are not a factor in the decline of the Plk activity level. These data contrast with those found for polo activity, which peaks at the anaphase/telophase stage during mitosis in syncytial *Drosophila* embryos, whereas overall Plk activity peaks at the onset of mitosis. Similar results were obtained for mitotically synchronized HeLa cells (data not shown).

Colocalization of Plk with CHO1/MKLP-1 during M phase. As judged by immunofluorescence studies, Plk is found 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 (data not shown and Fig. 7). However, we were not able to detect any significant signals when the Plk antibody was preincubated with its epitope peptide or when the secondary antibody was used alone. When the Plk antibody concentration was increased to 20 μg/ml (10-fold more concentrated than shown above), Plk signals became apparent in the interphase centrosomes and mitotic spindle poles (data not shown).

A previously characterized protein, CHO1/MKLP-1, has been shown to be localized to the interzone (30, 40) and associated with mitotic spindles prepared from CHO cells (40). Since Plk and CHO1/MKLP-1 show strikingly similar patterns of cellular localization during M phase, we examined whether Plk and CHO1/MKLP-1 are colocalized in cells doubly stained with Plk and CHO1 antibodies. Although it is not clear at the early stage of M phase, Plk and CHO1/MKLP-1 are colocalized as their signals become condensed after metaphase. From anaphase to cytokinesis, the short discrete signals of Plk were completely superimposable with those of CHO1/MKLP-1 when they were visualized on the same focal plane under a confocal microscope. Moreover, this colocalization between two proteins persists until the completion of M phase (Fig. 7). However, when the two secondary antibodies used for Plk and CHO1 were switched, no significant signals were detected (data not shown), indicating that the two secondary antibodies did not cross-react to contribute to the double staining.

CHO1/MKLP-1 is coimmunoprecipitated with Plk and is phosphorylated by Plk-associated kinase activity in vitro. Since Plk and CHO1/MKLP-1 are colocalized during late M phase, we examined whether CHO1/MKLP-1 can be coimmunoprecipitated by the Plk antibody and phosphorylated in the subsequent kinase reaction. Since the Plk immunocomplex prepared from the mitotic HeLa cells showed higher specific activity than those from mitotic NIH 3T3 or growing MEL cells (data not shown), we used the S150 fraction obtained from the nocodazole-arrested HeLa cells. Affinity-purified Plk antibody was able to coimmunoprecipitate CHO1/MKLP-1. However, the equivalent volume of neither the preimmune serum nor the Plk antibody preincubated with its epitope was able to coimmunoprecipitate CHO1/MKLP-1. A comparison of the amounts of Plk and CHO1/MKLP-1 present in the S150 fraction with those in the Plk immunoprecipitates showed that the affinity-purified Plk antibody coimmunoprecipitated CHO1/MKLP-1 with about 20% of the efficiency with which Plk immunoprecipitated (Fig. 8A). This observation suggests a direct interaction of Plk and CHO1/MKLP-1 during the colocalization detected in the mitotic NIH 3T3 cells.

Plk immunoprecipitation and subsequent kinase reaction revealed a phosphorylated signal of a 100-kDa protein (Fig. 8C), which is identical in molecular weight to CHO1/MKLP-1 detected in HeLa cells (30). This phosphorylated signal was undetectable in the assays done with the equivalent volume of preimmune serum or the Plk antibody preincubated with its epitope (data not shown). Since this signal was superimposable with CHO1/MKLP-1 in the subsequent Western analyses (data not shown), we examined whether this phosphorylated signal is

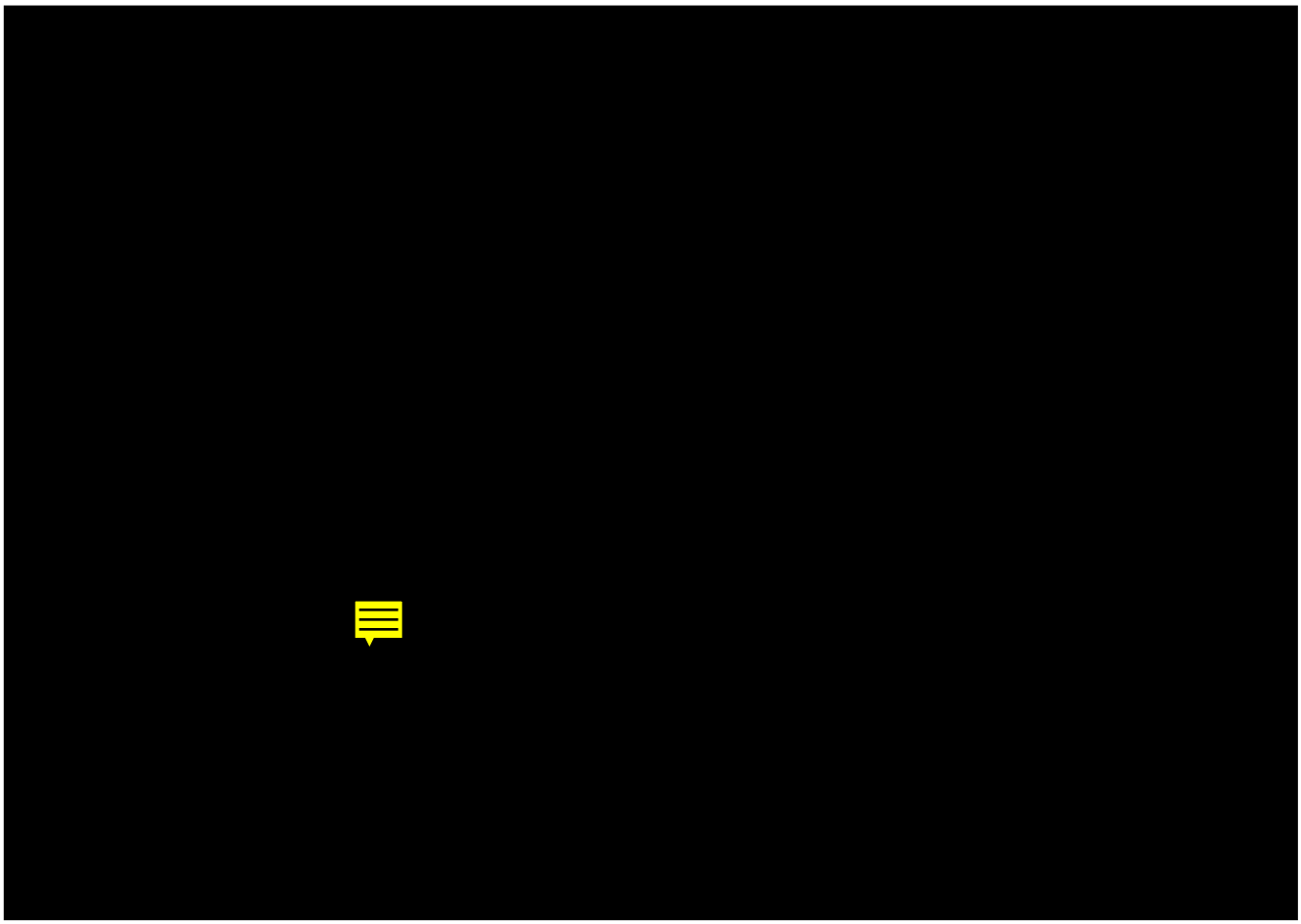

FIG. 7. Colocalization of Plk and CHO1/MKLP-1. Double-immunofluorescence localization of Plk and CHO1/MKLP-1 was performed in NIH 3T3 cells. Plk and CHO1/MKLP-1 were visualized with fluorescein isothiocyanate-conjugated and Texas red-conjugated secondary antibodies, respectively. Samples were viewed by confocal microscopy. Two different cells at anaphase (A) and telophase (B) are shown. Images of both Plk (green) and CHO1/MKLP-1 (red) were obtained at the same focal plane. Plk + CHO1 denotes overlapping images of Plk and CHO1/MKLP-1. Each image is the Kalman-averaged product of about 10 scans, and all images are at the same scale. Scale bar, 5 μm.

from CHO1/MKLP-1. Subsequent immunoprecipitation with the CHO1 antibody revealed that the coimmunoprecipitated and phosphorylated protein is indeed CHO1/MKLP-1. Moreover, the Plk antibody did not precipitate this phosphorylated band (Fig. 8C), indicating that coimmunoprecipitation of CHO1/MKLP-1 is not due to cross-reactivity of the Plk antibody with this protein. Similar results were obtained with NIH 3T3 cells (data not shown). Taken together, the data suggest that CHO1/MKLP-1 interacts with Plk in vivo and is phosphorylated by Plk-associated kinase activity in vitro.

DISCUSSION

Plk is an M-phase-specific protein kinase. In this study, we examined the expression of *PLK* in murine cells. *PLK*, together with *SNK*, belongs to a subfamily of protein kinase genes that includes *CDC5* and *polo*. We show that unlike the *SNK* transcript, the *PLK* transcript is not immediately inducible by mitogen treatment but is expressed at the late stage of the cell cycle. The cell cycle-associated fluctuations of *PLK* mRNA appear to be regulated posttranscriptionally, since the rates of *PLK* mRNA synthesis have been reported to be relatively constant at different stages of the cell cycle (22). In addition, the same concentration of cycloheximide which potentiated the mitogen induction of the *SNK* transcript prevented *PLK* mRNA expression in mitogen-stimulated T lymphocytes (14). Taken together, these results show that *PLK* is not an immediate-early gene and is regulated in a manner distinct from *SNK*, suggesting that Snk and Plk have unique functions in the cell cycle.

We show here that the expression level of the *PLK*-encoded product corresponds to mRNA expression in serum-stimulated NIH 3T3 cells (Fig. 2A and B). The timing of Plk protein accumulation at G_2 in continuously cycling cells correlates closely with that of the 2.2-kb *PLK* transcript described by Lake and Jelinek (22). The quantities of both *cdc2* and *SNK*-encoded products also correlate with mRNA levels after mitogen stimulation (9, 23, 24a, 42).

The enzymatic activity of Plk is also regulated in part at a posttranslational level. We observed that Plk-associated protein kinase activity increased sharply about 26-fold during a period when its protein level showed only a modest 4-fold increase. To date, we have no direct evidence for the mechanism of this rapid change in activity, although it is likely to involve a phosphorylation event by an as yet unidentified protein kinase. After this report was submitted for review, Golsteyn et al. (9a) reported on Plk enzymatic activity but did not resolve the issue of its regulation.

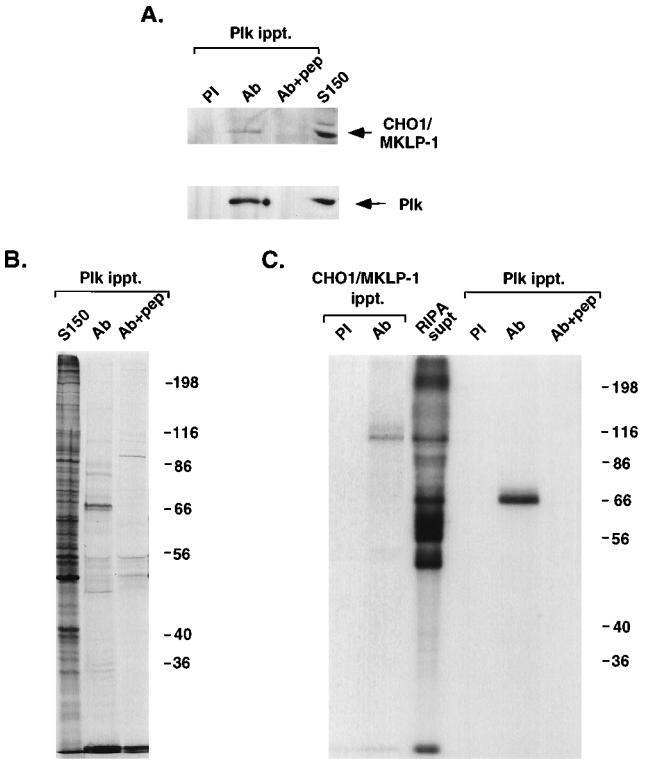

FIG. 8. Interaction of Plk and CHO1/MKLP-1. (A) Coimmunoprecipitation of CHO1/MKLP-1 by affinity-purified Plk antibody. Mitotic HeLa cells prepared by nocodazole treatment for 16 h were lysed in a TBSN buffer. The S150 fraction was divided into three aliquots and subjected to immunoprecipitation (ippt.) with preimmune serum, affinity-purified Plk antibody, or Plk antibody preincubated with its epitope peptide for 1 h as described in Materials and Methods. An aliquot of the S150 fraction was loaded in a separate lane to compare the efficiency of CHO1/MKLP-1 coimmunoprecipitation with that of Plk immunoprecipitation. Western analysis was carried out with the CHO1 antibody (top) or the Plk antibody (bottom). PI, preimmune serum; Ab, affinity-purified Plk antibody; pep, epitope peptide of the Plk antibody. (B) Plk immunoprecipitates from [^{35}S]methionine-labeled cell lysates. Mitotic HeLa cells metabolically labeled with [^{35}S]methionine were lysed in TBSN buffer, and the S150 fraction was subjected to immunoprecipitation with affinity-purified Plk antibody or Plk antibody preincubated with its epitope peptide under the same conditions as in panel A. The Plk antibody immunoprecipitates a 66-kDa protein, while the antibody preincubated with its epitope peptide does not. An aliquot of the S150 fraction was loaded on a gel to show proteins present prior to immunoprecipitation. Ab, affinity-purified Plk antibody; pep, epitope peptide of the Plk antibody. (C) CHO1/MKLP-1 is phosphorylated by Plk-associated kinase activity. Plk immunoprecipitates prepared under the conditions used for panel A were subjected to an in vitro kinase reaction as described in Materials and Methods. Reactions were terminated by the addition of SDS to a final concentration of 1% and boiled for 5 min to disrupt the interaction of associating proteins. Samples were diluted to RIPA buffer conditions as described in Materials and Methods and centrifuged briefly to remove the beads. The resulting RIPA supernatant (supt) was subjected to immunoprecipitations with indicated antibodies. The rabbit polyclonal CHO1 antibody immunoprecipitates the phosphorylated 100-kDa protein observed in the Plk immunocomplex kinase reaction, while its preimmune serum does not. Plk antibody immunoprecipitates Plk but not CHO1/MKLP-1.

Concurrently with cyclin B-associated cdc2 kinase activity, Plk activity peaks at the onset of M phase when the protein level is maximal. polo protein kinase activity peaks at the anaphase/telophase stage during mitosis in syncytial *Drosophila* embryos, however (8). It is possible that this discrepancy in timing of polo and Plk kinase activities is caused by a different regulation mechanism for M-phase progression in the highly synchronized embryonic cell cycle or that Plk is not the true homolog of polo kinase. Cyclin B1 is known to be degraded at the metaphase/anaphase transition (29, 35). It is therefore noteworthy that significant Plk activity is still detected after the metaphase-anaphase transition, which occurs 30 min after nocodazole release (about 50% of initial activity at the 30-min release and about 30% at the 40-min release). It should be noted that we can measure only the overall activity of Plk. Since Plk is highly localized in the midbody during anaphase/telophase, which has a very short duration, the relative local Plk activity may be high. The Plk activity measured here is obtained with immunocomplex kinase assays from the S150 fractions. Since mitotic microtubules begin to polymerize after nocodazole release, the overall Plk kinase activity may be underestimated if Plk associates with some cellular structures such as spindles.

Plk activity is not directly regulated by cdc2 or MAP kinase. The onset of mitosis is controlled by activation of the cyclin B-cdc2 protein kinase complex at the G_2/M transition (reviewed in reference 32). The cyclin B-cdc2 complex is localized to cytoplasmic microtubules and centrosomes (34) and to the spindles during mitosis and meiosis (2, 3, 33, 35, 36). Studies with cell extracts of *Xenopus* eggs revealed that the interphase/M-phase transition of microtubule dynamics is induced by phosphorylation reactions mediated by the maturation-promoting factor (46) and by mitogen-activated protein (MAP) kinase (12) functioning downstream of the maturation-promoting factor (11). MAP kinase is stably activated at metaphase and localized to the mitotic spindle poles and microtubule-organizing centers during meiotic maturation of mouse oocytes (47). We therefore examined whether cdc2 or MAP kinase can directly phosphorylate and regulate Plk kinase activity, using immunoaffinity-purified HA-Plk and its kinase-inactive form, HA-PlkK82M. Neither p13^{suc1}-precipitated cdc2 kinase from mitotic HeLa cells nor bacterially expressed glutathione *S*-transferase–Erk1 (p44^{MAPK}) activated by Raf-1 and Mek1 upstream activators was able to phosphorylate kinase-inactive HA-PlkK82M or influence the kinase activity of HA-Plk (data not shown). Similar results were obtained with Sf9 cells coexpressing human cdc2 and glutathione *S*-transferase–cyclin B with HA-Plk or with HA-PlkK82M (data not shown). These data strongly suggest that Plk does not serve as a substrate for these kinases.

CHO1/MKLP-1 colocalizes and interacts with Plk in vivo. We observed that Plk localizes to the interzone in anaphase and concentrates at the midbody during telophase and cytokinesis. Previously, others (10) have reported that in HeLa cells, Plk is abundant in mitotic cells and is localized to the region of the postmitotic bridge in cells that had just divided, and Plk is concentrated near the midbody, in contrast to tubulin staining, which extended further into the cytoplasmic bridge.

Although it is difficult to detect clear colocalization between Plk and CHO1/MKLP-1 before the beginning of anaphase, these two proteins are colocalized during late M phase (Fig. 7). It appears that both Plk and CHO1/MKLP-1 tightly associate with the midbody during early anaphase, resulting in a strong signal at this stage. Since CHO1/MKLP-1 is present in the midbody matrix prepared from isolated CHO spindles (40) and binds to microtubules in vitro (20, 30), colocalization of Plk and CHO1/MKLP-1 suggests that Plk is also associated with the midbody microtubules.

As is the case for Plk, CHO1/MKLP-1 also accumulates, although less dramatically, during G_2 and M phases (data not shown). Determination of the native molecular weight of intrinsic CHO1/MKLP-1 in cultured HeLa cells suggested that additional molecules associate with this motor protein in vivo (20). The colocalization of Plk and CHO1/MKLP-1 along with a much larger intrinsic molecular weight of the latter led us to investigate whether CHO1/MKLP-1 coimmunoprecipitated

with Plk. To examine this possibility, we used the S150 fraction obtained from mitotically arrested HeLa cells by nocodazole treatment for two reasons. First, both Plk and CHO1/MKLP-1 are enriched at M phase. Second, because most, if not all, of the mitotic microtubules might be depolymerized or shortened in the presence of nocodazole, the possibility of indirect interaction between these two proteins through microtubules will be greatly diminished. We observed that CHO1/MKLP-1 was coimmunoprecipitated with about 20% of the efficiency with which Plk was immunoprecipitated under physiologically relevant conditions but not after CHO1/MKLP-1 was denatured. This finding suggests the colocalization of Plk and CHO1/MKLP-1 results from a direct interaction in the spindle midzone or midbody structure. However, we cannot rule out the possibility of an indirect interaction mediated by a third protein present in the Plk immunoprecipitates.

Since cdc2 kinase plays important roles at the onset of mitosis, and its potential phosphorylation motif is present in CHO1/MKLP-1 (30), we examined whether they coimmunoprecipitated. Therefore, cdc2 was immunoprecipitated with either a cdc2 C-terminal domain antibody, a cdc2 PSTAIR antibody, or a cyclin B1 antibody. None of the cdc2 immunoprecipitates contained detectable amounts of CHO1/MKLP-1 (data not shown), indicating that no strong interactions occur between cdc2 and CHO1/MKLP-1. A number of other proteins, including autoantigens (reviewed in reference 7) and kinesin-like motor proteins (48, 51), have also been demonstrated to be present in the midbody region of the spindle. Preliminary analysis showed that CENP-E is not present in the Plk immunoprecipitates (data not shown). A search for other proteins interacting with Plk is in progress.

The observation that CHO1/MKLP-1 is phosphorylated by Plk-associated kinase activity may have important functional implications. During the transition from interphase to mitosis, a dramatic reorganization of the microtubule network occurs to form the mitotic spindles. The reversible phosphorylation of microtubule-associating proteins, such as MAP4 and Tau, is known to be important for the proper function of microtubules (6, 44). Since mutations in *polo* cause an aberrant spindle structure (25, 43) and depletion of Cdc5 blocks the completion of chromosome separation (18), Plk kinase activity may be required for the proper formation and function of the normal spindle apparatus.

Data accumulated from phosphorylation experiments conducted on conventional kinesin (37) and CENP-E (24) have allowed us to hypothesize that reversible phosphorylation of CHO1/MKLP-1 modulates its capacity to interact with microtubules and/or its associated proteins, leading to an altered subcellular distribution pattern of the motor protein in dividing cells. Given that CHO1/MKLP-1 is a motor protein present in the midzonal region of the spindles during late M phase, we speculate that Plk activity may play an important role in the process of separating two sister chromatids and directing them toward opposite spindle poles by activating the plus-end-directed motor activity of this protein. Although such a simple model is attractive, the mechanism generating force for chromosome separation might be more complicated. Two vertebrate kinesin-like proteins, CENP-E (51) and Xklp1 (48), which are structurally distinct from CHO1/MKLP-1, are also known to be localized to the spindle interzone and midbody during late M phase. The role of protein kinases such as Plk in the regulation of these motor proteins during the complex mechanochemical process in M phase awaits further investigation.

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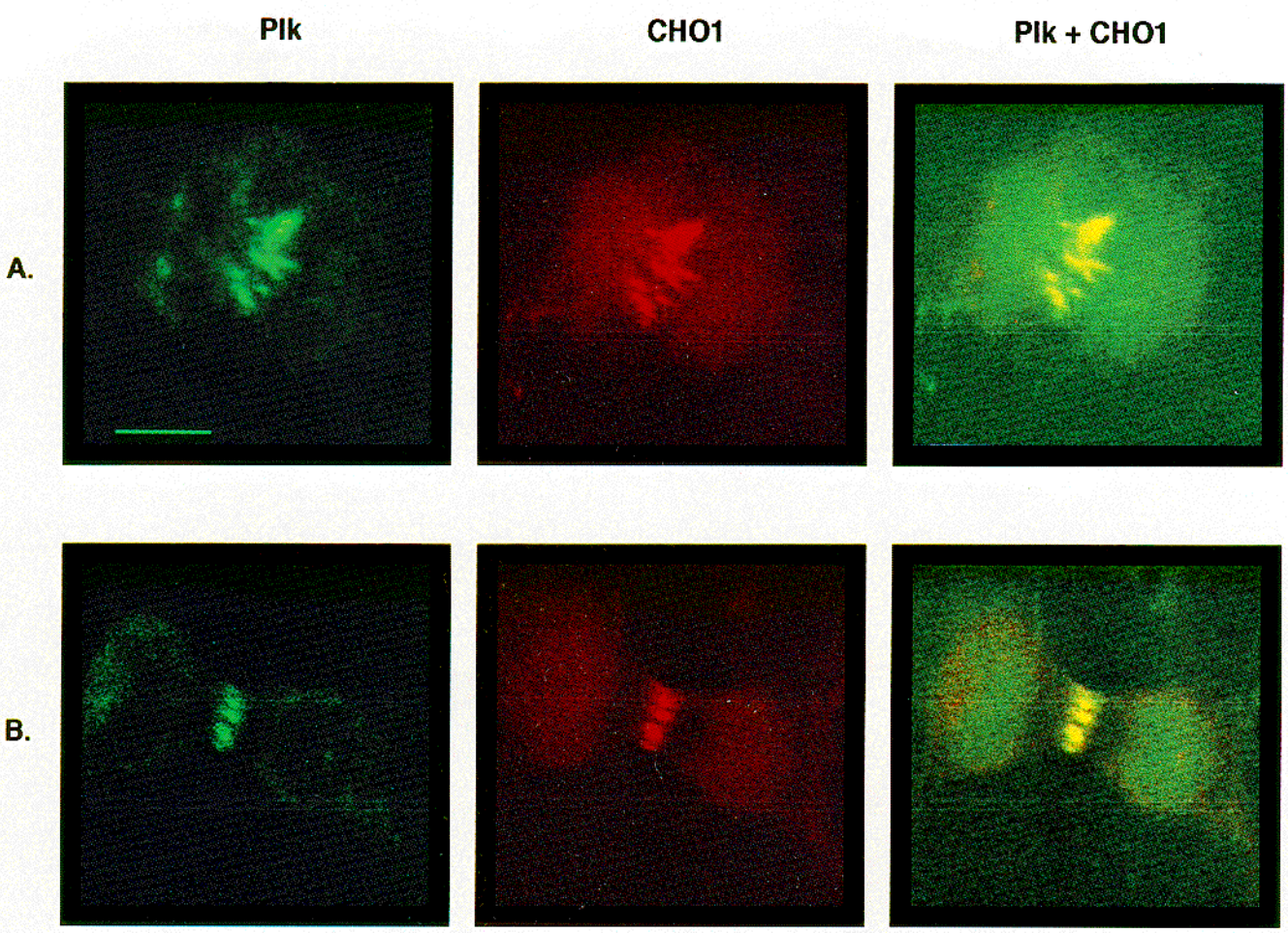
Plk
CHO1
Plk + CHO1
A.
B.