Cell Cycle Regulation of the Activity and Subcellular Localization of PLK1, a Human Protein Kinase Implicated in Mitotic Spindle Function

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Abstract. Correct assembly and function of the mitotic spindle during cell division is essential for the accurate partitioning of the duplicated genome to daughter cells. Protein phosphorylation has long been implicated in controlling spindle function and chromosome segregation, and genetic studies have identified several protein kinases and phosphatases that are likely to regulate these processes. In particular, mutations in the serine/threonine-specific *Drosophila* kinase polo, and the structurally related kinase Cdc5p of *Saccharomyces cerevisae*, result in abnormal mitotic and meiotic divisions. Here, we describe a detailed analysis of the cell cycle–dependent activity and subcellular localization of Plk1, a recently identified human protein kinase with extensive sequence similarity to both *Drosophila* polo and *S. cerevisiae* Cdc5p. With the aid of recombinant baculoviruses, we have established a reliable in vitro assay for Plk1 kinase activity. We show that the activity of human Plk1 is cell cycle regulated, Plk1 activity being low during interphase but high during mitosis. We further show, by immunofluorescent confocal laser scanning microscopy, that human Plk1 binds to components of the mitotic spindle at all stages of mitosis, but undergoes a striking redistribution as cells progress from metaphase to anaphase. Specifically, Plk1 associates with spindle poles up to metaphase, but relocalizes to the equatorial plane, where spindle microtubules overlap (the midzone), as cells go through anaphase. These results indicate that the association of Plk1 with the spindle is highly dynamic and that Plk1 may function at multiple stages of mitotic progression. Taken together, our data strengthen the notion that human Plk1 may represent a functional homolog of polo and Cdc5p, and they suggest that this kinase plays an important role in the dynamic function of the mitotic spindle during chromosome segregation.

DURING mitosis, replicated chromosomes (sister chromatids) segregate such that each daughter cell receives one complete copy of the genome. Chromosome segregation is a highly complex and dynamic process that relies on the assembly and function of a microtubule-based mitotic spindle apparatus (for reviews see McIntosh and Koonce, 1989; McIntosh and Hering, 1991; Karsenti, 1991; Hyman and Mitchison, 1992; Gorbsky, 1993; Wadsworth, 1993; Rieder and Salmon, 1994; Koshland, 1994). Biochemical, immunocytochemical, and genetic studies concur to demonstrate that phosphorylation plays an important role in controlling spindle assembly and function. For instance, biochemical studies have revealed that mitosis is accompanied by a substantial increase in protein phosphorylation (Karsenti et al., 1987), and inhibitors of protein kinases block the formation of taxol-stabilized microtubule asters (Verde et al., 1991), as well as chromosome-spindle interactions (Nicklas et al., 1993). Furthermore, phosphorylation was shown to control both the rate of nucleation of microtubules at the centrosome and their dynamic behavior (Verde et al., 1990, 1992; Gotoh et al., 1991; Buendia et al., 1992). Most recent studies indicate that reversible phosphorylation may control the mitotic function of microtubule-based motor proteins (Liao et al., 1994; Blangy, A., H. Lane, M. Kress, and E. A. Nigg, manuscript in preparation), and it is possible that phosphorylation of kinetochore-associated motors may determine the orientation of chromosome movements (Hyman and Mitchison, 1991). Immunocytochemical data also provide support for a role of protein phosphorylation in the regulation of spindle function. In fact, antibodies directed against phosphorylated epitopes, such as MPM-2 (Davis et al., 1983) or 3F3/2 (Cyert et al., 1988), strongly stain components of the mitotic spindle (Vandré et al., 1984; Vandré and Burry, 1992; Gorbsky and Ricketts, 1993). Finally, genetic studies performed with fungi and flies have identified multiple protein kinases

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and phosphatases with a possible role in spindle function and chromosome segregation (e.g., Ohkura et al., 1989; Doonan and Morris, 1989; Axton et al., 1990; Llamazares et al., 1991; Stone et al., 1993; Mayer-Jaekel et al., 1993; Toyn and Johnston, 1994).

One prominent protein kinase implicated in controlling spindle function is the p34^{cdc2}/cyclin B complex (for review see Nurse, 1990; Nigg, 1993). This kinase is partly localized to the mitotic spindle (Bailly et al., 1989; Pines and Hunter, 1991; Rattner et al., 1992; Gallant and Nigg, 1992), and it is able to stimulate microtubule dynamics in *Xenopus* cell-free extracts (Verde et al., 1990, 1992; Buendia et al., 1992). The most direct evidence in support of a role of p34^{cdc2}/cyclin B in the regulation of spindle function stems from the recent identification of spindle-associated, kinesin-related motor proteins as likely physiological substrates of p34^{cdc2}/cyclin B (Liao et al., 1994; Blangy, A., H. Lane, M. Kress, and E. A. Nigg, manuscript in preparation). However, it would a priori seem very unlikely that the regulation of all mitotic transitions could be attributed uniquely to changes in the activity of the p34^{cdc2} kinase, and, as mentioned above, genetic analyses have identified numerous protein kinases and phosphatases that are required for progression through mitosis (for reviews see Forsburg and Nurse, 1991; Glover, 1991; Kinoshita et al., 1991; Yanagida et al., 1992).

Among the protein kinases implicated in controlling spindle function and chromosome segregation is polo, a serine/threonine–specific enzyme first identified in *Drosophila* (Llamazares et al., 1991). *Drosophila* embryos harboring mutant polo alleles show a broad range of spindle abnormalities including monopolar spindles, highly branched bipolar spindles, and overcondensed chromosomes (Sunkel and Glover, 1988; Llamazares et al., 1991). The activity of wild-type polo kinase was measured during the rapid cell cycles of syncytial *Drosophila* embryos and reported to be maximal during late anaphase and early telophase (Fenton and Glover, 1993). Interestingly, recent studies have revealed that the *CDC5* gene of *Saccharomyces cerevisiae* encodes a protein kinase with a high degree of sequence similarity to *Drosophila* polo (Kitada et al., 1993). When yeast cells harboring a *cdc5 ts* mutant allele are cultured at the nonpermissive temperature, they arrest as large budded cells with partially segregated nuclei (Byers and Goetsch, 1974; Sharon and Simchem, 1990; Kitada et al., 1993), and spindle abnormalities have been observed in homozygous diploids undergoing meiosis (Schild and Byers, 1980). Although it is difficult to directly compare phenotypes of mutants in very different organisms, these findings raise the possibility that *Drosophila polo* and *S. cerevisiae CDC5* may encode functional homologs.

Using an approach based on the PCR (Schultz and Nigg, 1993), we have recently identified a human protein kinase that displays a substantial degree of sequence identity with *Drosophila* polo and budding yeast Cdc5p and hence was termed polo-like kinase 1 (Plk1)[1] (Golsteyn et al., 1994). Independently, cDNAs encoding human and murine Plk1 (Clay et al., 1993; Lake and Jelinek, 1993; Hamanaka et al., 1994; Holtrich et al., 1994), as well as Plk1-related protein kinases (Simmons et al., 1992; Fode et al., 1994), have been cloned in other laboratories. Northern blot analyses revealed that Plk1 mRNA levels are highest in tissues with a sizeable proportion of proliferating cells, consistent with a role of Plk1 in mitosis (Clay et al., 1993; Lake and Jelinek, 1993; Golsteyn et al., 1994). In cultured cells, both Plk1 mRNA (Lake and Jelinek, 1993) and protein levels (Golsteyn et al., 1994) were low during G1 phase, but increased during S phase and reached maximal levels during G2 and M phases. By immunofluorescent staining with monoclonal antibodies, Plk1 was found to be diffusely distributed throughout interphase cells; in dividing cells, however, a striking association with postmitotic bridges was noted, suggesting that Plk1 might be discarded at the end of mitosis through shedding of the midbody into the culture medium (Golsteyn et al., 1994).

Further progress towards understanding the function of human Plk1 had been hampered by a lack of biochemical information on the activity of this kinase. In this study, we have used recombinant Plk1 to establish a reliable assay for measuring Plk1 activity, and have then carried out a detailed study of Plk1 activity during the cell cycle. The activity of Plk1 isolated from synchronized HeLa cells was found to be low at all interphase stages of the cell cycle but high during mitosis. Using a novel, highly specific antibody, we have also reexamined the subcellular distribution of human Plk1. We found that Plk1 localizes to distinct elements of the mitotic spindle at all stages of mitosis, but undergoes a remarkable redistribution as cells progress from metaphase to anaphase. Taken together, these results suggest that Plk1 functions in mammalian mitotic cells to control spindle dynamics and chromosome segregation.

Materials and Methods

Cell Culture and Synchronization

HeLa cells were grown in DMEM (GIBCO BRL, Gaithersburg, MD) supplemented with 5% heat-inactivated FCS and penicillin-streptomycin (100 i.u./ml and 100 μg/ml, respectively) in a 7% CO_2 atmosphere. For metabolic labeling of mitotic cells with [^{35}S]methionine/cysteine, HeLa cells were cultured for 12 h in normal growth medium containing nocodazole (100 ng/ml) and then washed with PBS (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.2), and incubated for 30 min in methionine-free MEM (Gibco) supplemented with 10% FCS (previously dialyzed against 100 mM NaCl), nocodazole (100 ng/ml), 1% glutamine and penicillin-streptomycin. Finally, they were cultured for 4 h in the above methionine-free MEM containing 80 μCi/ml of Trans^{35}S-label (ICN Biomedicals, Inc., Costa Mesa, CA).

Cells were synchronized at the G1/S boundary by a double thymidine-aphidicolin block, as described by Heintz et al. (1983). In brief, cells were plated onto multiple 10-cm dishes and cultured in the presence of thymidine (2 mM; Sigma Chem. Co., St. Louis, MO) for 14 h; then they were washed three times with normal growth medium, incubated for an additional 14 h under normal growth conditions, and finally arrested by the addition of aphidicolin (1 μg/ml, Sigma) for 14 h. At time zero, cells were washed three times with normal growth medium and placed under normal growth conditions. At regular intervals, cells were collected by trypsinization. Aliquots were subjected to flow cytometric analysis as described by Draetta and Beach (1988), using a FACS II (fluorescence-activated cell sorter) instrument (Becton-Dickinson Immunocytometry Sys., Mountain View, CA). The remaining cells were used for the preparation of whole cell extracts, as described below.

To arrest exponentially growing HeLa cells at prometaphase, nocodazole was added to final concentration of 100 ng/ml for 14 h. Mitotic cells

1. *Abbreviation used in this paper*: Plk1, polo-like kinase 1.

were collected by mechanical shake-off, rinsed twice in prewarmed growth medium, and replated into normal growth medium. Immediately before (time zero) or at various intervals after the removal of nocodazole, cells were collected and extracted as described below. To synchronize cells in the absence of cell cycle arresting drugs, HeLa suspension cells were size-fractionated by centrifugal elutriation as described previously (Draetta and Beach, 1988; Golsteyn et al., 1994). All fractions were then used for flow cytometric analysis and for the preparation of whole cell extracts.

Site-directed Mutagenesis and Expression of Recombinant Plk1 in Insect Cells

To prepare a catalytically inactive Plk1 mutant, codon 82 (AAG, coding for lysine) was mutated to AGG (coding for arginine), using reagents and instructions supplied in the Transformer Site–Directed Mutagenesis Kit (Clontech Laboratories, Palo Alto, CA), and the oligonucleotides GCGGGCAGGATTGTGCCTAAG and AATTCGAGCTCAGTACCCGG as mutagenesis and selection primers, respectively. To ensure that no additional mutations were unintentionally introduced, a 352 bp BglII fragment encompassing this mutation was sequenced and replaced into the original Plk1-pGEM plasmid (Golsteyn et al., 1994). The mutated plasmid was named Plk1-K82R-pGEM.

A baculovirus encoding wild-type human Plk1 was constructed by subcloning an EcoRI fragment excised from Plk1-pGEM into the pBlueBac transfer vector (Invitrogen, San Diego, CA). In the resulting construct (Plk1-pBlueBac), the authentic Plk1 ATG is used for initiation of translation. Recombinant virus was then generated by homologous recombination in vivo, following cotransfection of Sf9 insect cells with Plk1-pBlueBac and baculovirus DNA. The transfection supernatant was used to infect Sf9 cells for standard plaque assays, and recombinant virus was purified by three rounds of plaque assays. Purity of the recombinant virus was confirmed by the absence of polyhedrin occlusion bodies. A baculovirus encoding the K82R mutant of Plk1 was generated by subcloning Plk1-K82R into the transfer vector pVL1392, cotransfection of this DNA (Plk1-K82R-pVL1392) together with Baculogold DNA (Pharmingen, San Diego, CA), and subsequent amplification of viral DNA according to the manufacturer's instructions (Pharmingen). All procedures relating to Sf9 cell growth and baculovirus handling were performed as described in O'Reilly et al. (1992).

Immunochemical Techniques

A polyhistidine-tagged 25-kD fusion protein (termed C-termPlk1) coding for the COOH terminus of Plk1 (residues 402-603) was expressed in *E. coli* and purified as described previously (Golsteyn et al., 1994). Rabbits were immunized with C-termPlk1 according to standard protocols (Krek and Nigg, 1991), until an adequate titer was obtained. The immune serum of rabbit 32 (R32) was used for immunoprecipitation experiments, and the preimmune serum obtained from the same rabbit was used for controls. For affinity purification of anti-Plk1 immunoglobulins (AR32) from serum R32, C-termPlk1 protein was isolated from bacteria by the preparation of inclusion bodies (Harlow and Lane, 1988), before nickel column chromatography (Qiagen). 1 mg of purified C-termPlk1 protein was then bound to CNBr-activated Sepharose 4B, as described by the manufacturer (Pharmacia LKB Biotechnology, Piscataway, NJ). Antibodies were purified on this affinity matrix as described by Harlow and Lane (1988), and AR32 immunoglobulins were used at 350 ng/ml for immunoblotting and at 3.5 μg/ml for immunofluorescent staining.

Immunoprecipitation experiments were performed by adding R32 serum (at 1:100; vol/vol) or AR32 immunoglobulins (to a final concentration of 7.5 μg/ml) to whole cell lysates and incubating the samples on ice for 1 h. After a 5-min centrifugation at 10,000 *g*, supernatants were transferred to new tubes, and immune complexes were collected by incubation for 30 min with protein A–Sepharose beads (Pharmacia LKB Biotechnology). Beads were washed three times in Bead Buffer (50 mM Tris pH 7.5, 0.1% NP-40, 250 mM NaCl, 5 mM NaF, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl phosphate, 1 μg/ml each of soybean trypsin inhibitor, leupeptin and pepstatin), as described by Meijer et al. (1989). For analysis of immune complexes by SDS-PAGE, washed beads were again transferred to new tubes, gel sample buffer was added, and samples were heated for 5 min to 95°C. For protein kinase assays, beads were also transferred to new tubes, washed once in the appropriate kinase assay buffer (see below), and stored on ice until used.

Preparation of Cell Extracts and Protein Kinase Assays

HeLa or Sf9 insect cells were collected and washed twice in PBS. Then, they were resuspended to a density of 5,000 cells/μl and incubated for 30 min on ice, in either NP-40 lysis buffer or histone H1 kinase buffer (see below), depending on the assay to be performed: since Plk1 was equally active under both lysis conditions, cells were lysed in histone H1 kinase buffer when both Plk1 and cyclin-dependent kinase (CDK) activities were to be determined. NP-40 lysis buffer was 50 mM Hepes pH 7.4, 1% NP-40, 100 mM NaCl, 25 mM NaF, 25 mM sodium β-glycerophosphate, 1 μg/ml each of soybean trypsin inhibitor, leupeptin and pepstatin, and 30 μg/ml of DNase I and RNase A. Histone H1 kinase buffer was 1% NP-40, 60 mM β-glycerophosphate, 10 mM $MgCl_2$, 10 mM EGTA, 1 mM ATP, 1 mM phenylmethylsulfonyl phosphate, 1 μg/ml each of soybean trypsin inhibitor, leupeptin and pepstatin, and 30 μg/ml of DNase I and RNase A. Subsequently, samples were passed five times through a 27-gauge needle and centrifuged for 5 min at 10,000 *g* (4°C). Supernatants were stored at −80°C until used for kinase assays.

To measure Plk1 activity, Plk1 immunoprecipitates were washed once in Plk1 wash buffer (20 mM Hepes, pH 7.4, 150 mM KCl, 10 mM MCl_2, 1 mM EGTA, 0.5 mM DTT, and 5 mM NaF) and stored on ice. To start the reaction, 20 μl of Plk1 assay buffer was added to the beads and samples were incubated for 15 min at 30°C. Plk1 assay buffer was Plk1 wash buffer supplemented with 10 μM ATP, 4 μCi of [γ-^{32}P]ATP (10 mCi/ml), and 0.5 mg/ml of dephosphorylated casein (Sigma). In some experiments, casein was substituted by different exogenous substrates, notably enolase, histone H1, myelin basic protein, phosvitin, or MAP-2, each used at 0.5 mg/ml. Also, to determine the ability of Plk1 to use GTP as a phosphate donor, experiments were carried out in the presence of 10 μM GTP and 4 μCi of [γ-^{32}P]GTP (10 mCi/ml) instead of ATP. Reactions were stopped by the addition of an equal volume of 2.5 × gel sample buffer. Then samples were heated for 5 min to 95°C before analysis by SDS-PAGE and autoradiography.

To assay CDK-associated histone H1 kinase activity, CDK/cyclin complexes were collected using $p9^{suc1}$ beads, as described previously (Maridor et al., 1993). They were washed once with CDK wash buffer (50 M β-glycerophosphate, pH 7.5, 10 mM $MgCl_2$, 10 mM NaF, and 1 mM DTT) and stored on ice. To start the reaction, 20 μl CDK assay buffer was added and samples were processed further as described above for Plk1 assays. CDK assay buffer was CDK wash buffer supplemented with 10 μM ATP, 0.2 mCi/ml of [γ-^{32}P]ATP, and 0.4 mg/ml of histone H1 (Boehringer Mannheim Corp., Indianapolis, IN).

Quantitations of Plk1 and CDK activities were performed using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA) and a CCD camera and Bio-Print software (Vilber Lourmat, France). For Plk1 assays, exposure times for autoradiography or phosphorimaging were usually ~10 times longer than those required for optimal visualization of CDK assays.

Miscellaneous Techniques

Confocal laser scanning microscopy was performed on an MRC 600 instrument (BioRad Labs., Hercules, CA), as described by Palladino et al. (1993). AR32 antibody was detected with biotinylated goat anti-rabbit/streptavidin Texas red, as described by the manufacturer (Amersham Life Sciences, UK). In vitro transcription-translation experiments were carried out in the presence of [^{35}S]methionine/cysteine, using the TnT rabbit reticulocyte lysate system (Promega Corp., Madison, WI). Transient transfections of HeLa cells with Plk1-CMV and myc-tagged Plk1-CMV (Golsteyn et al., 1994) were carried out as described by Krek and Nigg (1991).

Results

Characterization of Anti-Plk1 Antibody

All results reported here were obtained using a novel rabbit antibody (R32) raised against the COOH-terminal 201 amino acids of human Plk1. Fig. 1 illustrates the specificity of this reagent. When used for immunoprecipitation experiments (Fig. 1 *A*), the anti-Plk1 antibody precipitated a major 68-kD protein from ^{35}S-labeled mitotic cell lysates (lanes *2* and *3*). This protein comigrated exactly with the

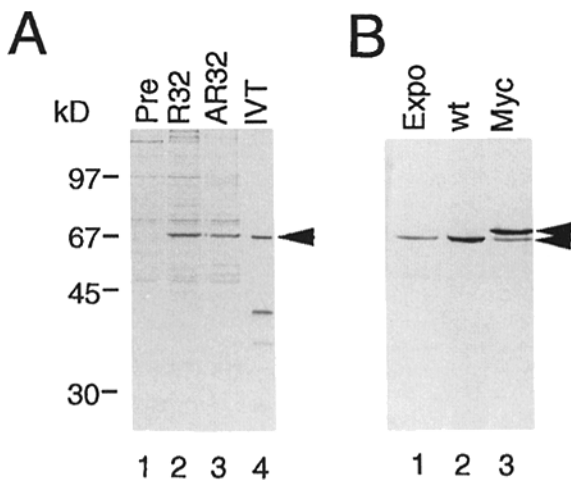

Figure 1. Characterization of R32 anti-Plk1 antibodies. (*A*) Determination of antibody specificity by immunoprecipitation. HeLa cells were cultured in the presence of nocodazole and [^{35}S]methionine/cysteine. A total cell lysate was then prepared and aliquots were incubated with either preimmune serum (*Pre*; lane *1*), anti-Plk1 immune serum (*R32*, lane *2*) or affinity-purified anti-Plk1 antibodies (*AR32*, lane *3*). Immune complexes were collected and analyzed by SDS-PAGE and autoradiography. For comparison, ^{35}S-labeled Plk1 was also prepared by in vitro transcription/translation of the Plk1-pGEM plasmid in a rabbit reticulocyte lysate and loaded onto the same gel (*IVT*, lane *4*). The 68-kD Plk1 protein is indicated by an arrowhead, and the molecular masses markers are indicated in kD. The 40-kD protein visible in lane *4* may arise from proteolysis or from internal initiation. (*B*) Determination of antibody specificity by immunoblotting. Total cell lysates were prepared from exponentially growing HeLa cells (*Expo*, lane *1*), and from HeLa cells transfected with cDNAs encoding either Plk1 (*wt*, lane *2*) or MycPlk1 (*Myc*, lane *3*). Extracts were then analyzed by immunoblotting, using the affinity-purified anti-Plk1 antibody AR32. Note the comigration of the endogenous protein detected in lanes *1* and *3* with the overexpressed Plk1 protein in lane *2* (*lower arrowhead*). The decreased mobility of the MycPlk1 protein detected in lane *3* is consistent with the presence of the 20–amino acid myc-tag at the NH_2 terminus of Plk1. Equal amounts of total cellular protein were loaded in each lane. We note that ectopically expressed Plk1 proteins were not detectable by Coomassie blue staining (not shown).

product obtained by in vitro translation of RNA transcribed from the human *plk1* cDNA (lane *4*), and it was not recognized by the preimmune serum (lane *1*). Likewise, when used for immunoblotting on a total cell lysate prepared from exponentially growing HeLa cells (Fig. 1 *B*), the anti-Plk1 antibody reacted with a single 68-kD protein (lane *1*). The identity of this protein was confirmed by transient transfection experiments: overexpression of the full-length Plk1 protein resulted in a markedly increased signal intensity (lane *2*), whereas expression of an myc-epitope tagged Plk1 protein resulted in the expected appearance of a second immunoreactive protein with a slightly increased molecular weight (lane *3*). In contrast to the anti-Plk1 monoclonal antibodies used previously (Golsteyn et al., 1994), the R32 antibodies did not cross-react with an unidentified 110-kD protein, and hence may be considered as monospecific for Plk1.

Recombinant Human Plk1 Is Active as a Casein Kinase

Previously, we had been unable to detect specific protein kinase activity associated with Plk1 immunoprecipitates (Golsteyn et al., 1994). A priori these negative results might have been due to the choice of inappropriate in vitro substrates or assay conditions, an inhibitory action of the anti-Plk1 antibodies, or a narrow window of activity of Plk1 during the cell cycle. To overcome this difficulty and make Plk1 amenable to a biochemical characterization, we constructed a recombinant baculovirus coding for wild-type Plk1. As a control, a virus coding for a catalytically inactive mutant Plk1 (K82R) was also made. Corresponding arginine for lysine substitutions in other protein kinases have previously been shown to interfere with phosphate transfer to the substrate without drastically altering the three-dimensional structure of the kinase (Taylor et al., 1992). Expression of human Plk1 in Sf9 insect cells could readily be visualized by immunoblotting, and maximal expression occurred at 30–40 h postinfection (Fig. 2 *A*). Wild-type and mutant Plk1 were expressed to comparable levels (Fig. 2 *B*, compare lanes *1* and *2*), and no signal was observed in control lysates (Fig. 2 *B*, lane *3*).

To determine whether recombinant Plk1 displayed protein kinase activity, immunoprecipitates were prepared from infected Sf9 insect cell lysates, washed extensively, and then incubated in vitro with [γ-^{32}P]ATP as a phosphate donor and casein as an exogenous substrate (Fig. 2 *C*). Casein was chosen as a substrate since both *Drosophila* polo and yeast Cdc5p have been reported to phosphorylate casein (Fenton and Glover, 1993; Kitada et al., 1993). Under the above conditions, kinase activity could readily be demonstrated for wild-type Plk1 (lane *4*), and phosphoamino acid analysis revealed that phosphorylation occurred on both serine and threonine residues (data not shown). Attesting to the specificity of the observed reaction, no casein kinase activity was seen in immunoprecipitates of the catalytically inactive mutant (lane *2*), or when immunoprecipitations were performed with the corresponding preimmune serum (lanes *1* and *3*). A phosphorylated protein migrating at the size of Plk1 was also detected, but, interestingly, this phosphoprotein was seen only in immunoprecipitates of wild-type Plk1 (lane *4*, *arrow*), suggesting that Plk1 may be able to undergo autophosphorylation. Using the above assay, several additional proteins were tested as potential substrates of Plk1. Whereas histone H1, enolase, and phosvitin were not phosphorylated to any significant extent, myelin basic protein and the microtubule-associated protein MAP-2 could be phosphorylated by Plk1, albeit to a much lower degree than casein (data not shown).

Since casein is a good in vitro substrate not only for Plk1, but also for other protein kinases, notably casein kinase II (Pinna, 1990) and p34^{cdc2}/cyclin B (Brizuela et al., 1989), we considered it important to compare the biochemical properties of these different casein kinases. To this end, we first tested the ability of Plk1 to use GTP as a phosphate donor. As shown in Fig. 3 *A*, Plk1 could use [γ-^{32}P]ATP (lane *1*) but not [γ-^{32}P]GTP (lane *3*), and the use of [γ-^{32}P]ATP was not inhibited by the presence of an excess of unlabeled GTP (lane *2*). In contrast, casein kinase II was able to use [γ-^{32}P]GTP as efficiently as [γ-^{32}P]ATP (compare

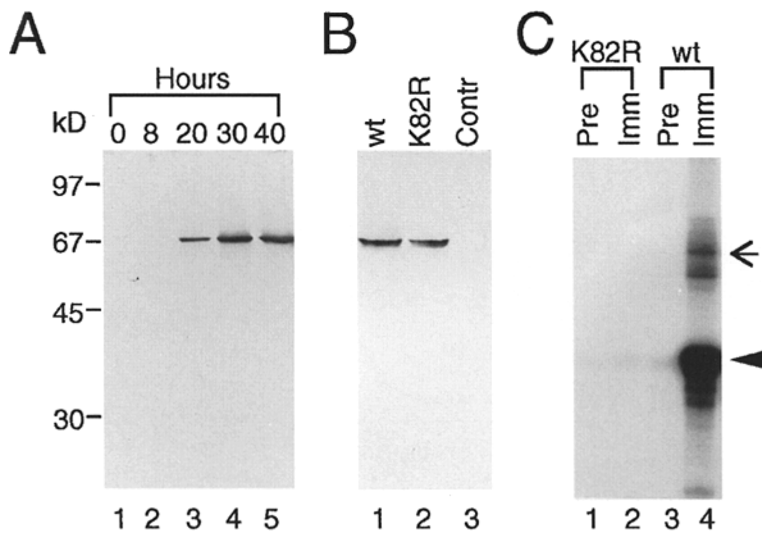

Figure 2. Production of recombinant Plk1 and K82R-Plk1 in baculovirus-infected Sf9 cells, and demonstration of Plk1 kinase activity. (*A*) Insect Sf9 cells were infected with recombinant baculovirus encoding Plk1. At the indicated intervals, extracts were prepared for analysis of Plk1 expression by immunoblotting with AR32 antibodies. Equal amounts of protein were loaded in each lane, and the position of molecular mass markers are indicated in kD. (*B*) Insect Sf9 cells were infected with either Plk1 baculovirus (*wt*, lane *1*), K82R-baculovirus (*K82R*, lane *2*) or a control baculovirus encoding a nonrelated protein (*Contr*, lane *3*). At 30 h postinfection, extracts were prepared and analyzed by immunoblotting with anti-Plk1 antibody AR32. (*C*) Insect Sf9 cells were infected with baculoviruses encoding either K82R-Plk1 (lanes *1* and *2*) or wild-type Plk1 (lanes *3* and *4*). At 30 h postinfection, extracts were prepared and incubated with either preimmune serum (*Pre*, lanes *1* and *3*) or anti-Plk1 immune serum (*Imm*, lanes *2* and *4*). The immune complexes were collected with protein A beads, washed three times with Bead buffer and once with kinase assay buffer, transferred to new tubes, and incubated in the presence of [γ-^{32}P]ATP and casein. The phosphorylated reaction products were analyzed by SDS-PAGE and autoradiography. The arrowhead marks the position of phosphorylated casein, whereas the arrow points to a phosphoprotein comigrating with Plk1.

lanes 4 and 6), and excess GTP interfered with the use of [γ-^{32}P]ATP (lane *5*). Hence, on the basis of a differential use of GTP, it is possible to distinguish Plk1 from casein kinase II. Also, in further experiments (data not shown), we found that Plk1 was unable to phosphorylate the peptide RRREEETEEE, a preferred substrate of casein kinase II (Kuenzel et al., 1987), and that Plk1 was not inhibited by heparin (up to 500 μg/ml), a potent inhibitor of casein kinase II (Pinna, 1990). Fig. 3 *B* shows that the casein kinase activity of Plk1 could also readily be distinguished from that associated with p34^{cdc2}/cyclin B: whereas Plk1 phosphorylated almost exclusively α-casein (lane *2*) p34^{cdc2}/cyclin B acted preferentially on β-casein (lane *1*).

Plk1 Activity Peaks during Mitosis

Having optimized experimental conditions for assaying Plk1 activity, we proceeded to carry out a detailed analysis of Plk1 kinase activity during the cell cycle. HeLa cells were either synchronized using drug arrest-release protocols (Figs. 4 and 5) or fractionated according to size by centrifugal elutriation (Fig. 6), and Plk1 casein kinase activity was measured in immunoprecipitates. To provide a marker for the timing of mitosis, the histone H1 kinase activity of CDKs (particularly p34^{cdc2}) was also determined for each sample. In these experiments, it was convenient to use p9^{suc1} beads for the isolation of p34^{cdc2} (Meijer et al.,

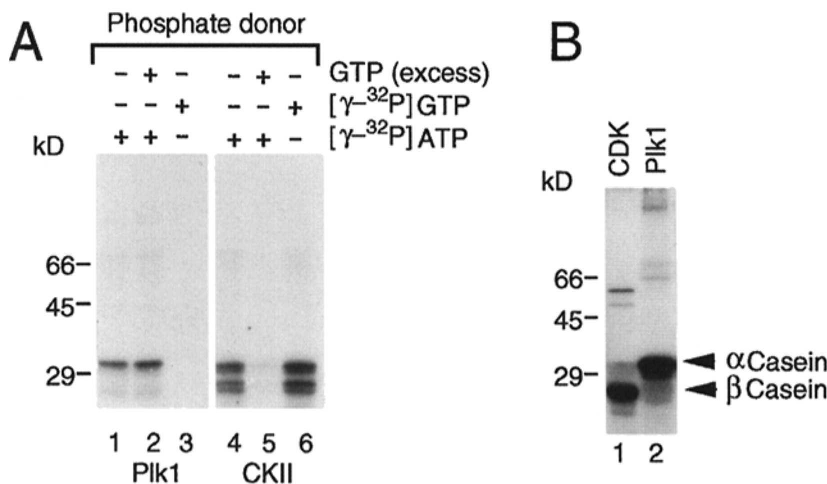

Figure 3. Comparison of Plk1 with casein kinase II and p34^{cdc2}. (*A*) The use of GTP provides a means to distinguish Plk1 activity from casein kinase II activity. Plk1 was immunoprecipitated from an extract of Plk1-baculovirus–infected Sf9 cells, and immune complexes were used for phosphorylating casein in the presence of the phosphate donors indicated above each lane (lanes *1–3*). In parallel, the ability of casein kinase II (Promega) to phosphorylate casein was assayed under identical conditions (lanes *4–6*). [γ-^{32}P]ATP or [γ-^{32}P]GTP were provided at 4 μCi, in the presence of 10 μM unlabeled nucleotide triphosphate, whereas excess unlabeled GTP was added at 1 mM. The reaction products were analyzed by SDS-PAGE and autoradiography. The positions of molecular mass markers are indicated in kD. (*B*) Plk1 preferentially phosphorylates α-casein, whereas p34^{cdc2}/cyclin B prefers β-casein. The casein kinase activities of p34^{cdc2}/cyclin B (lane *1*) and Plk1 (lane *2*) were assayed in parallel and analyzed by SDS-PAGE and autoradiography. The positions of α- and β-casein were determined by Coomassie blue staining of the gel before autoradiography. The p34^{cdc2}/cyclin B complex used in these experiments was isolated from mitotically arrested HeLa cells, using p9^{suc1} beads (Maridor et al., 1993; Golsteyn et al., 1994).

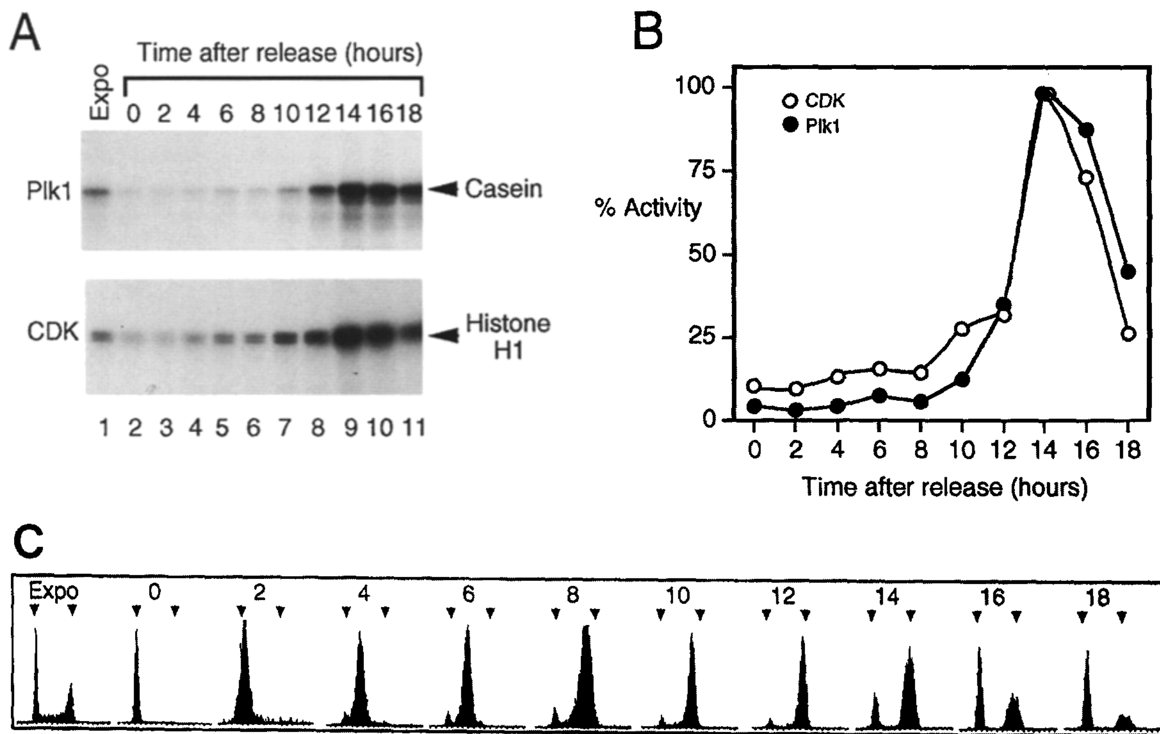

Figure 4. Cell cycle–dependent activation of Plk1 after release from a thymidine/aphidicolin block at G1/S. (*A*) HeLa cells were cultured for 14 h in the presence of 2 mM thymidine, and then for 14 h in fresh medium without thymidine, and finally, for 14 h in the presence of 1 μg/ml of aphidicolin. At time 0, cells were washed and replaced into fresh medium. Extracts were prepared from aphidicolin-arrested cells (time *0*, lane *2*), and at 2-h intervals after release from the aphidicolin block (lanes *3–11*). For comparison, an extract was prepared also from exponentially growing cells (*Expo*, lane *1*). Plk1 and CDKs were isolated from all extracts by immunoprecipitation and absorption on $p9^{suc1}$ beads, respectively. Immunoprecipitated Plk1 activity was assayed using casein as a substrate (*top panel*), whereas CDK activity associated with $p9^{suc1}$ beads was determined using histone H1 as a substrate (*bottom panel*). The reaction products were analyzed by SDS-PAGE and autoradiography. Only the relevant portions of each autoradiogram are shown. (*B*) The amounts of [^{32}P] incorporated into casein and histone H1, reflecting the activities of Plk1 and CDKs, respectively, were quantitated by using a phosphorimager. They were plotted as a function of time after release from the aphidicolin block; for both substrates, values were normalized relative to the maximal phosphorylation (100%) observed at 14 h after release. The Plk1-associated casein kinase activity is represented using filled symbols, whereas the CDK-associated histone H1 kinase activity is shown using open circles. (*C*) In parallel to the preparation of extracts for obtaining the data shown in panels *A* and *B*, a fraction of each cell population was used for FACS analysis. For each sample, the positions of the G1 phase peak and the G2/M phase peak are marked by small arrows.

1989; Maridor et al., 1993; Golsteyn et al., 1994). These beads bind also to other members of the CDK family, but it appears safe to attribute the bulk of the measured activity to $p34^{cdc2}$. During the cell cycle, complexes between this kinase and mitotic cyclins show at least tenfold higher histone H1 kinase activity than any other CDK/cyclin complex (Gabrielli et al., 1992). Furthermore, qualitatively very similar results were obtained when histone H1 kinase activities were measured in $p34^{cdc2}$ immunoprecipitates (data not shown).

In a first experiment (Fig. 4), HeLa cells were synchronized at the G1/S boundary, using a thymidine/aphidicolin double block procedure adapted from Heintz et al. (1983). After release from drug arrest, cells were collected at regular intervals, and Plk1 and CDK activities were measured, using casein and histone H1 as substrates, respectively (Fig. 4, *A* and *B*). In parallel, the DNA content of each sample was analyzed by flow cytometry (Fig. 4 *C*). This experiment allowed us to survey a period corresponding to nearly one complete cell cycle. It revealed that the Plk1-associated casein kinase and the CDK-associated histone H1 kinase displayed a very similar cell cycle dependency. Both activities were low in cells at early stages of the cell cycle (0–8 h after release from the G1/S block), but increased drastically to reach maximal levels as cells passed through mitosis (12-16 h after release from the G1/S block). By 18 h after release, when the bulk of the cell population had completed mitosis and reentered G1 phase, both Plk1 and CDK activities decreased again.

To corroborate the above results, Plk1 and CDK activities were also measured following release of cells from a prometaphase block imposed by nocodazole (Fig. 5). Under the experimental conditions used here, cells traversed mitosis within ~2 h, and daughter cells flattened onto the substratum by ~3–4 h (data not shown). As shown in Fig. 5, both Plk1-associated casein kinase and CDK-associated histone H1 kinase activities were high in nocodazole-arrested cells (lane *2*), stayed high for ~60 min after removal of the drug (lanes *3–5*), and declined with similar kinetics as cells exited from mitosis (lanes *6–8*). We note that three proteins coprecipitating with Plk1 (labeled *a*, *b*, and *c*) were reproducibly phosphorylated in these as-

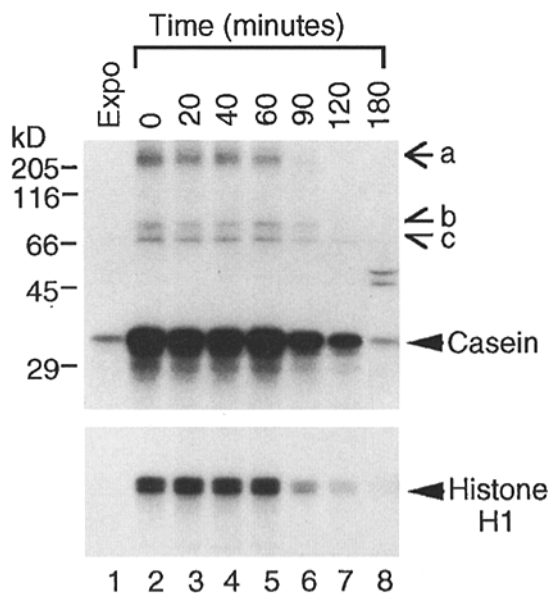

Figure 5. Cell cycle–dependent inactivation of Plk1 after release from a nocodazole block at prometaphase. HeLa cells were cultured for 14 h in the presence of nocodazole and mitotic cells were collected by mechanical shake-off. Extracts were prepared at time 0 (lane *2*), as well as at the indicated times (*in minutes*) after the release from the nocodazole block (lanes *3–8*). For comparison, an extract was prepared also from exponentially growing cells (*Expo*, lane *1*). The casein kinase activity of immunoprecipitated Plk1 (*top panel*) and the histone H1 kinase activity of p9[suc1]-absorbed CDKs (*bottom panel*) were then determined as described in the legend to Fig. 4, and the reaction products were analyzed by SDS-PAGE and autoradiography. The positions of molecular mass markers are indicated in kD. Arrows *a, b,* and *c* point to potential substrates coprecipitating with Plk1.

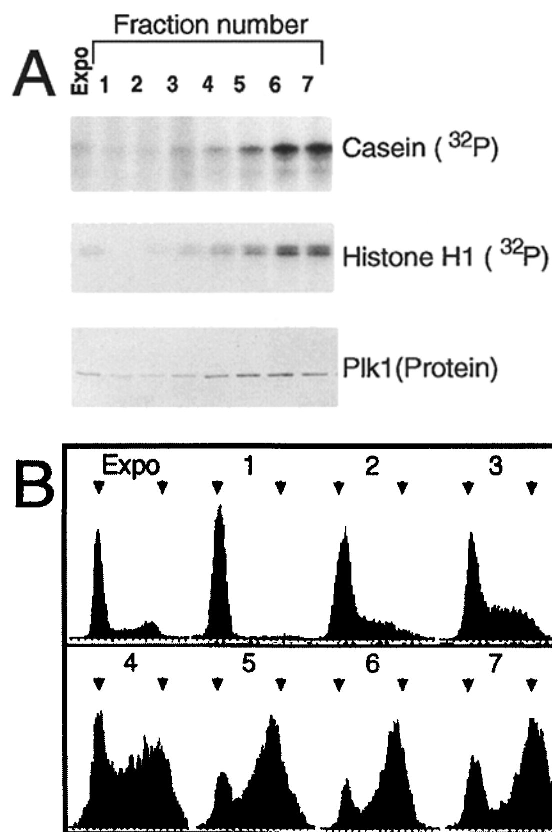

Figure 6. Cell cycle–dependent activity of Plk1 in cells synchronized by centrifugal elutriation. (*A*) Exponentially growing HeLa cells were synchronized by centrifugal elutriation, as described previously (Draetta and Beach, 1988; Golsteyn et al., 1994). Plk1 and CDKs were then isolated from each sample, as well as from exponentially growing cells, and the activities of these kinases were assayed as described in the legend to Fig. 4, using casein as a substrate for Plk1 (*top panel*) and histone H1 as a substrate for CDKs (*middle panel*). To determine the abundance of Plk1 in each sample, each extract was also probed by immunoblotting with AR32 anti-Plk1 antibodies (*bottom panel*). Only the relevant parts of the autoradiograms and the immunoblot are shown. (*B*) In parallel to the preparation of extracts for obtaining the data shown in *A*, a fraction of each cell population was used for FACS analysis. For each sample, the positions of the G1 phase peak and the G2/M phase peak are marked by small arrows.

says. One of these (protein *c*) may correspond to Plk1 itself, but it will be interesting to identify the others and determine whether they are physiologically relevant substrates of Plk1.

To exclude potential artefacts arising from the use of drugs for synchronization, HeLa cells were subjected to size fractionation by centrifugal elutriation (Fig. 6). Each sample was then used for assaying the activities of immunoprecipitated Plk1 and p9[suc1]-adsorbed CDKs (Fig. 6 *A*, *top* and *middle panel*, respectively), and analyzed for DNA content by flow cytometry (Fig. 6 *B*). In addition, the amount of Plk1 protein in each fraction was determined by immunoblotting (Fig. 6 *A*, *bottom panel*). Consistent with the data shown in Figs. 4 and 5, we found that both Plk1 and CDK activities were low in cell populations consisting predominantly of G1 and S phase cells (Fractions *1–3*), but increased substantially as more and more cells progressed to the G2 and M phases of the cell cycle (fractions *4–7*). On the basis of these results we conclude that the human protein kinase Plk1 is maximally active during mitosis.

We have shown previously that Plk1 protein levels fluctuate during the cell cycle, being low during G1, and increasing progressively to reach a peak during mitosis (Golsteyn et al., 1994). This finding is confirmed by the immunoblotting data shown in Fig. 6 *A* (*bottom panel*). Interestingly, however, a quantitative analysis of these data revealed that the increase in Plk1 protein cannot fully account for the increase in kinase activity (data not shown). This suggests that posttranslational events may be required for maximal activation of Plk1.

Association of Plk1 with the Mitotic Spindle Apparatus

Previous immunolocalization studies carried out with monoclonal antibodies against Plk1 had revealed that this kinase is diffusely distributed in both cytoplasm and nucleus during interphase stages of the cell cycle, but concentrated within postmitotic bridges in dividing cells (Golsteyn et al., 1994). Prompted by the exquisite specificity of the R32 rabbit anti-Plk1 antibody described here (see Fig. 1), we reexamined the subcellular localization of Plk1 using con-

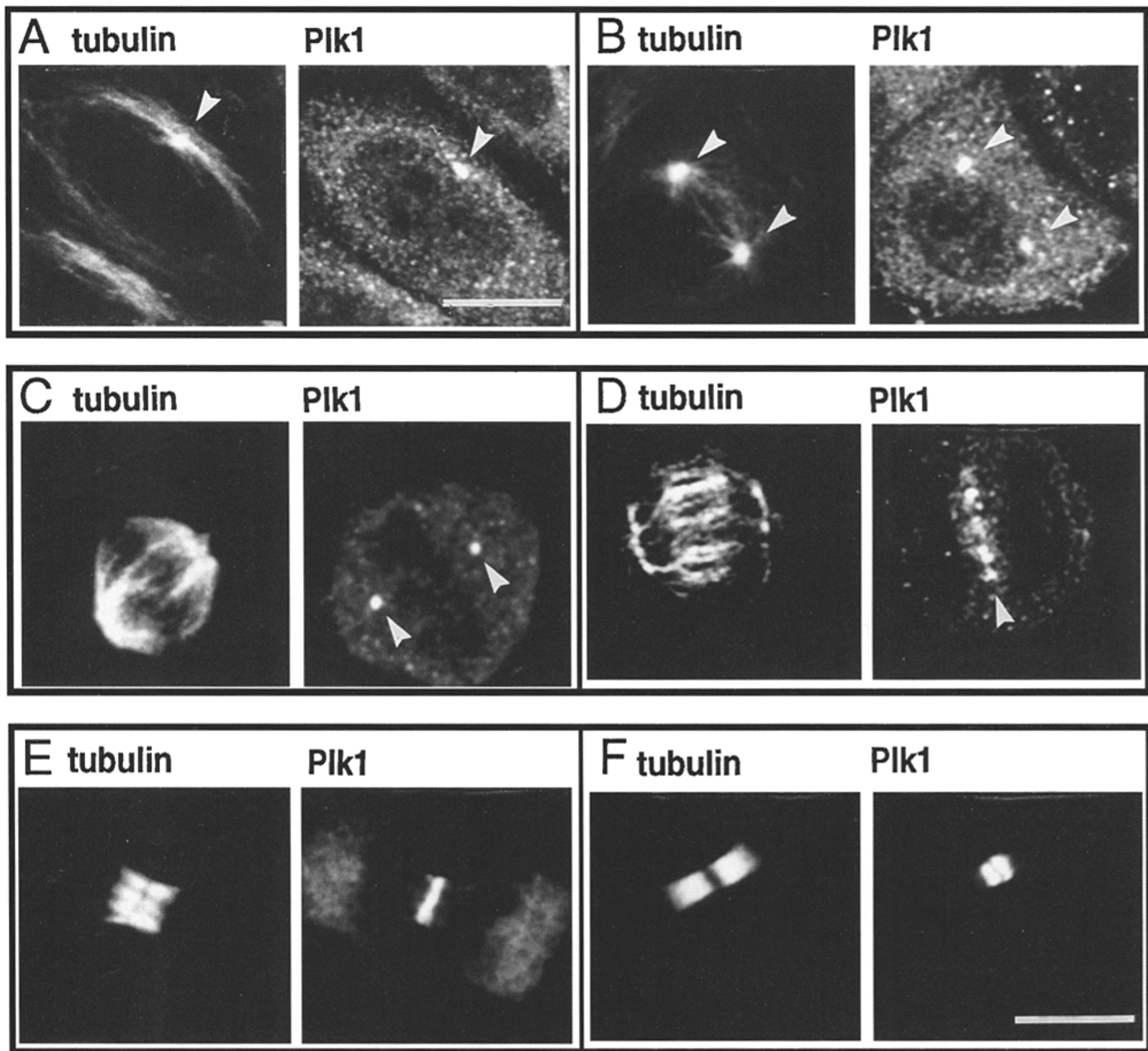

Figure 7. Subcellular localization of Plk1 in mitotic HeLa cells. HeLa cells were cultured on glass coverslips. Exponentially growing cells were treated, at room temperature, for 5 min with 3% paraformaldehyde, 2% sucrose in PBS, and then for 5 min in 0.5% Triton X-100 in 20 mM Hepes, pH 7.4, 3 mM $MgCl_2$, 50 mM NaCl, 300 mM sucrose, 0.02% NaN_3, and finally, at −20°C, for 5 min with methanol. Then, they were washed with PBS, and sequentially incubated with the rat monoclonal anti-tubulin antibody YOL1/34 (Serotec, Oxford, UK), followed by fluorescein-conjugated goat anti–rat IgG antibody, and with AR32 anti-Plk1 antibody, followed by biotinylated goat anti–rabbit IgG antibody and Texas red–conjugated streptavidin. Confocal microscopy was performed with an MRC 600 instrument (BioRad Labs). In each panel, tubulin staining is shown on the left, and Plk1 staining on the right. *A*, interphase cell with single centrosome; *B*, prophase cell with duplicated centrosomes; *C*, metaphase cell; *D*, anaphase cell; *E*, telophase cell; *F*, daughter cell pair in late telophase-early G1 phase. Bar in *A* indicates the magnification for panels *A*, *B*, *C*, and *D* and represents 10 μm. Bar in *F* represents 5 μm.

focal laser scanning microscopy. Exponentially growing HeLa cells were stained with affinity-purified rabbit R32 anti-Plk1 antibodies (Fig. 7; *right hand panels*), and, in parallel, with the rat monoclonal anti-tubulin antibody YOL/34 (Fig. 7; *left hand panels*). These experiments confirmed that Plk1 is diffusely distributed throughout interphase cells (*A*) and associated with postmitotic bridges in dividing cells (*F*). More interestingly, however, they also revealed that Plk1 undergoes a remarkable redistribution as cells progress through mitosis. In particular, we observed a prominent association of Plk1 with centrosomes and spindle poles at early stages of mitosis (*A–C*), followed by a concentration of Plk1 in the equatorial region of the spindle and in the cleavage plane at later stages of mitosis (*D–F*). Plk1 was detected at centrosomes already in interphase cells (*A*), and it persisted at spindle poles during prophase (*B*) and metaphase (*C*). During anaphase, however, Plk1 immunoreactivity disappeared completely from the spindle poles (*D*). Instead, intense staining for Plk1 arose in an equatorial plane corresponding to the spindle microtubule overlap zone (*D*). Plk1 then persisted in a region corresponding to the cleavage plane throughout telophase (*E*),

and concentrated close to the midbody in the postmitotic bridges connecting the dividing cells (*F*; see also Golsteyn et al., 1994). At this time, staining of Plk1 in the surrounding cytoplasm was barely detectable, suggesting that either all Plk1 had accumulated close to the midbody or that cytoplasmic Plk1 had been degraded. In additional experiments, cells were extracted with detergent before fixation with methanol and acetone (Nislow et al., 1993), with very similar results (data not shown). Attesting to the specificity of the R32 anti-Plk1 antibody, we also note that very similar staining patterns were observed after transfection of HeLa cells with myc-epitope tagged Plk1, and subsequent visualization of the ectopically expressed protein with anti-myc antibodies (data not shown).

Discussion

A Novel Protein Kinase Family Implicated in Cell Cycle Progression

Human Plk1 is a member of a newly emerging family of protein kinases. The founding member of this family is encoded by *polo*, a *Drosophila* gene identified in the course of studies on mutants displaying mitotic arrest phenotypes (Sunkel and Glover, 1988; Llamazares et al., 1991). More recently, the *S. cerevisiae* gene *CDC5* was found to encode a polo-related protein kinase (Kitada et al., 1993), and mutations in this gene cause yeast cells to arrest during late mitotic division (Byers and Goetsch, 1974; Schild and Byers, 1980; Kitada et al., 1993). Furthermore, when diploid cells homozygous for a temperature-sensitive *cdc5* mutant allele were subjected to the restrictive temperature during meiosis, they arrested at a stage following spindle pole body duplication with an aberrant meiosis I spindle (Schild and Byers, 1980). Hence, genetic evidence suggests that polo-related kinases are required for both mitotic and meiotic divisions.

Several laboratories have independently isolated cDNAs encoding a mammalian protein kinase, Plk1, that may represent a functional homolog of the *Drosophila* polo and yeast Cdc5p (Clay et al., 1993; Lake and Jelinek, 1993; Golsteyn et al., 1994; Hamanaka et al., 1994; Holtrich et al., 1994). Over the entire protein, Plk1 displays 52% sequence identity with *Drosophila* polo, and sequence conservation is not confined to the catalytic domain (65% identity) but extends to several motifs within the noncatalytic COOH-terminal domain. Other mammalian polo-related protein kinases displaying a lower degree of sequence similarity have also been described, notably the murine kinases Snk (Simmons et al., 1992) and Sak-a and Sak-b (Fode et al., 1994). Interestingly, the expression of Snk is strongly induced upon mitogenic stimulation of cells, suggesting that this kinase might play a role at early stages of the cell cycle (Simmons et al., 1992). Hence, it is possible that different members of the polo-like kinase family may all function in relation to cell proliferation, but may carry out distinct roles during the cell cycle, somewhat reminiscent of the functional specialization of cyclin-dependent protein kinases.

Recombinant Plk1 Is Active as a Casein Kinase

No data have so far been available on the biochemical properties of any of the polo-related kinases identified in mammals. To obtain this indispensable information, we have raised a rabbit antibody (R32) that is exquisitely specific for Plk1, and have constructed recombinant baculoviruses for expressing both wild-type and catalytically inactive Plk1 in insect cells. Using these tools, we were then able to develop an in vitro kinase assay that allows us to reliably measure Plk1 activity. When several commonly used protein kinase substrates were tested for their ability to be phosphorylated by Plk1, casein was found to be a preferred substrate. This result falls in line with previous studies showing that *Drosophila* polo and yeast Cdc5p also phosphorylate casein preferentially over histone H1 (Fenton and Glover, 1993; Kitada et al., 1993). More importantly, it provides the first biochemical evidence for a functional relationship between mammalian Plks and structurally related kinases from lower eukaryotes.

Human Plk1 Is Maximally Active during Mitosis

Using both drug-arrest release protocols for cell cycle synchronization and centrifugal elutriation for size-fractionation of HeLa cells, we found that Plk1 activity was low during interphase, but high as cells progressed through mitosis. We emphasize that possible contamination of Plk1 immunoprecipitates by casein kinase II or $p34^{cdc2}$ cannot be invoked to explain these results: casein kinase II activity has previously been shown to be virtually constant throughout the cell cycle (Litchfield et al., 1992; Krek, W., and E. A. Nigg, unpublished results) and $p34^{cdc2}$ phosphorylates β-casein rather than α-casein (see Fig. 3 *B*). Parallel measurements of Plk1 and $p34^{cdc2}$ kinase activities indicate that these two kinases display a very similar pattern of cell cycle–dependent activation and inactivation, at least within the temporal resolution afforded by our cell synchronization protocols.

Our findings raise the question of how Plk1 activity is regulated. Although nuclear run-off experiments suggested that Plk1 transcription rates may be fairly constant, mRNA levels were shown to fluctuate during the cell cycle, reaching a peak at the G2/M transition (Lake and Jelinek, 1993). In line with these results, we have shown previously (Golsteyn et al., 1994) and confirmed here (Fig. 6), that Plk1 protein levels increase as cells progress from G1 to M phase. Interestingly however, this increased expression cannot fully account for the increased activity of Plk1 during mitosis. In fact, we found that Plk1 isolated from mitotically arrested cells was ~4–6 times more active than an equivalent amount of Plk1 from interphase cells (data not shown, but see Fig. 6). Thus, the activity of Plk1 may be regulated by posttranslational mechanisms, and preliminary results indicate that Plk1 is phosphorylated during mitosis (Mundt, K. E., R. M. Golsteyn, and E. A. Nigg, unpublished results). In this context, it is noteworthy also that when recombinant Plk1 was immunoprecipitated from insect cells, the wild-type protein was phosphorylated in vitro, whereas the catalytically inactive K82R mutant was not (Fig. 2 *C*). Similarly, a phosphorylated protein of ~68 kD was observed after performing protein kinase assays with Plk1 isolated from mitotic human cells (Fig. 5, *arrow c*). These data suggest that Plk1 might undergo autophosphorylation.

On the basis of studies performed with *Drosophila* early syncytia embryos, which undergo synchronous and very rapid nuclear divisions, *Drosophila* polo was reported to be maximally active during late anaphase and telophase (Fenton and Glover, 1993). Hence, we were surprised to find that human Plk1 was activated already at the G2/M transition (Figs. 4 and 6). In fact, the kinetics of activation of Plk1 at the onset of mitosis were indistinguishable from those determined for the $p34^{cdc2}$ protein kinase. Likewise, upon release of cells from a prometaphase block Plk1 and $p34^{cdc2}$ displayed very similar patterns of inactivation (Fig. 5). No synchronization protocol presently available for cultured cells affords a degree of cell cycle synchrony that would match the synchrony observed in the early embryos of species such as *Drosophila*. Hence, we cannot exclude that a sharp spike of Plk1 activity at the anaphase-telophase transition might have gone undetected in our studies. On the other hand considering the very similar activation patterns observed for Plk1 and $p34^{cdc2}$, it appears difficult to escape the conclusion that Plk1 is active before the onset of anaphase. At present, we cannot readily reconcile our data with those reported for *Drosophila* polo, and it would clearly be interesting to study Plk1 activity in other organisms displaying natural cell cycle synchrony, e.g., sea urchins (Meijer et al., 1989). This, however, will have to await the generation of appropriate tools.

Plk1 Associates with Multiple Components of the Mitotic Spindle

The intracellular localization of Plk1 suggests that this kinase may control processes related to spindle organization and chromosome segregation. Using monospecific anti-Plk1 antibodies for confocal laser scanning immunofluorescence microscopy, we observed that Plk1 associated with centrosomes already in interphase cells. The kinase then remained associated with duplicating centrosomes during prophase and with spindle poles during metaphase. However, at anaphase all Plk1 immunoreactivity disappeared from the spindle poles and instead, strong Plk1 staining was observed in the equatorial region of the cell where spindle microtubules emanating from opposite poles overlap. Finally, at the end of telophase intense Plk1 staining was seen within postmitotic bridges, where Plk1 protein became concentrated on both sides of the midbody.

Thus, during the period of its maximal activity, Plk1 undergoes multiple transient associations with different elements of the mitotic spindle. With present technologies, it is not possible to assay protein kinase activities in situ, and hence we do not know at which of the various described locations Plk1 is active. However, our data are consistent with the possibility that Plk1 may act upon as yet unidentified substrates already at the onset of mitosis, when it is associated with the centrosomes, and then later during metaphase, when it is bound to spindle poles. In this context, it is interesting that mutations in *Drosophila* polo resulted in a disruption of centrosome organization, as visualized by a loss of staining by an anti-centrosomal antibody (Llamazares et al., 1991). Thus, *Drosophila* polo may also be required not only during anaphase and telophase, when it was reported to be maximally active (Fenton and Glover, 1993), but already at earlier stages of mitosis.

With the onset of anaphase, centrosome staining by anti-Plk1 antibodies disappeared and was replaced by strong staining in the spindle midzone area. We cannot formally exclude that these results simply reflect a concomitant masking and unmasking of Plk1 epitopes, but the most straightforward interpretation of our data is that the Plk1 protein undergoes a profound redistribution at the metaphase to anaphase transition. Other proteins that ultimately collect in postmitotic bridges are also recruited to the spindle midzone at the metaphase to anaphase transition, but it is interesting that several of these proteins first localize to chromosome arms or kinetochores (Rattner, 1992; Nislow et al., 1992; Earnshaw and Pluta, 1994). Examples of this are provided by CENP-E (Yen et al., 1991), CENP-F (Rattner et al., 1993), and the inner centromere proteins (INCEPs; Cooke et al., 1987; Earnshaw and Cooke, 1991). In contrast, there is presently no evidence that Plk1 would associate with chromosomes at any stage of mitosis. Hence, it will be interesting to determine with what components of the mitotic spindle Plk1 associates, and what mechanisms control the observed redistribution from spindle poles to midzone. To approach this issue, we have constructed several Plk1 mutants for use in both transfection experiments and yeast two-hybrid screens.

Concerning the function of the redistribution of Plk1 from spindle poles to spindle midzone, two alternative, but not mutually exclusive, possibilities come to mind. On the one hand, Plk1 may continue to be active during anaphase and telophase, and hence may phosphorylate substrates in the vicinity of the equatorial spindle microtubules. On the other hand, it is possible also that Plk1 translocates to the miotic spindle in preparation for its own destruction. We have argued previously that Plk1 protein levels might be reduced at the end of cell division, simply by shedding of postmitotic bridges (Golsteyn et al., 1994). Alternatively, or in addition, it is possible that Plk1 protein may be subject to degradation at the end of mitosis. Plk1 levels drop later in mitosis than those of cyclins A and B (Murray and Hunt, 1993), but this does not exclude that Plk1 might be degraded by a similar type of proteolytic mechanism. In support of this possibility, it has recently been proposed that the motor protein CENP-E is specifically destroyed at the end of mitosis (Brown et al., 1994).

In future studies, it will be interesting to determine how Plk1 activity is regulated during the cell cycle, and to establish possible links between Plk1 and known regulatory elements of mitotic control, particularly the CDK/cyclin complexes. Furthermore, it will be important to identify the physiological substrates of Plk1, *Drosophila* polo, and yeast Cdc5p. In this regard, the information on the activity and subcellular localization of Plk1, as well as the availability of active recombinant Plk1, should be very helpful.

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