Antibody Microinjection Reveals an Essential Role for Human Polo-like Kinase 1 (Plk1) in the Functional Maturation of Mitotic Centrosomes

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Abstract. Mammalian polo-like kinase 1 (Plk1) is structurally related to the *polo* gene product of *Drosophila melanogaster*, Cdc5p of *Saccharomyces cerevisiae*, and plo1$^+$ of *Schizosaccharomyces pombe*, a newly emerging family of serine-threonine kinases implicated in cell cycle regulation. Based on data obtained for its putative homologues in invertebrates and yeasts, human Plk1 is suspected to regulate some fundamental aspect(s) of mitosis, but no direct experimental evidence in support of this hypothesis has previously been reported. In this study, we have used a cell duplication, microinjection assay to investigate the in vivo function of Plk1 in both immortalized (HeLa) and nonimmortalized (Hs68) human cells. Injection of anti-Plk1 antibodies (Plk1$^+$) at various stages of the cell cycle had no effect on the kinetics of DNA replication but severely impaired the ability of cells to divide. Analysis of Plk1$^+$-injected, mitotically arrested HeLa cells by fluorescence microscopy revealed abnormal distributions of condensed chromatin and monoastral microtubule arrays that were nucleated from duplicated but unseparated centrosomes. Most strikingly, centrosomes in Plk1$^+$-injected cells were drastically reduced in size, and the accumulation of both γ-tubulin and MPM-2 immunoreactivity was impaired. These data indicate that Plk1 activity is necessary for the functional maturation of centrosomes in late G2/early prophase and, consequently, for the establishment of a bipolar spindle. Additional roles for Plk1 at later stages of mitosis are not excluded, although injection of Plk1$^+$ after the completion of spindle formation did not interfere with cytokinesis. Injection of Plk1$^+$ into nonimmortalized Hs68 cells produced qualitatively similar phenotypes, but the vast majority of the injected Hs68 cells arrested as single, mononucleated cells in G2. This latter observation hints at the existence, in nonimmortalized cells, of a centrosome-maturation checkpoint sensitive to the impairment of Plk1 function.

CELL cycle progression in all eukaryotes depends on the periodic activation and inactivation of cyclin-dependent kinases (for reviews see Norbury and Nurse, 1992; Morgan, 1995; Nigg, 1995). However, it is becoming increasingly clear that protein kinases structurally distinct from cyclin-dependent kinases also play important roles in the regulation of cell cycle events. One prominent, newly emerging family of serine/threonine-kinases with a likely role in cell cycle control is represented by the *polo* gene product of *Drosophila melanogaster* (Sunkel and Glover, 1988; Llamazares et al., 1991) and its putative homologues Cdc5p of *Saccharomyces cerevisiae* (Kitada et al., 1993), plo1$^+$ of *Schizosaccharomyces pombe* (Ohkura et al., 1995), and polo-like kinase 1 (Plk1, also referred to as Plk)[1] of mouse and human (Clay et al., 1993; Lake and Jelinek, 1993; Golsteyn et al., 1994, 1995, 1996; Hamanaka et al., 1994; Holtrich et al., 1994). All these kinases exhibit a high degree of sequence similarity, particularly within their amino-terminally located catalytic domains. Moreover, regions of sequence identity are also present within the carboxy-terminal noncatalytic domains, and a consensus motif comprising ~30 amino acids may constitute a characteristic signature of polo-like kinases (Clay et al., 1993; Hamanaka et al. 1994; Golsteyn et al.; 1994, 1996). In addition to Plk1, two murine kinases more distantly related to *Drosophila* polo have also been discovered. These kinases, termed Snk (Simmons et al., 1992) and Fnk (Donohue et al., 1995), are transcriptionally induced in response to mitogens, suggesting that they may play a role in progression through the G1 phase of the cell cycle. Thus, the family of polo-like kinases may comprise multiple members, at least in vertebrates, and it is an attractive possibility that different polo-like kinases may function at multiple stages of the cell cycle. Yet another polo-related murine kinase, termed Sak, is also thought to be involved in mitotic and meiotic cell division (Fode et al., 1994).

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1. *Abbreviations used in this paper*: BrdU, bromodeoxyuridine; MPM-2, mitotic protein monoclonal 2; Plk1, polo-like kinase 1.

However, although Sak resembles *Drosophila* polo over its catalytic domain, it lacks the polo-consensus motif in the carboxy-terminal domain.

The most detailed information about the properties and biological function of polo-like kinases has been obtained for *Drosophila* polo, the founding member of the family. During *Drosophila* development, increased levels of *polo* transcripts are observed in tissues with extensive mitotic activity, and polo protein expression appears to be required for all proliferative stages (Llamazares et al., 1991). In syncytial embryos, polo kinase activity was reported to peak during late anaphase–telophase (Fenton and Glover, 1993), and the phenotype of *polo* mutants strongly suggests a role for this kinase in mitosis (Sunkel and Glover, 1988; Llamazares et al., 1991). *Drosophila* mutants homozygous for a strong mutant *polo* allele ($polo^2$) die as larvae. However, flies with a weaker mutant allele ($polo^1$) produce embryos unable to undertake the syncytial mitotic cycles (Sunkel and Glover, 1988). These embryos display condensed chromosomes associated with branched, highly irregular microtubule arrays, and they appear to lack organized centrosomes (Sunkel and Glover, 1988). In larval neuroblasts, $polo^1$ mutants display a range of abnormal mitoses, which include overcondensed and polyploid chromosome complements and monopolar mitotic spindles. During male meiosis, chromosome disjunction is also disturbed (Sunkel and Glover, 1988; Llamazares et al., 1991).

The putative yeast homologues of *polo* have also been studied by genetic approaches. In *S. cerevisiae*, mutations in the *CDC5* gene produce abnormalities in both mitotic and meiotic divisions (Hartwell et al., 1973; Byers and Goetsch, 1974; Schild and Byers 1980; Sharon and Simchem, 1990). *CDC5* is essential for viability (Kitada et al., 1993), and *cdc5* mutants arrest in late mitosis as large, budded, dumbbell-shaped cells, with partially segregated nuclei on an elongated spindle (Kitada et al., 1993). In the case of *S. pombe*, loss of $plo1^+$ function has two distinct consequences: it leads to either a mitotic arrest in which condensed chromosomes are associated with a monopolar spindle or, following the completion of nuclear division, to a failure in septation (Ohkura et al., 1995). Overexpression of wild-type $plo1^+$ results in the formation of monopolar spindles and multiple septa, and, interestingly, multiple septa are formed even when $plo1^+$ is overexpressed in G1-arrested cells (Ohkura et al., 1995). These results suggest that the fission yeast $plo1^+$ kinase may play a dual role, being involved in both the establishment of a bipolar spindle and in septum formation.

To what extent *Drosophila* polo, budding yeast Cdc5p, and fission yeast $plo1^+$ perform exactly corresponding functions is not clear at this time. Likewise, the biological role of vertebrate Plk1 remains to be established, and as yet, information pertaining to mammalian Plk1 function is mainly indirect. Plk1 transcripts are highly expressed in tissues and cells exhibiting a high mitotic index (Clay et al., 1993; Lake and Jelinek, 1993; Golsteyn et al., 1994; Holtrich et al., 1994), including tumors and immortalized cell lines (Holtrich et al., 1994). Similar to budding yeast *CDC5* (Kitada et al., 1993), mammalian Plk1 mRNA shows a cell cycle–dependent expression, accumulating to maximal levels during G2 and M phases (Lake and Jelinek, 1993; Lee et al., 1995). Corresponding cell cycle oscillations have also been observed for the Plk1 protein (Golsteyn et al., 1994, 1995; Hamanaka et al., 1995; Lee et al., 1995) and Plk1-associated casein kinase activity (Golsteyn et al., 1995; Hamanaka et al., 1995; Lee et al., 1995). Most interestingly, Plk1 associates with the mitotic spindle apparatus throughout mitosis (Golsteyn et al., 1994, 1995; Lee et al., 1995). Specifically, the kinase localizes to spindle poles during prophase and metaphase, redistributes to the spindle equatorial plane (the midzone) as cells enter anaphase, and finally, concentrates at the midbody during telophase (Golsteyn et al., 1994, 1995; Lee et al., 1995).

The data described above support the view that vertebrate Plk may play a role during mitotic progression, much like its purported homologues in lower organisms. However, we emphasize that data on the biological function of mammalian Plk1 remain scarce, and there is no direct evidence for a mitotic role. Instead, results obtained with a sense–antisense RNA approach were interpreted to indicate that Plk1 functions somehow in the regulation of DNA replication (Hamanaka et al., 1994). In these experiments, microinjection of in vitro transcribed Plk1 sense mRNA into serum-starved murine NIH 3T3 cells was reported to induce DNA synthesis, whereas full-length antisense RNA appeared to block thymidine incorporation in growing cells (Hamanaka et al., 1994).

The goal of the present study was to directly explore the in vivo function of Plk1 in human cells. To this end, highly specific anti-Plk1 antibodies were microinjected into both immortalized (Hela) and nonimmortalized (Hs68) human cells at various stages of the cell cycle, and the ability of the injected cells to perform cell cycle functions was monitored. No evidence was obtained to support a role for Plk1 in S phase progression. Instead, our data clearly demonstrate that Plk1 function is required for both immortalized and nonimmortalized cells to proceed normally through mitosis. Cells injected with anti-Plk1 antibodies displayed striking defects in their ability to assemble a bipolar spindle. As indicated by the use of antibodies against several centrosomal antigens, these defects most probably arise from a failure of centrosomes to undergo critical structural changes at the G2 to M transition. Additional roles for Plk1 at later stages of mitosis are not excluded, although they could not be revealed by antibody microinjection. Finally, microinjection of anti-Plk1 antibodies into nonimmortalized Hs68 cells revealed that the majority of these cells failed to enter mitosis in the absence of Plk1 function. This observation raises the intriguing possibility that the activation of Cdc2 kinase in nonimmortalized cells may depend on Plk1 function and/or proper centrosome maturation.

Materials and Methods

Cell Culture, Synchronization, and Extraction

Hs68 human foreskin fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and grown for not more than eight passages. All cells were cultured in DME (GIBCO BRL, Gaithersburg, MD) supplemented with 5% (HeLa) or 10% (Hs68) FCS and penicillin-streptomycin (100 i.u./ml and 100 μg/ml, respectively) at 37°C in 7% CO_2. G0-arrested Hs68 fibroblasts were obtained by incubating 50% confluent cultures for 36 h in DME without FCS. After this period, <1% of the cell population incorporated bromodeoxyuridine (BrdU) during an incubation for 24 h, as detected by immunofluorescence microscopy (see below). This result correlated well with cell cycle analysis by flow cytometry, using

a FACS II® fluorescence-activated cell sorter, (Becton-Dickinson Immunocytometry Systems, Mountain View, CA), which demonstrated that >95% of the cells presented a 2 N DNA content. To arrest exponentially growing HeLa cells at prometaphase, nocodazole (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 50 ng/ml for 17 h. Mitotic cells were collected by mechanical shake-off, rinsed twice in prewarmed medium, and replated in standard HeLa growth medium. To arrest HeLa cells at the G1/S boundary, aphidicolin (1 μg/ml; Sigma Chemical Co.) was added for 19 h. This treatment resulted in an accumulation of cells that were >80% arrested in late G1/early S phase with a 2 N DNA content, as assessed by flow cytometry.

Cell extracts were prepared in NP-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 1% NP-40, 20 mM NaF, 1 mM EDTA, 5 mM EGTA, 20 mM β-glycerol phosphate, 0.1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 0.1 mM aprotinin) essentially as outlined in Golsteyn et al. (1995), except that lysates were centrifuged at 12,000 *g* (4°C) for 20 min immediately after extraction. Total protein concentrations were determined using the BioRad Protein Assay system (Hercules, CA) and BSA as a calibration standard.

Immunochemical Techniques

Anti-Plk1 IgG ($Plk1^+$) were affinity purified from serum R32 as previously reported (Golsteyn et al., 1995). A negative control antibody ($Plk1^-$) was prepared by passing the flow through the affinity column, depleted of all Plk1-specific antibodies, over a protein A–Sepharose column (Pharmacia LKB Biotechnology, Piscataway, NJ) and eluting with 100 mM glycine-HCl, pH 3.0, according to manufacturers instructions. Purified $Plk1^+$ and $Plk1^-$ IgG preparations, as well as nonimmune IgG (Sigma Chemical Co.), were dialyzed extensively against PBS, concentrated using a Centricon 30 unit (Amicon, Beverly, MA) and frozen in small aliquots at −70°C. Affinity-purified IgG specific for the human kinesin-related motor protein HsEg5 ($Eg5^+$; Blangy et al., 1995) was also prepared as above.

For immunoblotting, 10 μg of HeLa or Hs68 whole cell extracts or 3 μg of an extract from Sf9 cells overexpressing recombinant Plk1 (see Golsteyn et al., 1995) were resolved on a 10% acrylamide SDS-PAGE gel, transferred to nitrocellulose membranes, and incubated with $Plk1^+$ or $Plk1^-$ IgG (112 ng/ml in PBS containing 0.5% Tween 20), followed by alkaline phosphatase–conjugated secondary antibodies (1/5,000 in PBS containing 0.5% Tween 20; Promega Corp., Madison, WI), as previously described (Krek and Nigg, 1991).

Microinjection and Immunofluorescence Microscopy

For microinjection of asynchronous cells, HeLa and Hs68 cells were grown on acid-washed coverslips until 50% confluent and injected with antibodies using glass capillary needles (Clark Electromedical Instruments, Reading, UK) pulled on a micropipette puller (Flaming/Brown; Sutter Instruments Co., Novato, CA). Alternatively, cells were injected after serum starvation for 30–36 h (Hs68 G0 injections), after a nocodazole block and a 6–8 h release (HeLa G1 injections), or after an aphidicolin block and a 5–6 h release (HeLa late S phase injections). All coverslips were transferred into fresh medium shortly before injection of the appropriate IgG preparation (1.7 and 2 mg/ml for $Plk1^+$ and $Eg5^+$ IgG, respectively, in 75% PBS) into the cytoplasm of interphase or metaphase cells. Injections were either clustered for DNA synthesis experiments or widely scattered (one cell per microscopic field) so that any effects on the ability of injected cells to divide could be clearly observed. After injection, coverslips were placed into fresh medium containing 5% FCS (for HeLa cells), 10% FCS (for Hs68 asynchronous injections), or 20% FCS (for Hs68 G0 injections). For measurements of DNA synthesis, bromodeoxyuridine/fluorodeoxyuridine (Amersham Corp., Arlington Heights, IL) was added to the medium (1/1,000) for the duration of the experiment or for the times specified in the text.

Cells were fixed at room temperature for 10 min with 3.7% formaldehyde in PBS and permeabilized for 30 s with 100% acetone at −20°C. Injected cells were located by incubating coverslips with biotinylated anti-rabbit IgG antibodies (1/50 in PBS containing 1% BSA [PBS/BSA]; Amersham Corp.) for 1 h, followed by a mixture of Texas red–conjugated streptavidin (1/200 in PBS/BSA; Amersham Corp.) and Hoechst 33258 (0.5 μg/ml in PBS/BSA; Calbiochem-Novabiochem, La Jolla, CA) for 30 min. For analysis of DNA synthesis, fixed and permeabilized cells were treated for 10 min at room temperature with 1.5 M HCl followed by extensive washing with PBS. BrdU incorporation into nuclei was revealed as above, except that an anti-BrdU mouse mAb (Amersham Corp.) was used in the primary incubation, followed by FITC-conjugated anti-mouse IgG antibodies (Sigma Chemical Co.).

For costaining of injected antibodies and microtubules, cells were first incubated with undiluted rat anti-tubulin YOL1/34 hybridoma supernatant (Serotec Ltd., Oxford, UK) for 1 h, followed by biotinylated anti-rat IgG antibodies (1/100 in PBS/BSA; Amersham Corp.) for 30 min and, finally, Texas red–conjugated streptavidin (1/200 in PBS/BSA), Hoechst 33258 (0.5 μg/ml in PBS/BSA), and FITC-conjugated anti-rabbit IgG antibodies (1/100 in PBS/BSA; Sigma Chemical Co.) for 30 min. For staining of centrosomes with the mouse mAb CTR453, cells were fixed either as above or in −20°C methanol for 6 min, as described previously (Bailly et al., 1989). For γ-tubulin staining, a rat antipeptide polyclonal antibody raised against a synthetic peptide (Joshi et al., 1992) was used, kindly provided by Val Scott and Keith Gull (University of Manchester, UK), and cells were preextracted in detergent and fixed as previously described (MacRae et al., 1990). Before staining with the MPM-2 mAb, cells were preextracted before methanol fixation, as previously described (Vandré et al., 1984). CTR453, γ-tubulin, and MPM-2 epitopes were revealed using the tubulin staining protocol, except that either CTR453 (diluted 1/5 in PBS/BSA), γ-tubulin (diluted $1/_{100}$ in PBS/BSA), or MPM-2 (diluted 1/1,000 in PBS/BSA) antibodies were used for the primary incubation, followed by biotinylated anti–mouse or anti–rat IgG antibodies (1/50 in PBS/BSA; Amersham Corp.). Coverslips were routinely mounted in 80% glycerol, 3% *n*-propyl gallate (in PBS), but were mounted in 90% glycerol, 100 mM Tris-HCl, pH 9.0, for DNA synthesis experiments. Cells were observed with a microscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY) using 63 or 100× oil immersion objectives. In some cases, confocal laser scanning microscopy was performed on an invert laser scan microscope (model LSM 410; Carl Zeiss, Inc.), with a 63× plan-APOCHROMAT oil immersion objective, according to manufacturers instructions. Excitation wavelengths used were 488 nm for FITC (argon laser) and 543 nm for Texas red (helium/neon laser), with absorption windows of 510–525 nm and >570 nm, respectively. Confocal images presented in this paper represent projections of Z-series scans.

Results

A Polyclonal Antibody Highly Specific for Plk1 in HeLa and Hs68 Cells

A rabbit polyclonal antibody (R32) was raised against the carboxy-terminal 202 amino acids of human Plk1 (Golsteyn et al., 1995). For the purpose of the present microinjection study, Plk1-specific IgG ($Plk1^+$) were affinity purified from R32 serum. As a control, nonspecific IgG ($Plk1^-$) were prepared from the flow through of the affinity column. The specificity of these reagents is demonstrated in Fig. 1 (see also Golsteyn et al., 1995). Upon immunoblotting, $Plk1^+$ decorated a single ~66-kD protein in asynchronous HeLa and Hs68 cell extracts (Fig. 1, lanes *2* and *3*). This protein comigrated exactly with recombinant Plk1 expressed in baculovirus-infected Sf9 insect cells (lane *1*). It is noteworthy that the expression level of Plk1 protein was approximately twofold lower in Hs68 than in HeLa cells (compare lanes *2* and *3*), probably reflecting the lower mitotic index of nonimmortalized cell populations. Also, no Plk1 protein could be detected in extracts derived from G0-arrested Hs68 cells (lane *4*), consistent with previous analyses of Plk1 mRNA levels (Lake and Jelinek, 1993; Holtrich et al., 1994; Lee et al., 1995). The $Plk1^-$ IgG did not recognize Plk1 in any of the samples (Fig. 1, lanes *5–7*), indicating that this preparation had indeed been successfully depleted of all Plk1-specific antibodies and could thus be used as an innocuous control.

$Plk1^+$ Injection Does Not Affect DNA Synthesis in HeLa or Hs68 Cells

On the basis of antisense experiments, Plk1 had previously

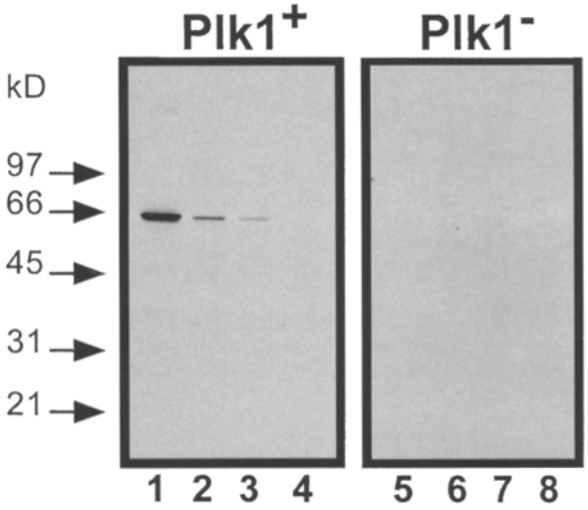

Figure 1. Specificity of the Plk1$^+$ antibody. Extracts of Sf9 cells expressing recombinant Plk1 (lanes *1* and *5*), asynchronous HeLa cells (lanes *2* and *6*), asynchronous Hs68 cells (lanes *3* and *7*), and G0 serum–arrested Hs68 cells (lanes *4* and *8*) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with Plk1$^+$ (lanes *1–4*) or Plk1$^-$ (lanes *5–8*) IgG. Molecular mass standards are indicated on the left.

been implicated in the control of DNA replication (Hamanaka et al., 1994). Therefore, we wished to determine whether microinjection of Plk1-specific antibodies would interfere with DNA synthesis. Equal concentrations of Plk1$^+$ and Plk1$^-$ IgG (1.7 mg/ml) were injected into the cytoplasm of both asynchronous HeLa and G0-arrested Hs68 cells. Then, BrdU was added to the injected cells, and these were either further cultured for 20 h (HeLa) or released into the cell cycle by readdition of serum for 40 h (Hs68). After fixation, the ability of injected cells to incorporate BrdU into nuclei was assessed using an anti-BrdU mAb for indirect immunofluorescence microscopy. In both cell types, very similar proportions of cells had incorporated BrdU, regardless of whether they had been injected with Plk1$^+$ or Plk1$^-$ antibodies, or had not been injected at all (Fig. 2 *A*).

The above experiment argued that Plk1$^+$ antibodies did not block DNA synthesis, but it remained possible that they might have influenced the kinetics of either entry into or exit from S phase. To examine this possibility, both HeLa and Hs68 cells were synchronized before injection with Plk1$^+$ antibodies. HeLa cells were arrested in prometaphase with nocodazole and then released and microinjected in the subsequent early G1 phase. Hs68 cells, on the other hand, were arrested in G0 by serum starvation and then injected and released into the cell cycle by serum readdition. In both cases, entry into and exit from S phase were monitored by pulse labeling with BrdU. The results, summarized in Fig. 2 *B*, clearly show that cells microinjected with Plk1$^+$ entered and exited S phase with the same kinetics as the surrounding, noninjected cells. We conclude, therefore, that injection of Plk1-specific IgG has no effect on the ability of either HeLa or Hs68 cells to proceed through S phase. These results lend no support to the proposition that Plk1 plays a role in S phase events (Hamanaka et al., 1994; see Discussion).

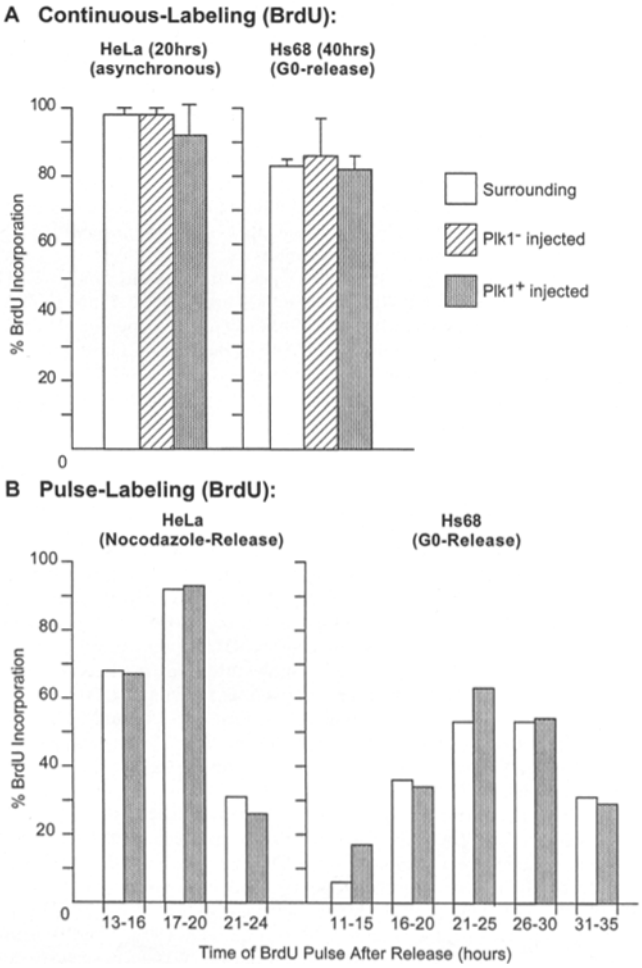

Figure 2. Plk1$^+$ injection has no effect on S phase progression in HeLa or Hs68 cells. (*A*) Asynchronously growing HeLa cells, and Hs68 cells induced to withdraw from the cell cycle by serum deprivation (G0), were microinjected into the cytoplasm with Plk1$^+$ or Plk1$^-$ IgG. Upon continued incubation (*HeLa*) or release into the cell cycle by serum readdition (*Hs68*), in the presence of BrdU, the cells were analyzed by immunofluorescence after 20 and 40 h, respectively. The percentage of cells incorporating BrdU into nuclei is presented. Results are averages with standard deviations derived from three (*HeLa*) and two (*Hs68*) independent experiments. A total of 227 (*HeLa*) and 85 (*Hs68*) cells were injected with Plk1$^+$, and 134 (*HeLa*) and 70 (*Hs68*) cells were injected with Plk1$^-$. (*B*) HeLa cells synchronized in G1 by nocodazole release and Hs68 arrested in G0 were injected with Plk1$^+$ IgG. Upon continued incubation (*HeLa*) or serum readdition (*Hs68*), cells were pulse-labeled with BrdU for the times indicated and then analyzed by immunofluorescence microscopy. The percentage of cells incorporating BrdU into nuclei during the pulse time is presented. At least 40 cells were injected for each time point.

Plk1$^+$ Injection into HeLa Cells Interferes with Mitotic Progression and Cell Division

During the course of the experiments described above, we had noticed a build-up of rounded, mitotic-like cells after Plk1$^+$ injection. To investigate the possibility that Plk1 might be required for progression through mitosis, we established a protocol of scattered microinjection. Accord-

ing to this protocol, only one cell per microscopic field is injected, and the fate of each cell is then monitored as a function of time. In a first series of experiments, Plk1$^+$ or control antibodies were microinjected into the cytoplasm of asynchronously growing HeLa cells, and after incubation for 20 or 40 h, cells were analyzed by fluorescence microscopy using secondary antibodies to visualize injected cells and Hoechst dye to monitor the state of the DNA. The results of these experiments are summarized in Fig. 3 and Table I. Of the Plk1$^-$-injected cells, >90% proceeded through mitosis to the two cell stage by 20 h, with only very few cells in mitosis (<1%), and with no significant proportion of abnormal phenotypes evident. After 40 h, most of these cells had proceeded through a second round of division, reaching the four cell stage (Fig. 3 *A* and Table I). Identical results were obtained after injection of a nonimmune IgG control (Table I). In contrast, only 30% of the cells injected with Plk1$^+$ had divided normally by 20 h, while 34% accumulated as rounded, mitotic-like cells (Table I). Hoechst staining of these cells revealed condensed, ball-like chromosomes that, in most cases, exhibited no obvious alignment in a metaphase plate (Fig. 3 *B*, *a*). Some 25% of the cells displayed additional abnormal phenotypes (Fig. 3 *B* and Table I). These included cells with strikingly fragmented nuclei (referred to as micronuclei; Fig. 3 *B*, *b*) or with multiple nuclei of unknown karyotype (Fig. 3 *B*, *c*), and cells that had undergone inappropriate nuclear division, as characterized by the appearance of DNA strands between divided cells (Fig. 3 *B*, *d*). By 40 h after injection of Plk1$^+$, the number of abnormal mitotic cells had dropped to 5%, with a concomitant increase in the number of micronucleated and multinucleated cells to 40% (Table I).

The described phenotypes suggested a function for Plk1 specifically during mitosis. One could legitimately argue, however, that Plk1 might actually be required earlier in the cell cycle, for example during S phase when centrosomes are duplicated, but that the consequences of impaired Plk1 function might only become apparent in the subsequent mitosis. To rule out this possibility, HeLa cells were synchronized at the G1/S border, using aphidicolin, and Plk1$^+$ or Plk1$^-$ IgG were injected 5–6 h after release from this block, i.e., when cells were in late S phase/early G2 (as assessed by flow cytometry; data not shown). After an additional incubation for 7, 14, or 24 h, cells were fixed and their mitotic index and ability to divide recorded (Fig. 4). By 13 h after the release from the G1/S block, the time at which we found the wave of mitotic cells to reach its highest level in the control samples, 23 and 19% of noninjected and Plk1$^-$-injected cells, respectively, were in mitosis (Fig. 4 *A*). At this early time, the number of Plk1$^+$ cells in mitosis was somewhat reduced (14%; Fig. 4 *A*), and comparatively few of the injected cells had completed division (19% for Plk1$^-$ and 12% for Plk1$^+$; Fig. 4 *B*). The impairment of Plk1 function did, however, produce striking effects by 20 h; at this time, 77% of Plk1$^-$-injected cells had divided (Fig. 4 *B*), and, concomitantly, the mitotic index of these as well as the surrounding noninjected cells was strongly reduced (Fig. 4 *A*). In marked contrast, comparatively few of the Plk1$^+$-injected cells (26%) had completed division (Fig. 4 *B*), and instead, many (45%) had accumulated as rounded, mitotic-like cells (Fig. 4 *A*). After 30 h, virtually all Plk1$^-$-injected cells had completed division (87%), but there was no significant further increase in the number of Plk1$^+$-injected cells that had divided (29%; Fig. 4 *B*). At this late time, up to 50% of the Plk1$^+$-injected cells were either micronucleated or multinucleated (data not shown). From the data shown in this section, we conclude that abrogation of Plk1 function in HeLa cells results in a strong inhibition of progression through mitosis, which ultimately culminates in a lack of cytokinesis and a failure to segregate chromosomes correctly.

Microinjection of Plk1$^+$ into HeLa Cells Leads to Nucleation of Abnormal Microtubule Arrays from Small, Duplicated but Unseparated Centrosomes

As a first step towards understanding the molecular basis for the observed requirement for Plk1 function during mitosis, Plk1$^+$-injected, mitotically blocked cells (of the type shown in Fig. 3 *B*, *a*) were analyzed by confocal and conventional microscopy, using antitubulin and antipericentrosomal mAbs for immunofluorescent staining (Fig. 5). Whereas Plk1$^-$-injected cells were able to set up a normal bipolar spindle (Fig. 5 *A*, *left*), most of the Plk1$^+$-injected cells failed to do so. Instead, 68% of all mitotic cells examined displayed arrays of comparatively thin microtubule bundles nucleating from a single organizing center, more reminiscent of interphase microtubules than of spindle fibres (Fig. 5 *A*, *right*). Structures resembling bipolar spindles could be observed in the remaining cells, but only few of these looked completely normal (data not shown).

Colocalization of microtubules and the CTR453 antigen, a pericentrosomal protein (Bailly et al., 1989), revealed that the monoastral microtubule arrays frequently contained two centrosomes that had clearly duplicated but had failed to separate sufficiently for bipolar spindle formation (Fig. 5 *B*, *left*). At first glance, this phenotype appears similar to that previously observed after microinjection of antibodies against HsEg5, a centrosome-associated, kinesin-related motor protein (Blangy et al., 1995). However, although centrosome separation was indeed inhibited in both cases, a direct comparison of the phenotypes induced by Plk1$^+$ or anti-HsEg5 antibodies (Eg5$^+$) revealed striking differences: compared to Eg5$^+$-injected cells, Plk1$^+$-injected cells displayed much smaller centrosomes, and the density of microtubules nucleating from these centrosomes appeared to be decreased (Fig 5 *B*, compare *left* and *right*). To confirm the apparent reduction in centrosome size, HeLa cells were injected in the exact same experiment with Plk1$^+$ or Eg5$^+$ IgG's. After 20 h, centrosomes were stained with CTR453 and the sizes of centrosomes were recorded using conventional fluorescence microscopy and identical photographic conditions. As can be seen from Fig. 5 *C*, the centrosomes in Eg5$^+$-injected cells (*right*) were indistinguishable in their sizes from those of uninjected mitotic HeLa cells (*left*), whereas those of Plk1$^+$-injected cells were substantially reduced in size (*middle*). These data indicate that Plk1 function is required for centrosomes to undergo a functional maturation that is typically reflected by an apparent increase in centrosome size (Vorobjev and Nadehzdina, 1987; Kimble and Kuriyama, 1992; Kalt and Schliwa, 1993).

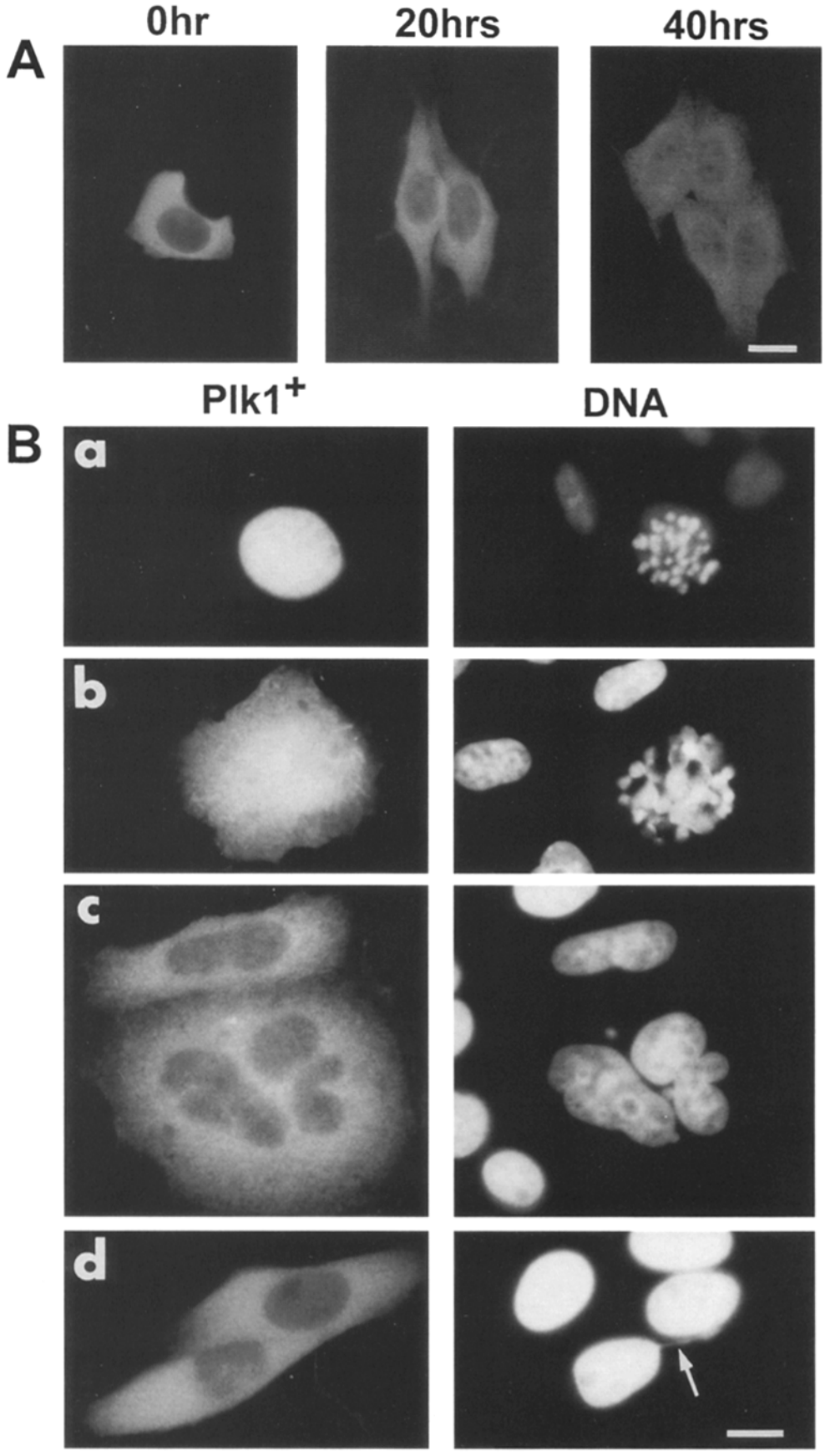

Figure 3. Injection of HeLa cells with $Plk1^+$ inhibits mitotic progression and causes abnormal division phenotypes. Widely scattered HeLa cells were injected with $Plk1^+$ or $Plk1^-$ IgG, fixed, and analyzed by immunofluorescence microscopy after 20 and 40 h of incubation. (*A*) $Plk1^-$ control-injected cells showing normal progression through the first (20 h) and second (40 h) cell division. (*B*) Commonly observed phenotypes caused by $Plk1^+$ injection: rounded mitotic cells with ball-like DNA condensation (*a*), micronucleated interphase cells (*b*), multinucleated interphase cells (*c*), and divided cells still connected by a DNA strand (*d*). Bars, 10 μm.

Table I. Effect of Anti-Plk1 Antibodies on HeLa Cell Division

Antibody injected	Plk1$^+$		Plk1$^-$		Nonimmune IgG	
20 h	%		%		%	
Single interphase cells	11	(32/291)‡	3	(5/147)	4	(8/191)
Normally divided cells	30	(87/291)	94	(138/147)	92	(176/191)
Mitotic cells*	34	(98/291)	0.5	(1/147)	1	(2/191)
Micro-/Multinucleated cells	20	(58/291)	2.5	(4/147)	2	(4/191)
Divided cells with connecting DNA strand	5	(16/291)		(0/147)	1	(1/191)
Total	100		100		100	
40 h						
Single interphase cells	1	(2/201)	1	(1/78)	0	(0/66)
Normally divided cells§	51	(102/201)	91	(71/78)	94	(62/66)
Mitotic cells*	5	(10/201)	0	(0/78)	0	(0/66)
Micro-/Multinucleated cells	40	(81/201)	8	(6/78)	6	(4/66)
Divided cells with connecting DNA strand	3	(6/201)	0	(0/78)	0	(0/66)
Total	100		100		100	

Asynchronously growing interphase HeLa cells were microinjected with Plk1$^+$, Plk1$^-$, or nonimmune IgG. After 20 and 40 h incubation, cells were fixed and analyzed by immunofluorescence microscopy. Microscopic scanning of the glass coverslip allowed the calculation of the percentage of injected cells with the phenotypes listed. The total results of four independent experiments with the appropriate controls are shown.

*Mitotic cells injected with Plk1$^+$ display abnormal DNA condensation as described in the text (see also Fig. 3 *B*, *a*).

‡Total number of cells displaying phenotype/total number of injected cells.

§Cells that have gone through at least one normal cell division. For simplicity, cells that have divided once or twice are grouped together. In control-injected cells, at least 80% have gone to the four cell stage.

Plk1$^+$ Injection Inhibits the Recruitment of γ-Tubulin and MPM-2 Epitopes to Mitotic Centrosomes

To analyze the centrosome maturation defect in more detail, HeLa cells were injected with either Plk1$^+$ or Eg5$^+$ IgG's and 20 h later were stained with antibodies recognizing either γ-tubulin (Fig. 6 *A*) or the MPM-2 phosphoepitope (Fig. 6 *B*). γ-Tubulin is a highly conserved centrosomal protein required for microtubule nucleation (Oakley and Oakley, 1989; Oakley et al., 1990; Stearns et al., 1991; Zheng et al., 1991, Joshi et al., 1992; Félix et al., 1994; Stearns and Kirschner, 1994; Shu and Joshi, 1995; Sunkel et al., 1995; Moritz et al., 1995*a*,*b*), whereas MPM-2 immunoreactivity appears on multiple proteins upon entry of cells into mitosis (Davis et al., 1983; Vandré et al., 1991), most prominently on mitotic spindle poles (Vandré et al., 1984, 1986, 1991). Thus, in HeLa cells the accumulation of both γ-tubulin and MPM-2 immunoreactivity can be considered as excellent markers for the functional maturation of centrosomes at the G2 to M transition.

In line with previous results (Zheng et al., 1991; Lajoie-Mazenc et al., 1994), we found that centrosome staining with anti–γ-tubulin antibodies was comparatively weak in interphase HeLa cells, but increased substantially as cells entered mitosis (Fig. 6 *A*, compare *left panels*). Within monoastral spindles of Plk1$^+$-injected cells, the centrosomes displayed very weak γ-tubulin staining, reminiscent of interphase cells, whereas in Eg5$^+$-injected mitotic cells, staining was as intense as in noninjected mitotic cells (Fig 6 *A*, compare *right panels*). Although the lack of immunoreactivity cannot be considered as rigorous proof for the absence of the corresponding antigen, the most likely interpretation of these results is that, during the G2 to M transition, Plk1$^+$ antibodies specifically inhibited the recruitment of γ-tubulin to centrosomes, whereas Eg5$^+$ antibodies were without effect. Next, we investigated whether the injection of Plk1$^+$, or Eg5$^+$ for control, would interfere with the appearance of MPM-2 reactivity. 20 h after injection, HeLa cells were stained with MPM-2 (Fig. 6 *B*, *upper*) and, for comparison, with antibodies against α-tubulin (Fig. 6 *B*, *lower*). In agreement with previous results (Vandré et al., 1984, 1986, 1991), the MPM-2 antibody stained spindle poles and spindle microtubules in HeLa cells; in addition, it stained a number of dots in the spindle mid-zone, most likely representing kinetochores (Fig. 6 *B*, *upper left*). After injection of Plk1$^+$ antibodies, however, MPM-2 reactivity was rearranged. Most typically, spindle poles showed little if any staining, and instead, MPM-2 reactivity was confined to surrounding microtubules (Fig. 6 *B*, *upper middle*). In contrast, MPM-2 reactivity could readily be discerned on the duplicated centrosomes as well as on the astral microtubules in Eg5$^+$-injected cells (Fig. 6 *B*, *upper right*). We reproducibly observed also that kinetochore staining by MPM-2 antibodies was impaired after the microinjection of Plk1$^+$ but not Eg5$^+$ antibodies, although this result is very difficult to document photographically (see Fig. 6 *B*). These latter data indicate that the accumulation of MPM-2 reactivity at centrosomes, and possibly at kinetochores, is inhibited by Plk1$^+$ but not Eg5$^+$ antibodies.

Plk1$^+$ Injection Blocks Entry into Mitosis in Nonimmortalized Hs68 Fibroblasts

As many immortalized tumor cell lines, including HeLa, are known to be defective in cell cycle checkpoint functions (Hartwell and Weinert, 1989; Murray, 1992; Hartwell and Kastan, 1994), we considered it important to extend our studies to diploid, nonimmortalized human cells. Specifically, we examined the effects of Plk1$^+$ injection on the cell cycle progression of human foreskin fibroblasts (Hs68). Plk1$^+$ and Plk1$^-$ IgG were injected into the cytoplasm of widely scattered, asynchronously growing interphase Hs68 cells, and 36 or 48 h later, cells were fixed and examined

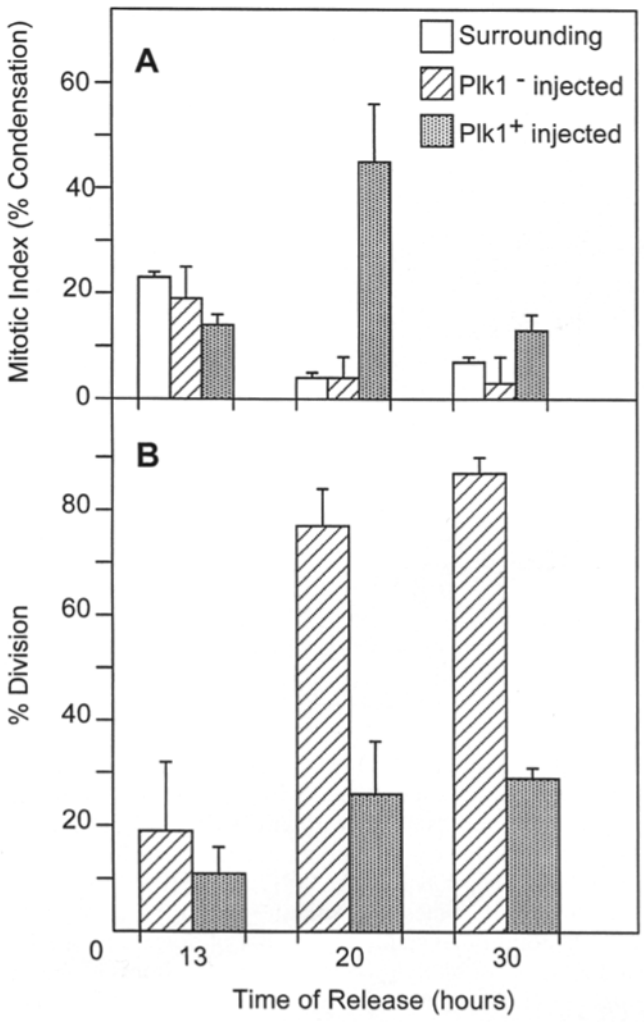

Figure 4. Plk1$^+$ injection of HeLa cells in late S phase inhibits mitotic progression. HeLa cells, synchronized at G1/S by aphidicolin treatment, were released into the cell cycle for 5–6 h before Plk1$^+$ or Plk1$^-$ injection into the cytoplasm; at that time, they were in late S/early G2 phase. Injections were scattered for accurate counting, and upon continued incubation, cells were fixed and analyzed by immunofluorescence microscopy at the times (post G1/S release) indicated. Indicated are in (*A*) the mitotic index (i.e., the percentage of cells showing chromosome condensation) and in (*B*) the percentage of injected cells that had proceeded through one cell division. At least 30 cells were injected for each time point in each experiment, and the averages of three independent experiments with standard deviations are shown.

microscopically. Whereas 52 and 67% of the Plk1$^-$-injected cells had divided normally after 36 and 48 h, respectively, with no abnormal phenotypes evident, only 5% of the Plk1$^+$-injected cells had progressed to the two cell stage by these times (Table II). Furthermore, only 16% of the Plk1$^+$-injected cells had accumulated as rounded, mitotic-like cells by 36 h (Table II). These abnormal cells displayed the same ball-like chromatin condensation as seen with HeLa cells (Fig. 7 *A*, *right*). Further analysis of these cells by immunofluorescence and confocal microscopy demonstrated a complete absence of bipolar mitotic spindles (Fig. 7 *A*, *left*). Centrosomes, although duplicated, were unseparated and drastically reduced in size, as described above for HeLa cells (data not shown). By 48 h, the number of mitotically blocked cells decreased to 2%, with a concomitant increase in the proportion of micro- and multinucleated cells (from 8 to 18%; Table II), consistent with the idea that these late phenotypes may represent the end result of aborted mitoses.

These results show that Plk1$^+$ antibody injection into Hs68 cells could produce aberrant mitotic phenotypes that were qualitatively similar to those observed in HeLa cells. Rather unexpectedly, however, we found that the vast majority of the Plk1$^+$-injected Hs68 cells (75%) persisted as single, interphase cells with apparently normal interphase microtubule networks. As shown in Fig. 7 *B*, these Plk1$^+$-injected cells displayed duplicated centrosomes (*middle*), the sizes of which were comparable to those of uninjected interphase cells (*left*), but significantly smaller than those of mitotic cells (*right*). It would seem, therefore, that most of the Plk1$^+$-injected Hs68 cells did not even attempt entering mitosis, but instead arrested in late interphase. In comparison, only 32% of Plk1$^-$-injected Hs68 cells, and only 1% of Plk1$^+$-injected HeLa cells, persisted as single cells (Tables I and II). One intriguing interpretation of these results is that nonimmortalized cells contain a checkpoint monitoring the proper maturation of centrosomes (see Discussion).

Plk1$^+$ Injection Does Not Affect Mitotic Events Subsequent to Bipolar Spindle Formation

In a final series of experiments, we asked whether Plk1 function might also be essential during later stages of mitosis, particularly during cytokinesis. To this end, Plk1$^+$ and Plk1$^-$ IgG were injected into cells that had already established a mitotic spindle. These were selected amongst exponentially growing HeLa and Hs68 cells, using phase contrast microscopy to identify cells with clearly visible metaphase chromosomes. After 15–20 h, 87 and 97% of control-injected HeLa and Hs68 cells had completed cytokinesis and proceeded to the two-cell stage, respectively (Table III). Remarkably, a similar percentage of Plk1$^+$-injected cells (88 and 91% of HeLa and Hs68, respectively) had also divided, with no significant percentages of cells displaying abnormal phenotypes (Table III). These results indicate that Plk1 function is no longer sensitive to inhibition by microinjected antibodies beyond the metaphase state. They do not prove that Plk1 is not involved in later stages of mitosis (see Discussion), but they provide an exquisite control and attest to the specificity of the block to mitotic progression observed after antibody injection into interphase cells.

Discussion

In this study, we have explored the function of human Plk1, using highly specific anti-Plk1 antibodies for microinjection experiments. We have obtained no evidence to support a role for Plk1 during DNA replication. Instead, our data clearly establish that Plk1 function is required for progression through mitosis. A detailed analysis of the phenotypes of antibody-injected cells indicates that mammalian Plk1 is required for the functional maturation of centrosomes, and hence for the assembly of a bipolar spin-

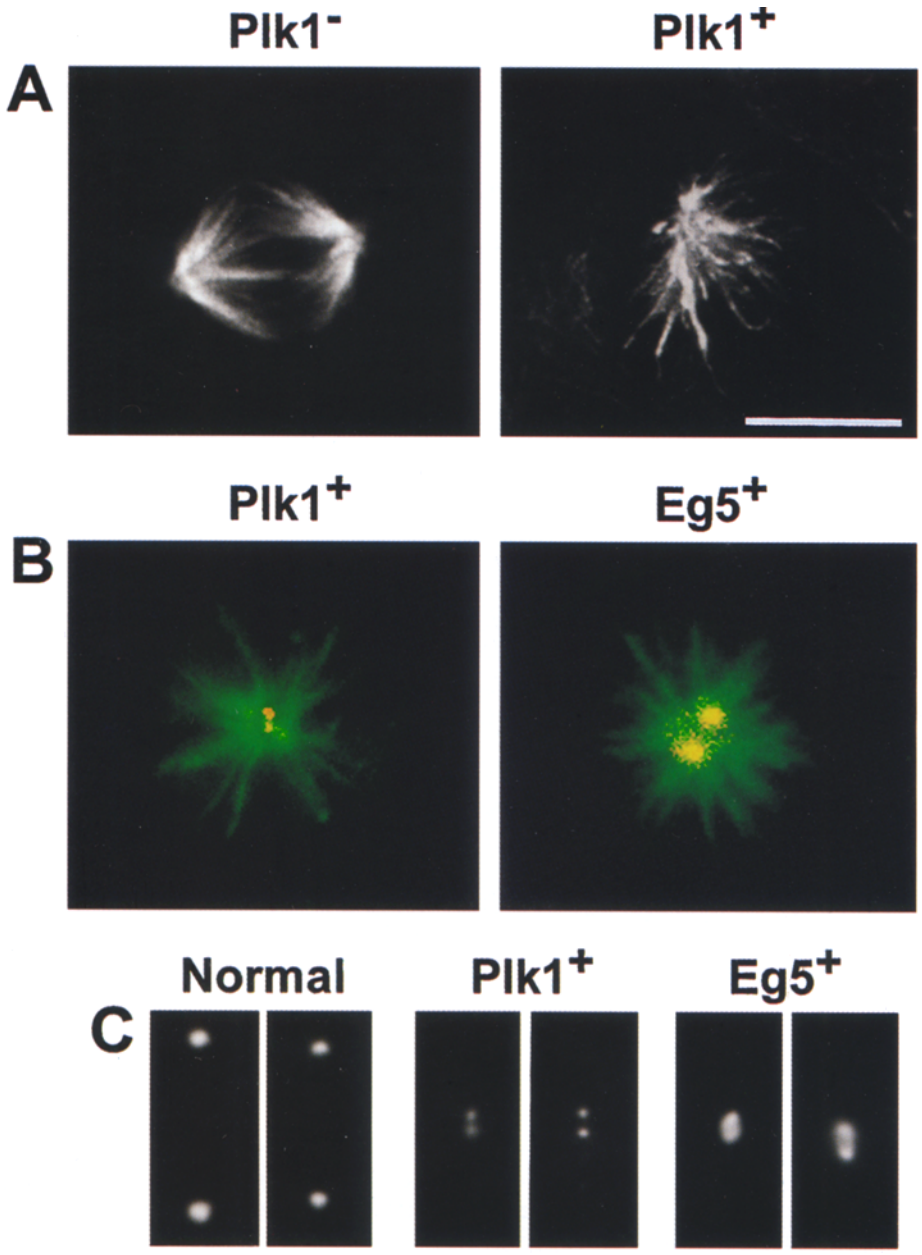

Figure 5. Plk1$^+$ injection into HeLa cells causes nucleation of abnormal microtubule arrays from tiny, unseparated centrosomes. Asynchronous interphase HeLa cells were injected with Plk1$^+$, Plk1$^-$, or Eg5$^+$ antibodies, fixed, and analyzed by immunofluorescence microscopy. (*A*) Confocal projection of the microtubules of mitotic cells injected with Plk1$^-$ or Plk1$^+$. (*B*) Overlays of confocal projections of microtubules (stained with anti–α-tubulin; *green*) and centrosomes (stained with CTR453; *yellow*) in mitotic cells injected with either Plk1$^+$ or Eg5$^+$. (*C*) Centrosomes, stained with CTR453, of uninjected mitotic cells (*Normal*), Plk1$^+$-injected mitotic cells (Plk1$^+$), and Eg5$^+$-injected mitotic cells (Eg5$^+$). Bar, 10 μm.

dle. Additional functions at later stages of mitosis are not excluded. We also found that Plk1$^+$-induced inhibition of cell cycle progression is more pronounced in diploid, nonimmortalized cells (Hs68) than in karyotypically abnormal carcinoma cells (HeLa). Thus, nonimmortalized cells may depend on Plk1 function and/or may monitor the proper maturation of centrosomes, before entering mitosis. This G2 checkpoint appears to be lost in at least some immortalized cell lines.

Plk1 is Specifically Required for Entry into Mitosis

We found that microinjection of anti-Plk1 antibodies resulted in a significant accumulation of abnormal mitotic cells and in a severe block to cell division. Many injected cells displayed ball-like, condensed chromatin and monoastral microtubule arrays reminiscent of interphase microtubules. These were nucleated from a single organizing center that contained duplicated but small, unseparated centrosomes. At later times after injection of Plk1$^+$ antibodies, we also observed an accumulation of multinucleated and micronucleated cells. These phenotypes may represent end products of aborted mitoses. Alternatively, they might reflect additional requirements for Plk1 function during later stages of cell division. Antibody injection had no effect on the kinetics of either entry into S phase or exit from S phase. Although negative results need to be interpreted with caution, these data lend no support to a previous study attributing to Plk1 an important S phase function (Hamanaka et al., 1994). Considering our present data, together with the timing of cell cycle–dependent expression and activation of Plk1 (Lake and Jelinek, 1993; Golsteyn et al., 1994, 1995; Lee et al., 1995; Hamanaka et al., 1995), it appears unlikely that Plk1 should perform a ma-

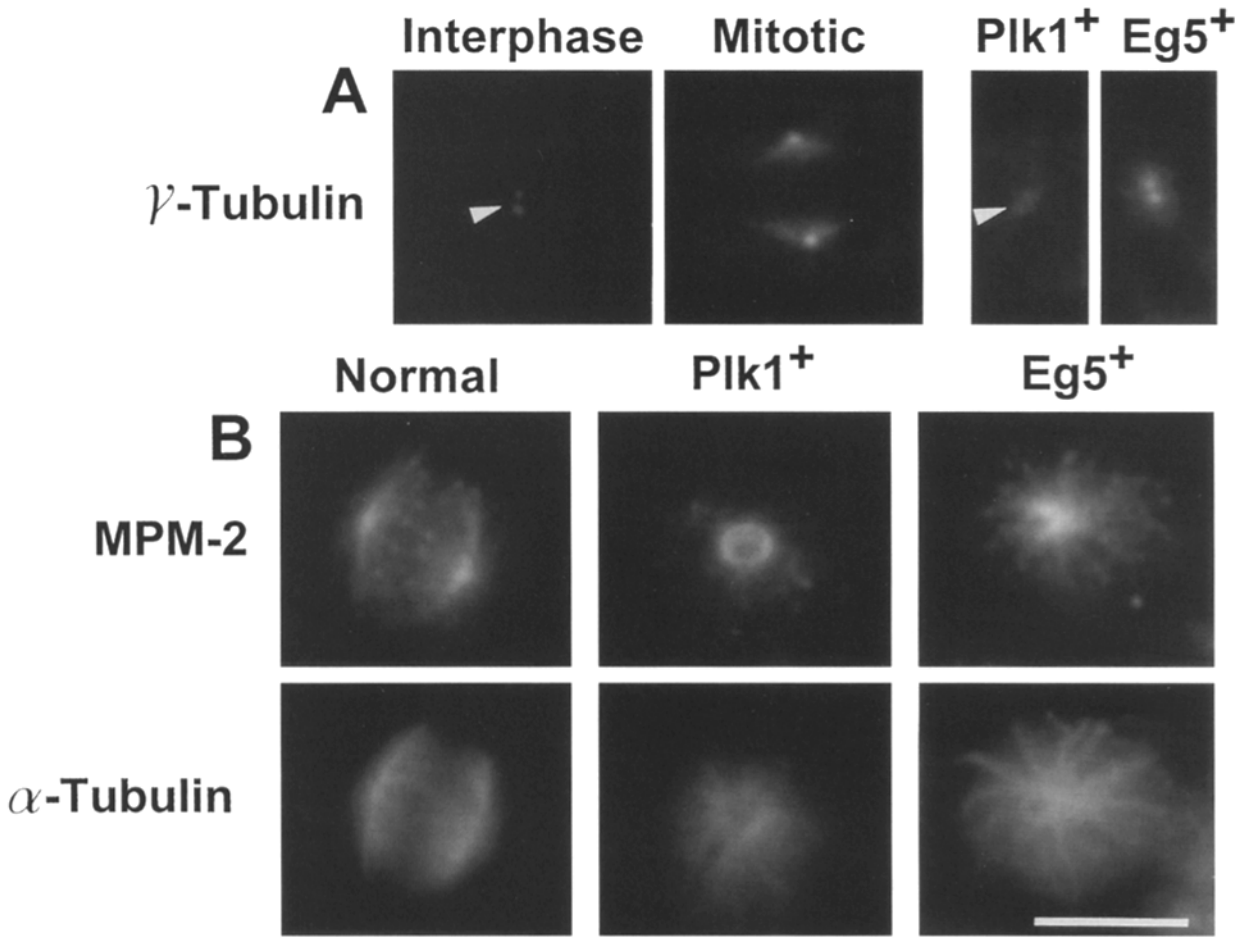

Figure 6. Injection of HeLa cells with Plk1$^+$ inhibits the accumulation of γ-tubulin and MPM-2 reactivity characteristic of mitotic centrosomes. Asynchronously growing HeLa cells were injected with Plk1$^+$ or Eg5$^+$ and analyzed by immunofluorescence microscopy after 20 h. (*A*) γ-tubulin staining of uninjected interphase and mitotic HeLa cells (*left*), and of Plk1$^+$- and Eg5$^+$-injected mitotic HeLa cells (*right*). (*B*) Staining of the MPM-2 phospho-epitopes (*MPM-2*) and microtubules (*α-tubulin*) of, from left to right, an uninjected mitotic cell (*Normal*), a Plk1$^+$-injected mitotic cell (*Plk1$^+$*) and an Eg5$^+$-injected mitotic cell (*Eg5$^+$*). Bar, 10 μm.

jor function during S phase. This does not exclude, however, that other polo-like kinases, such as Snk or Fnk, might play important roles during G1, the G1/S transition, or S phase (Simmons et al., 1993; Donohue et al., 1995).

Although it is difficult to directly compare the phenotypes produced by Plk1$^+$ antibody injection to those resulting from mutations of the putative Plk1 homologues in *Drosophila* and yeasts (for references see Introduction), our present data fall in line with some of the phenotypes observed for the *polo^1* mutant of *Drosophila* and for *plo1$^+$* gene disruptants in fission yeast. In contrast, *cdc5* mutants of budding yeast arrest in late nuclear division with separated chromosomes and thus appear to retain the ability to construct a bipolar spindle. Considering the differences in the temporal and spatial choreography of mitotic events in the above organisms, it is not entirely unexpected that the inhibition of polo homologues may produce distinct phenotypic consequences in different species. Also, we emphasize that our data do not necessarily exclude additional functions for mammalian Plk1 at later stages of mitotic progression. Both HeLa and Hs68 cells divided normally when Plk1$^+$ antibodies were injected during metaphase, but, considering the rapidity with which mitosis proceeds, it remains possible that antibodies injected during metaphase might not have reached their epitopes in time. Also, Plk1 function may be redundant at later stages of mitosis.

Plk1 Is Required for the Functional Maturation of Centrosomes and Bipolar Spindle Formation

The centrosomal and spindle abnormalities observed after Plk1$^+$ injection strongly suggest a specific role for human Plk1 in the establishment of a bipolar spindle. Normally, centrosomes increase in size shortly before the onset of mitosis, and concomitantly, they acquire the ability to nucleate highly dynamic spindle microtubules (Vorobjev and Nadehzdina, 1987; Karsenti, 1991; Kimble and Kuriyama, 1992). This increase in size is thought to reflect a functional maturation of centrosomes that can be visualized by monitoring the accumulation of multiple proteins (Kalt and Schliwa, 1993; Kellogg et al., 1994; Lange and Gull, 1995). In HeLa cells, these include γ-tubulin (Zheng et al., 1991; Lajoie-Mazenc et al., 1994) and MPM-2 reactive phosphoepitopes (Vandré et al., 1986). As shown by immunofluorescent staining with several antibodies, the centrosomes in Plk1$^+$-injected cells were much smaller than

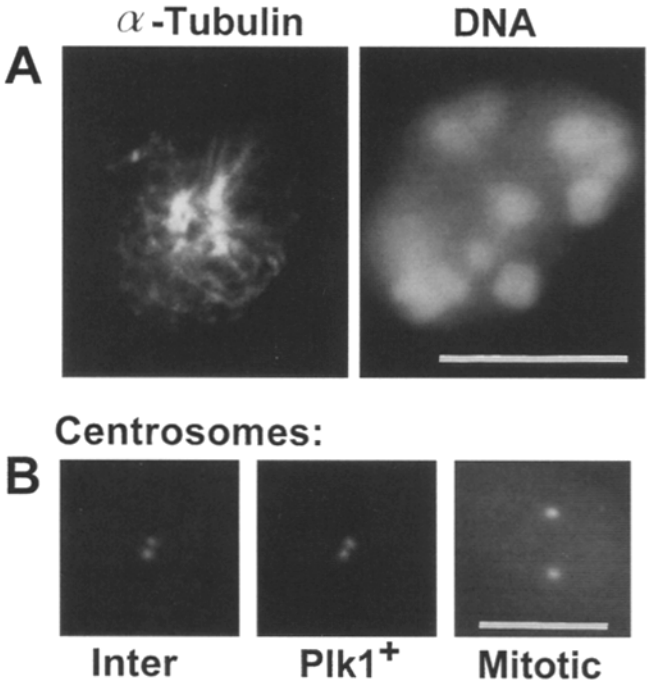

Figure 7. Plk1$^+$ injection of Hs68 cells causes mitotic microtubule abnormalities, and in the majority of cells, an interphase arrest with duplicated but small, unseparated centrosomes. Hs68 cells were injected with Plk1$^+$ and incubated for 36 or 48 h. Then, cells were fixed and analyzed by immunofluorescence microscopy. (*A*) Confocal projection of microtubules (α-*Tubulin*) and DNA of a Plk1$^+$-injected mitotic cell after 36 h incubation. (*B*) CTR453 staining of the centrosomes of an uninjected interphase (*Inter*) and mitotic (*Mitotic*) Hs68 cell compared to a Plk1$^+$-injected interphase cell (*Plk1$^+$*) after 48 h incubation. Bars, 10 μm.

normal mitotic spindle poles, and furthermore, contained reduced amounts of γ-tubulin and MPM-2 immunoreactivity, two markers for HeLa mitotic centrosomes. Attesting to the specificity of the observed effects, no changes in

Table II. Effect of Anti-Plk1 Antibodies on Cell Division in Human Diploid Fibroblasts (Hs68)

Antibody injected	Plk1$^+$		Plk1$^-$	
	%		%	
36 h				
Single interphase cells	72	(67/93)‡	46.5	(64/138)
Normally divided cells§	4	(4/93)	52	(72/138)
Mitotic cells*	16	(15/93)	1.5	(2/138)
Micro-/Multinucleated cells	8	(7/93)	0	(9/138)
Total	100		100	
48 h				
Single interphase cells	75	(67/89)	32	(37/116)
Normally divided cells§	5	(4/89)	67	(78/116)
Mitotic cells*	2	(2/89)	0	(0/116)
Micro-/Multinucleated cells	18	(16/89)	1	(1/116)
Total	100		100	

Asynchronously growing interphase Hs68 cells were microinjected with Plk1$^+$ or Plk1$^-$ IgG. At the times specified, cells were fixed and analyzed by immunofluorescence microscopy. The coverslip was scanned as in Table I, and scored for cells with the listed phenotypes. The total results from four independent experiments are shown.
*Mitotic cells injected with Plk1$^+$ display abnormal DNA condensation as described in the text (see also Fig. 7 *A*).
‡Total number of cells displaying phenotype/total number of injected cells.
§Cells that have gone through at least one normal cell division.

Table III. Effect of Anti-Plk1 Antibodies on Cell Division in HeLa and Hs68 Cells Injected at Metaphase

Antibody injected	Plk1$^+$		Plk1$^-$	
	%		%	
HeLa				
Normally divided cells	88	(117/133)*	87	(106/122)
Hs68				
Normally divided cells	91	(91/100)	97	(94/97)

Widely scattered (one cell per microscopic field) metaphase HeLa and Hs68 cells were microinjected with Plk1$^+$ and Plk1$^-$ IgG. 15 (HeLa) or 20 (Hs68) h after injection, cells were fixed and analyzed by immunoflourescence microscopy. Coverslips were scanned, and the percentage of injected cells that had divided calculated. The total results from two independent experiments are shown.
*Number of normally divided cells/number of injected cells.

either centrosome size or marker acquisition were seen in monoastral microtubule arrays produced by the injection of antibodies against HsEg5, a human kinesin-related motor protein (Blangy et al., 1995). These findings place Plk1 upstream of HsEg5, and they imply that Plk1 functions before prometaphase, when HsEg5 (and its homologues) act to promote the migration of centrosomes (for review see Sawin and Endow, 1993; see also Heck et al., 1993; Blangy et al., 1995). Furthermore, they indicate that one of the early functions of Plk1 concerns the recruitment of specific proteins to centrosomes. In line with this conclusion, it has previously been reported that the centrosomal antigen CP190 (formerly called Bx63) is not recruited to centrosomes in *polo^1* mutant *Drosophila* embryos (Sunkel and Glover, 1988).

During late G2/early prophase, the microtubule nucleation capacity of centrosomes increases drastically (Snyder and McIntosh, 1975; Kuriyama and Borisy, 1981, Karsenti, 1991). The cell cycle regulation of this change in centrosomal activity remains poorly understood, but recent studies strongly implicate γ-tubulin as a critical element in centrosomal microtubule nucleation (Joshi et al., 1992; Félix et al., 1994; Stearns and Kirschner, 1994; Shu and Joshi, 1995; Sunkel et al., 1995; Moritz et al. 1995*a*,*b*). Our finding that anti-Plk1 antibodies inhibit the recruitment of γ-tubulin to centrosomes raises the possibility that Plk1 may participate in promoting the increased centrosomal nucleation activity at the G2 to M transition. This notion is supported by the relatively low density of microtubules in the monoasters of Plk1$^+$-injected cells (Fig. 5), and the apparent absence of centrosomal MPM-2 phosphoepitope reactivity in these structures (Fig. 6 *B*). MPM-2 reactive phosphoproteins have been found at mitotically activated microtubule organizing centers of all eukaryotic cell types examined (e.g., Vandré et al., 1984, 1986, 1991), and dephosphorylation of MPM-2 reactive centrosomal components directly blocks microtubule nucleation in vitro (Centonze and Borisy, 1990).

Our data raise the possibility that Plk1 may itself be an MPM-2 kinase. Alternatively, one or several distinct MPM-2 kinase(s) may depend on Plk1 for their activity. In direct support of the former interpretation, a putative Plk1 homologue from *Xenopus* eggs was recently shown to be able to phosphorylate the Cdc25 phosphatase, thereby generating MPM-2 reactive sites and stimulating Cdc25 phosphatase activity (Kumagai and Dunphy, 1996). Considering that Cdc25C phosphatase is a key activator of

Cdc2, these findings have two important implications for the interpretation of our results: firstly, they bear directly on our finding that anti-Plk1 antibodies inhibit entry into mitosis in nonimmortalized Hs68 cells (see below). Secondly, they raise the possibility that Plk1 might in part regulate centrosomal structure and nucleation activity through the activation of Cdc25 phosphatase, and consequently, Cdc2 kinase. In support of this view, there is compelling evidence that Cdc2 kinase plays an important role in the activation of centrosomal microtubule nucleation (Buendia et al., 1992; Verde et al., 1992), and both Cdc2 and B-type cyclins accumulate at the centrosome during interphase (Riabowol et al., 1989; Bailly et al., 1989, 1992; Pines and Hunter, 1991; Gallant and Nigg, 1992).

The molecular identity of all MPM-2 antigens has not yet been established, but it is intriguing that, in addition to Cdc25, several other mitotic regulators are MPM-2 antigens. These include Wee1, Myt1, Cdc27, and NIMA (Kuang et al., 1994; King et al., 1995; Mueller et al., 1995*a*,*b*; Ye et al., 1995). It will clearly be interesting to search for additional Plk1 substrates amongst these proteins. In particular, it seems plausible that Plk1 might phosphorylate the Wee1 and/or Myt1 kinases, thereby down-regulating Cdc2-inhibitory pathways. Likewise, it is attractive to speculate that Plk's might phosphorylate the Cdc27 gene product and perhaps other components of the anaphase promoting complex (Irniger et al., 1995; King et al., 1995; Tugendreich et al., 1995), and thereby contribute to the regulation of anaphase onset.

A Checkpoint Monitoring Centrosome Maturation?

In an elegant study, microsurgical removal of centrosomes from cultured mammalian cells was shown to prevent all aspects of mitosis, including the activation of Cdc2 kinase (Maniotis and Schliwa, 1991). This observation strongly suggested a link between centrosomes and Cdc2 activation, but the nature of this link remained unknown (for discussion see Bailly and Bornens, 1992). A priori, centrosomes might exert control over Cdc2 via a positive signal (emitted by mature centrosomes), via a negative signal (emitted by immature centrosomes), or both. The data by Maniotis and Schliwa (1991) argue in favor of a positive signal, but do not exclude the existence of inhibitory signals. A role of Plk1 in stimulating the Cdc25 phosphatase has recently been established (Kumagai and Dunphy, 1996), and a possible role for this kinase in counteracting inhibitory pathways involving Wee1 and/or Myt1 kinase is worth investigating. Most intriguingly, we have shown here that nonimmortalized diploid fibroblasts (Hs68) respond to the inhibition of Plk1 function by a G2 arrest. In contrast, karyotypically abormal cells (HeLa) attempt to proceed through mitosis despite the loss of Plk1 function, indicating that they do not depend on Plk1 for the activation of Cdc2. Such a scenario appears plausible, since tumor cells frequently display deregulated expression of cell cycle regulators, including Cdc25 (Hunter and Pines, 1994; Galaktionov et al., 1995). Thus, we propose that normal cells may exhibit a checkpoint that causes a G2 arrest in response to the lack of Plk1 function and/or the resulting lack of functionally mature mitotic centrosomes. This centrosome maturation–related checkpoint would appear to be absent from HeLa cells. Loss of such a checkpoint might be common among tumor cells and conceivably contribute to the development of aneuploidies (Murray, 1992; Hartwell and Kastan, 1994).

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