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Tyrosine Phosphorylations Specific to Mitosis in Human and Hamster Cells

Robert Schlegel, M. Olivia Harris, and Glenn S. Belinsky

Department of Molecular and Cellular Toxicology Harvard School of Public Health, Boston, Massachusetts 02115

Abstract Changes in protein tyrosine phosphorylation are known to be important for regulating cell cycle progression. With the aim of identifying new proteins involved in the regulation of mitosis, we used an antibody against phosphotyrosine to analyze proteins from synchronized human and hamster cells. At least seven proteins were found that displayed mitosis-specific tyrosine phosphorylation in HeLa cells (pp165, 205, 240, 250, 270, 290, and ~400) and one such protein in hamster BHK cells (pp155). In synchronized HeLa and BHK cells, all proteins except HeLa pp165, pp205, and pp250 were readily detectable only in mitosis. Tyrosine phosphorylation of pp165, pp205, and pp250 was apparent during arrest in S phase, suggesting that cell cycle perturbations can affect the phosphorylation state of some of these proteins. In a related finding in BHK cells, pp155 underwent tyrosine phosphorylation when cells were forced into premature mitosis by caffeine treatment. Only one protein (pp135 in HeLa cells) was found to be dephosphorylated on tyrosine during mitosis. The above findings may prove helpful for isolating new cell cycle proteins that are important for both the normal regulation of mitosis and the mitotic aberrations associated with cell cycle perturbations and chemical treatments.

Key words: cell cycle, cell division, protein phosphorylation, phosphotyrosine, caffeine

It is estimated that less than 0.1% of protein phosphorylation occurs on tyrosine residues in normal cells [Hunter and Sefton, 1980], with the vast majority present on serine and threonine. These infrequent tyrosine phosphorylations play an important role in signal transduction and cell growth control, as evidenced by the numerous growth factor receptors and oncogenes that are tyrosine kinases [Hunter, 1989; Ullrich and Schlessinger, 1990; Cantley et al., 1991].

The onset of mitosis is partially controlled by tyrosine phosphorylation. Cell division is initiated in all eukaryotic cells by a protein complex called maturation promoting factor (MPF). MPF contains a serine/threonine kinase catalytic subunit, $p34^{cdc2}$ [Gautier et al., 1988; Dunphy et al., 1988], and a cyclin B regulatory subunit [Labbé et al., 1989; Gautier et al., 1990]. Genetic experiments using a temperature-sensitive mutant of $p34^{cdc2}$ [Th'ng et al., 1990] and microinjection experiments with an antibody against $p34^{cdc2}$ [Riabowol et al., 1989] indicate that this kinase is essential for entry of mammalian cells into mitosis. The phosphorylation state and activity of $p34^{cdc2}$ is cell cycle regulated [for reviews see Norbury and Nurse, 1992; Nishimoto et al., 1992]. Upon complex formation with cyclin B at the late S/G_2 phase of the cell cycle, $p34^{cdc2}$ is maintained in an inactive form by phosphorylation of $p34^{cdc2}$ at tyrosine-15. Genetic studies in *Schizosaccharomyces pombe* have shown that the kinases Wee1 and Mik1 are responsible for this inhibitory phosphorylation [Russell and Nurse, 1987; Featherstone and Russell, 1991; Lundgren et al., 1991; Parker et al., 1992]. Activation of $p34^{cdc2}$ at mitosis is accompanied by, and is thought to require, dephosphorylation at tyrosine-15. The tyrosine phosphatase cdc25 acts to remove this inhibitory phosphate and stimulates the serine/threonine kinase activity of MPF at the G_2/M transition [Gautier et al., 1991; Millar et al., 1991; Strausfeld et al., 1991; Lee et al., 1992].

The activity of other tyrosine kinases is elevated during mitosis and meiosis. For example, $pp60^{c\text{-}src}$ is activated during mitosis in mouse NIH 3T3 cells [Chackalaparampil and Shalloway, 1988] and tyrosine phosphorylation acti-

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Address reprint requests to Robert Schlegel, Harvard School of Public Health, Department of Molecular and Cellular Toxicology, 665 Huntington Avenue, Boston, MA 02115.

vates extracellular signal-regulated (ERK)/microtubule-associated protein (MAP) kinases during meiosis in *Xenopus* and clam oocytes [Posada et al., 1991; Ferrell et al., 1991; Shibuya et al., 1992] and during the G_0/G_1 and G_2/M transitions in mammalian cells [Tamemoto et al., 1992].

Recent evidence suggests that $p34^{cdc2}$ may not be the only kinase required for initiating mitosis in eukaryotic cells. In the filamentous fungus *Aspergillus nidulans,* the protein kinase NimA is activated at mitosis and, along with $p34^{cdc2}$, is essential for mitotic onset [Osmani et al., 1991a,b]. Neither NimA nor $p34^{cdc2}$ activation is sufficient by itself. In the budding yeast *Saccharomyces cerevisiae,* Sorger and Murray [1992] and Amon et al. [1992] found that cells arrested in S and G_2 contain activated MPF but do not enter mitosis, indicating an additional, but currently unidentified, regulatory pathway.

With the goal of identifying candidates for new mitotic regulatory components, we used an anti-phosphotyrosine antibody to analyze proteins from synchronized human and hamster cells. A number of proteins were identified that underwent mitosis-specific changes in tyrosine phosphorylation. In most cases, phosphorylation was tightly coupled with the mitotic phase of the cell cycle. Several of these proteins, however, underwent tyrosine phosphorylation when cells were arrested during DNA replication or when cells were treated with caffeine to initiate premature mitosis, indicating a sensitivity to cell cycle perturbations.

MATERIALS AND METHODS

Cell Culture and Synchronization

Syrian hamster fibroblast (BHK-21) cells and human HeLa-S3 cells were grown in Dulbecco's modified Eagle's medium (DME) containing 10% iron-supplemented calf serum (HyClone) and glutamine (4 mM) in a water-saturated 10% CO_2-90% air atmosphere. New cultures were started from frozen stocks every 4–6 weeks. Cells were *Mycoplasma*-free based upon fluorescent staining with Hoechst 33258.

Mitotic populations of HeLa and BHK cells were accumulated from asynchronously growing cultures treated for 8–12 h with 0.05 and 0.25 μg/ml nocodazole, respectively, and harvested by gentle physical detachment [Tobey et al., 1967]. Mitotic populations were at least 95% pure, as determined by chromosome analysis (see next section). Cells remaining attached to the culture flasks after mitotic selection were removed by treatment with 0.5 mM EDTA in phosphate-buffered saline (PBS) and used as the "adherent" interphase control group. Untreated interphase populations were prepared by physically detaching mitotic cells from asynchronously growing cultures and removing the attached cells with EDTA-PBS as before. Both interphase preparations were at least 97% pure. Synchronous populations of cells leaving mitosis were produced by first collecting mitotic populations as above, except that treatment with nocodazole was reduced to 6 h. Mitotic cells were then washed once in PBS, replated onto dishes, and extracts prepared at appropriate intervals from cells scraped from the dishes. The percentage of cells remaining in mitosis was monitored at each time point by chromosome analysis, as described below.

Synchronization of cells at various stages of S and G_2 was accomplished by arrest and subsequent release from G_1/S. For HeLa cells, reversible G_1/S arrest was produced by plating 3×10^6 cells in 175 cm^2 flasks and 24 h later adding 2 mM thymidine. After 14 h, thymidine was removed and replaced with normal media containing 24 μM deoxycytidine and thymidine [Heintz et al., 1983]. Nine hours later, 2 mM thymidine was added for an additional 12 h. Cells were released from G_1/S by removal of thymidine, and extracts were prepared at the indicated intervals, as described in the Immunoblotting section, next page. For a similar synchronization of BHK cells, cells were plated at 3×10^6 per 175 cm^2 flask and 24 h later the media was changed to isoleucine-deficient DME containing 5% dialyzed calf serum for 32 h [Hamlin and Pardee, 1976]. Cells were returned to normal media and 7 h later were treated with 0.5 μg/ml aphidicolin for 9 h. Aphidicolin was then removed and cell extracts prepared at the indicated times.

Premature mitosis was induced in BHK cells by first arresting cells in S phase with 2.5 mM hydroxyurea for 3 h and then treating cells with 5 mM caffeine for 8 h. Premature mitotic cells were accumulated in the presence of nocodazole (0.25 μg/ml) and selectively recovered by physical detachment, yielding populations that were approximately 95% pure. Control groups were composed of 1) adherent cells remaining after removal of caffeine-induced premature mitotic cells, and 2) untreated S-phase arrested cells.

Control populations contained less than 4% mitotic or premature mitotic cells.

Chromosome and Fluorescence-Activated Cell Sorting (FACS) Analyses

Mitotic indices were determined in chromosome spreads. HeLa and BHK cells were swelled in 75 mM KCl for 10 and 7 min, respectively. After centrifugation, cells were fixed in glacial acetic acid:methanol (1:3 v/v) for at least 10 min and dropped on slides. DNA was stained with Hoechst 33258 (1 μg/ml for 7 min). At each time point, at least 300 cells were examined at ×400 magnification by fluorescence microscopy to determine the percentage of cells in mitosis.

Cell cycle distribution was examined with an Ortho model 2150 FACS, using cell cycle analysis software by Cytomation, Inc. FACS analysis was conducted on trypsinized cell samples that were incubated for 15 min in PBS containing 0.6 mM EDTA and 0.1% Triton X-100 and stained for 1 h with 50 μg/ml propidium iodide; 5,000 cells were analyzed from each sample.

Immunoblotting

Proteins were extracted from whole cells in Laemmli sample buffer (2% SDS/5% 2-mercaptoethanol/10% glycerol/62.5 mM Tris, pH 6.8), heated at 100°C for 5 min, and separated in SDS polyacrylamide gels [Laemmli, 1970]. Protein concentrations were estimated by the modified Lowry assay described by Peterson [1977], using gamma-globulin as the standard. Proteins were transferred to immobilon-P membranes (Millipore) in a tank blotting system (5 h at 60 V in transfer buffer containing 25 mM Tris/192 mM glycine/20% methanol). Blots were rinsed three times in filtered Tris-buffered saline (pH 7.5) containing 0.3% Tween-20 (TBST) and subsequently incubated overnight at 4°C with a polyclonal antibody raised against N-succinyl-phosphotyrosine (1:1,000 dilution in TBST) provided by Dr. L. Levine [Levine et al., 1989]. Blots were washed for 30 min with three changes of TBST, incubated for 1.25 h in 1 μCi/ml ^{125}I-labeled protein A, and washed for 40 min with four changes of TBST. Phosphotyrosine-containing proteins were detected by indirect autoradiography using an intensifying screen and quantitated on autoradiograms with a Millipore BioImage scanner. Antibody competitions were conducted by incubating (1 h at 4°C) the diluted antibody with 250 μM phosphotyrosine or phosphoserine prior to blotting.

RESULTS

Changes in Tyrosine Phosphorylation During Mitosis in HeLa Cells

Protein extracts from populations of HeLa cells that were either in interphase or mitosis were immunoblotted with an anti-phosphotyrosine antibody that is approximately 10^5-fold more immunoreactive to phosphotyrosine than to phosphoserine, phosphothreonine, or unphosphorylated tyrosine [Levine et al., 1989]. Mitotic populations (at least 95% pure as determined by chromosome analysis) were collected by treating asynchronously growing cells with nocodazole and selectively shaking off mitotic cells. Two interphase populations were collected, each of which contained less than 3% mitotic cells. One was composed of adherent cells that remained after removal of mitotic cells. The other was derived from untreated asynchronously growing cultures that were partially depleted of mitotic cells by shaking.

The profile of immunoreactive proteins showed that mitotic HeLa cells contained at least seven phosphotyrosine-containing proteins that were absent or present in low abundance in interphase cells (Fig. 1A). Immunoreactivity of all of these proteins was eliminated by preincubation of the antibody with 250 μM phosphotyrosine (Fig. 1B) and was unaffected by antibody preincubation with 250 μM phosphoserine (Fig. 1C), confirming the specificity of the antibody to tyrosine phosphorylations. In addition, treatment of cell extracts with either potato acid or alkaline phosphatases abolished immunoreactivity while antibody preincubation with 250 μM phosphothreonine had no effect (Schlegel and Belinsky, unpublished results). No significant differences in tyrosine phosphorylation were noted between the two interphase populations, indicating that nocodazole treatment alone had no detectable effect. The mitosis-specific proteins were all of high relative molecular weight, ranging from approximately 165 to ~400 kDa (pp165, 205, 240, 250, 270, 290, and 400). In addition, a somewhat smaller protein of 135 kDa was found to contain phosphotyrosine only in interphase. No changes in tyrosine phosphorylation were seen in lower molecular weight proteins (~18 kDa and higher) that were re-

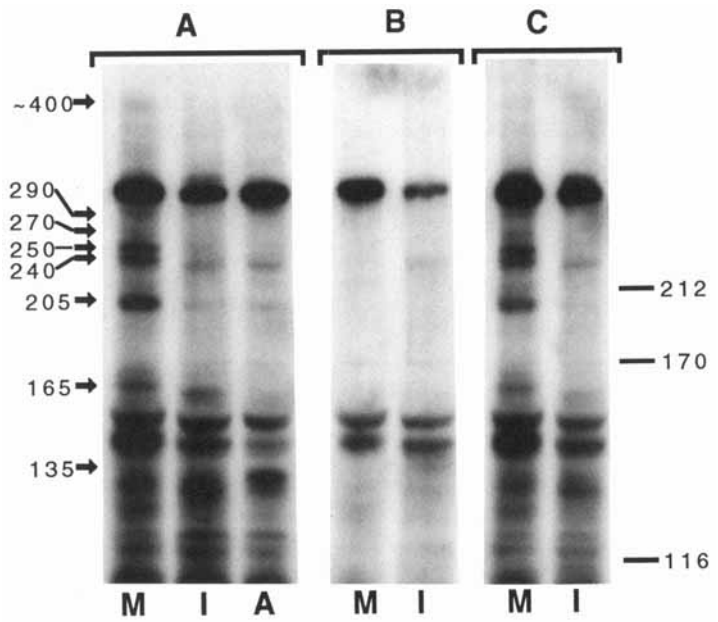

Fig. 1. Changes in tyrosine phosphorylation during mitosis in HeLa cells. **A:** Whole cell extracts from nocodazole-arrested mitotic cells (M), untreated interphase cells (I), and "adherent" interphase cells following removal of nococazole-arrested mitotics (A) were prepared, separated in 5% polyacrylamide gels and immunoblotted with phosphotyrosine antiserum, as described in Materials and Methods; 150 μg of protein was added to each lane. Mitotic (M) and interphase (I) extracts identical to those described above were immunoblotted after preincubating the phosphotyrosine antiserum with either 250 μM phosphotyrosine (**B**) or 250 μM phosphoserine (**C**). Mitosis-specific tyrosine phosphorylations are indicated with arrows and molecular weight markers with lines.

solved in gels containing higher percentages of polyacrylamide (data not shown).

Reversal of Tyrosine Phosphorylation Following Release From Nocodazole

To determine how rapidly the mitosis-specific tyrosine phosphorylations reverted to an interphase profile, protein extracts and chromosome preparations were made at 30 min intervals following release from nocodazole arrest. During the 2 h after release, pp290, pp250, pp240, and pp205 showed a gradual 4- to 10-fold reduction in immunoreactivity, but still displayed levels somewhat higher than those seen in asynchronous interphase cells (Fig. 2A). Proteins pp400, pp270, and pp165, however, lost essentially all immunoreactivity within 1.5 to 2 h, indicating a tight coupling of tyrosine phosphorylation to the mitotic phase of the cell cycle. A longer exposure of this autoradiogram shows more definitively the very close association between the percentage of cells remaining in mitosis and the extent of tyrosine phosphorylation on pp400 (Fig. 2B,C). As shown by chromosome analysis in Figure 2C, the frequency of mitotic cells dropped from an initial level of approximately 98% to approximately 10% within 1.5 h, with all of the decrease occurring in the one hour interval between 0.5 and 1.5 h after release. At 0.5, 1.0, and 1.5 h after nocodazole release, tyrosine phosphorylation of pp400 was approximately 100%, 39%, and 14% of that prior to release, closely paralleling the frequency of mitotic cells in the population. The only protein showing an increase in tyrosine phosphorylation as cells exited mitosis, pp135, was first detectable within one hour after release from nocodazole and remained at relatively constant levels at later time points.

Tyrosine Phosphorylation During Progression From S Phase to Mitosis

HeLa cells initially synchronized in early S phase by a double thymidine block were released and monitored for changes in tyrosine phosphorylation as cells progressed toward mitosis. Protein extracts were prepared and FACS analysis was conducted on cells at 2 h intervals following release from S phase arrest, representing cell populations in early, middle, and late S phase as well as G_2 phase. For comparison with earlier experiments, populations of mitotic and post-

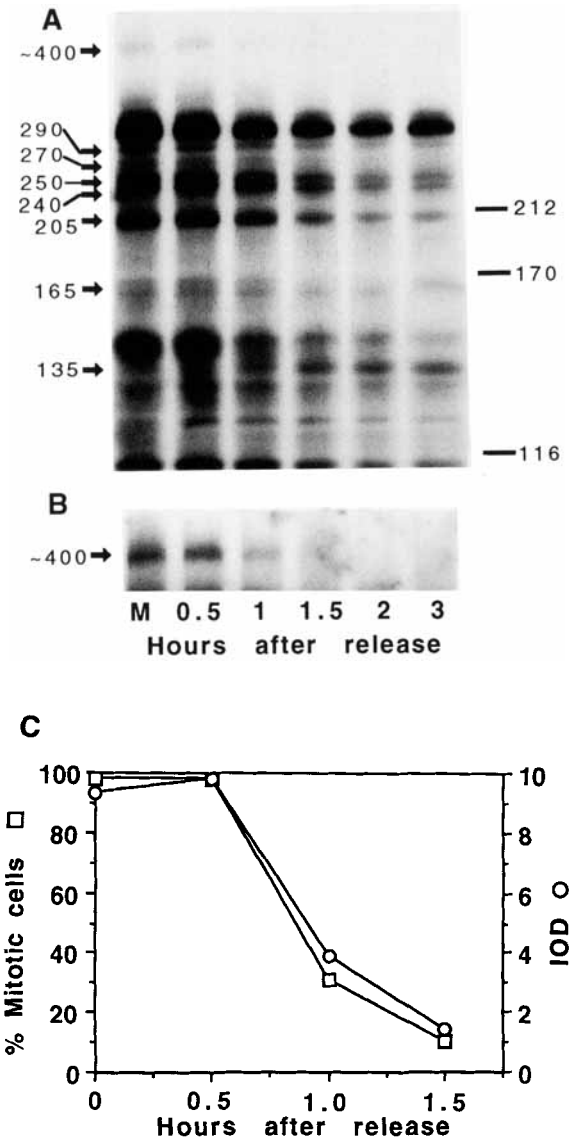

Fig. 2. Reversal of tyrosine phosphorylation following release from nocodazole. Mitotic HeLa cells were selectively recovered from nocodazole-treated cultures. Cells were replated in the absence of nocodazole and whole cell extracts and chromosome spreads were prepared from cells at the indicated times (hrs), as described in Materials and Methods. **A:** Proteins were resolved in 5% polyacrylamide gels and immunoblotted with phosphotyrosine antiserum as described in Materials and Methods. **B:** Longer exposure of the immunoblot shown in (A) to better illustrate changes in phosphotyrosine content of pp400. **C:** Mitotic index and integrated optical density (IOD) of pp400 following release from mitotic arrest.

mitotic cells were collected by exposing cells to nocodazole for 4 h (starting from the point where they entered G_2) and by harvesting cells 12 h after release from S phase arrest, respectively. Phosphotyrosine changes during cell cycle progression from S phase to the following postmitotic stage are illustrated in Figure 3A. FACS analysis for all groups is shown in Figure 3B.

Proteins pp400, pp290, pp270, and pp240 displayed low to undetectable levels of immunoreactivity throughout interphase, including G_2. Phosphotyrosine content rose dramatically between G_2 and mitosis, again linking these changes with the mitotic phase of the cell cycle. Tyrosine phosphorylation of pp250, pp205, and pp165 was already elevated during the S-phase arrest used for synchronization. This elevation persisted when cells were allowed to progress through interphase and into mitosis. Since pp250, pp205, and pp165 were absent or only weakly detectable in asynchronous interphase cultures (Fig. 1A), it appears that these three proteins undergo what is normally a mitosis-specific tyrosine phosphorylation when cells are arrested in S phase. This finding is consistent with other reports that either suggest, or show directly, that proteins and kinase activities associated with mitosis can accumulate during cell cycle perturbations such as arrest in S phase [Schlegel and Pardee, 1986; Yamashita et al., 1990; Steinmann et al., 1991; Schimke et al., 1991; Sorger and Murray, 1992; Amon et al., 1992]. The loss of phosphotyrosine from pp135 also occurs very close to the onset of mitosis. S and G_2 phase cells showed significant levels of this phosphoprotein while mitotic cells had little or no immunoreactivity. As illustrated in the last lane of Figure 3A, all proteins in postmitotic cells reverted to their normal interphase levels.

Changes in Tyrosine Phosphorylation During Mitosis in BHK Cells

To determine whether cell types other than HeLa cells showed mitosis-specific tyrosine phosphorylations, Syrian hamster (BHK) fibroblasts were also examined. In addition, certain chemicals can uncouple mitosis from the completion of DNA replication in hamster cells but not in human cells [Schlegel and Pardee, 1986; Schlegel and Craig, 1991; Steinmann et al., 1991]. This characteristic of hamster cells allowed us to determine whether chemically induced premature mitosis induced the same tyrosine phosphorylations as those seen during normal mitosis.

As shown in Figure 4A, a 155 kDa protein that was detected in nocodazole-arrested mitotic BHK

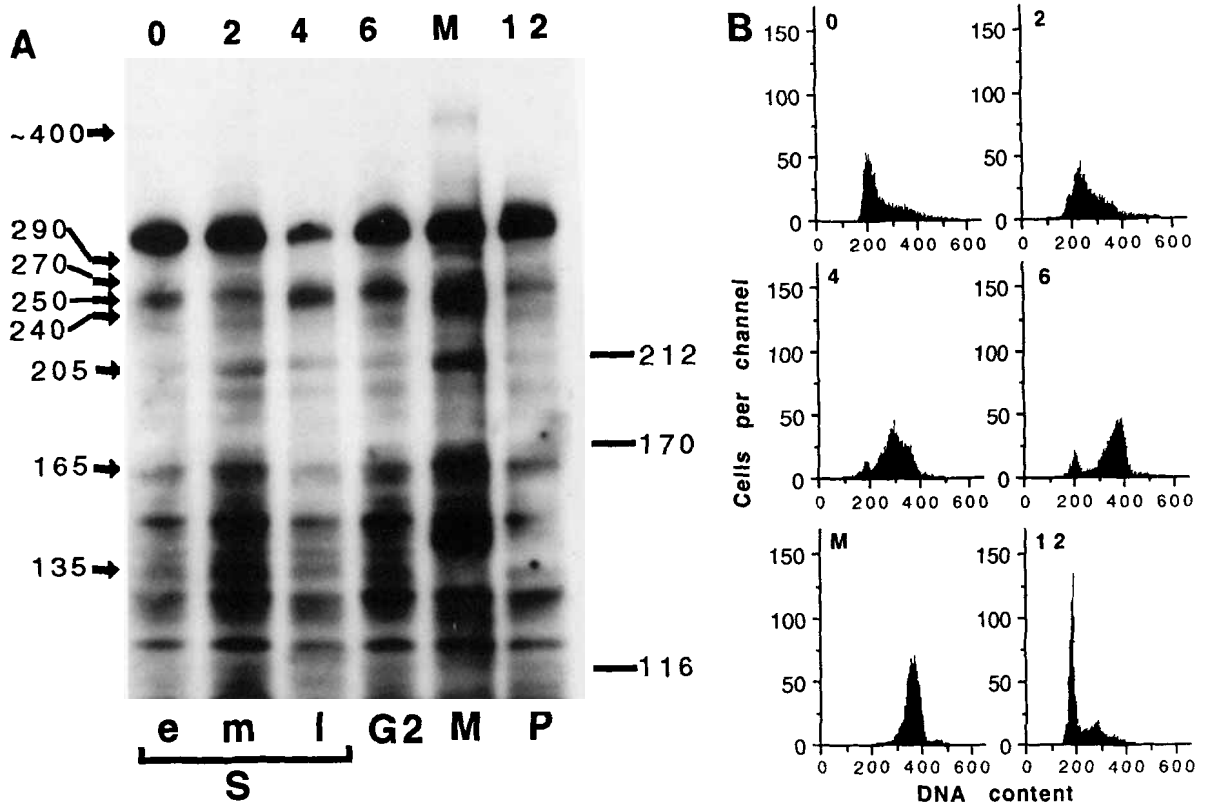

Fig. 3. Tyrosine phosphorylation during progression from S phase to mitosis. HeLa cells were synchronized and released from early S phase. FACS analysis was conducted and whole cell extracts were prepared and immunoblotted at the indicated times (h) after release (see Materials and Methods). **A:** Phosphotyrosine immunoblot of synchronized cells during early (e), middle (m) and late (l) S phase (S), G_2 phase (G_2), mitosis (M), and post-mitotic phase (P). Time (h) after release from thymidine is indicated above each lane. M identifies cells arrested in the subsequent mitosis with nocodazole; 150 μg of protein was added to each lane. **B:** FACS analysis of cells used for preparation of cell extracts in (A). Analyses are identified in the upper left corner by the same nomenclature as that used above each lane in (A).

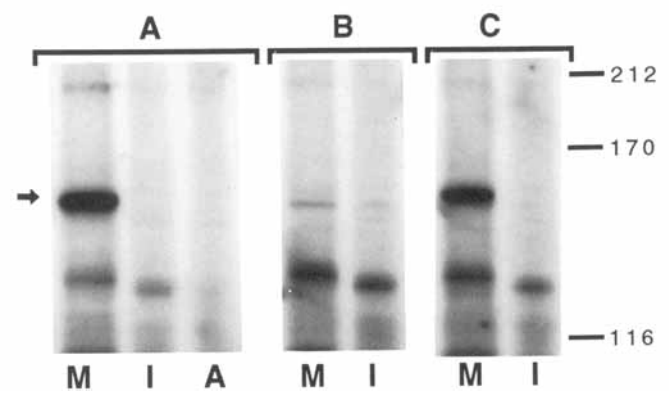

Fig. 4. Tyrosine phosphorylation of pp155 during mitosis in BHK cells. **A:** Whole cell extracts from nocodazole-arrested mitotic cells (M), untreated interphase cells (I), and "adherent" interphase cells following removal of nococazole-arrested mitotics (A) were prepared, separated in 5% polyacrylamide gels and immunoblotted with phosphotyrosine antiserum, as described in Materials and Methods; 150 μg of protein was added to each lane. Mitotic (M) and interphase (I) extracts identical to those described above were immunoblotted after preincubating the phosphotyrosine antiserum with either 250 μM phosphotyrosine (**B**) or 250 μM phosphoserine (**C**). Mitosis-specific tyrosine phosphoprotein pp155 is identified with an arrow. Molecular weight markers are indicated with lines.

cells was not seen in either interphase cells or in the adherent cells remaining after nocodazole-arrested cells were detached by shaking. Immunoreactivity was abolished by preincubating the antibody with 250 μM phosphotyrosine (Fig. 4B) and was unaffected by preincubation with 250 μM phosphoserine (Fig. 4C). Although regions of this and other immunoblots suggested the possibility of other mitosis-specific tyrosine phosphorylations, these other proteins were not consistently elevated in all experiments. As with HeLa cells, no differences were found in lower molecular weight proteins that were resolved in higher-percentage polyacrylamide gels (data not shown). It therefore appears that BHK cells also undergo mitosis-specific tyrosine phosphorylation, but to a lesser extent than do HeLa cells. The reason for this difference is not known.

pp155 Tyrosine Phosphorylation During Exit From and Entry Into Mitosis

BHK cells that were released from mitotic arrest by removal of nocodazole were examined for changes in tyrosine phosphorylation of pp155 (Fig. 5A). As shown in Figure 5B, BHK cells rapidly exited mitosis following released from nocodazole arrest, with approximately half of the cells entering G_1 within 20 min and about 90% having done so within 30 min. The loss of phosphotyrosine immunoreactivity of pp155 was tightly coupled with the return of cells to interphase, showing a great reduction within 20 min.

To determine whether phases of the cell cycle other than mitosis might express pp155, BHK cells were synchronized in early S phase and cell extracts were prepared at hourly intervals as cells were allowed to progress toward mitosis. pp155 was undetectable in all cell cycle stages other than mitosis, including cells in late G_2 (Fig. 6A). FACS analysis for this synchronization is shown in Figure 6B. The absence of pp155 in late G_2 cells, combined with the loss of tyrosine phosphorylation precisely at the time when cells exited mitosis, indicates that tyrosine phosphorylation of pp155 is very closely, and exclusively, linked to the mitotic stage of the cell cycle.

pp155 Is Phosphorylated on Tyrosine During Caffeine-Induced Premature Mitosis

Caffeine [Schlegel and Pardee, 1986], as well as certain protein kinase and phosphatase inhibitors [Schlegel et al., 1990; Yamashita et al., 1990; Steinmann et al., 1991; Tam and Schlegel, 1992], are able to override normal cell cycle checkpoints and induce premature mitosis in hamster cells that have not completed DNA replication. It was, therefore, of interest to determine whether pp155 also underwent tyrosine phosphorylation during caffeine-induced premature mitosis. BHK cells were arrested in S phase with hydroxyurea, exposed to caffeine, and premature mitotic cells collected by physical detachment. Populations of untreated S-phase arrested cells, as well as adherent cells remaining after removal of premature mitotic cells, were used as controls. Immunoblots of extracts from these cell populations indicated that pp155 underwent tyrosine phosphorylation during caf-

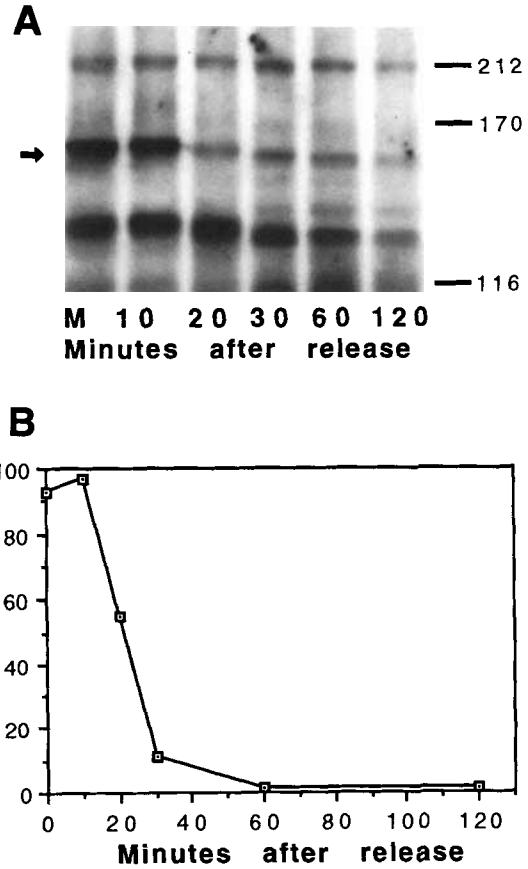

Fig. 5. Tyrosine phosphorylation of pp155 following release from nocodazole. Mitotic BHK cells were selectively recovered from nocodazole-treated cultures. Cells were replated in the absence of nocodazole and whole cell extracts and chromosome spreads were prepared from cells at the indicated times (min), as described in Materials and Methods. **A:** Proteins were resolved in 5% polyacrylamide gels and immunoblotted with phosphotyrosine antiserum as described in Materials and Methods. **B:** Mitotic index following release from nocodazole, as determined from chromosome spreads.

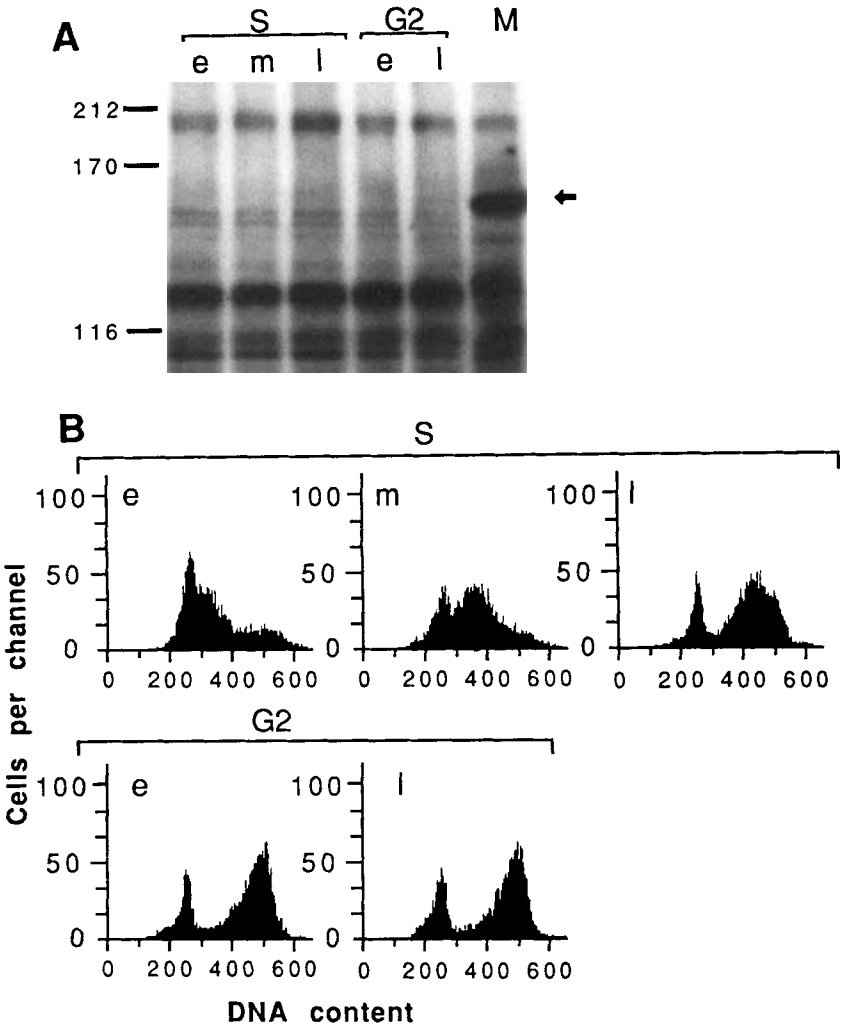

Fig. 6. Tyrosine phosphorylation of pp155 during progression from S phase to mitosis. BHK cells were synchronized and released from early S phase. FACS analysis was conducted and whole cell extracts were prepared and immunoblotted at the indicated times after release (see Materials and Methods). **A:** Phosphotyrosine immunoblot of synchronized cells during early (e), middle (m) and late (l) S phase (S), early (e) and late (l) G_2 phase (G_2), and mitosis (M). Mitotic cells were arrested in mitosis with nocodazole; 150 μg of protein was added to each lane. **B:** FACS analysis of cells used for preparation of cell extracts in (A). Analyses are identified in the upper left corner by the same nomenclature as that used above each lane in (A).

feine-induced premature mitosis (Fig. 7). pp155 was not phosphorylated on tyrosine in untreated cells that were arrested in S phase or in the fraction of cells that did not undergo premature mitosis in response to caffeine treatment. These results further support the mitosis-specific nature of pp155 tyrosine phosphorylation and provide an additional molecular marker showing the similarity between chemically induced premature mitosis and normal mitotic events.

DISCUSSION

We have found numerous changes in protein tyrosine phosphorylation as mammalian cells enter into and exit from mitosis. In all but one instance, entry into mitosis was accompanied by increasing tyrosine phosphorylation, while exit from mitosis resulted in tyrosine dephosphorylation. In addition, some of these changes were induced by cell cycle perturbations resulting from arrest in S phase and from exposure to caffeine. The tyrosine phosphorylation of HeLa proteins pp165, pp205, and pp250 during arrest in S phase provides additional molecular evidence that certain mitotic events may proceed normally even when cells are prevented from completing DNA replication. These findings are consistent with previous work showing that cyclin B can accumulate prematurely in hamster cells

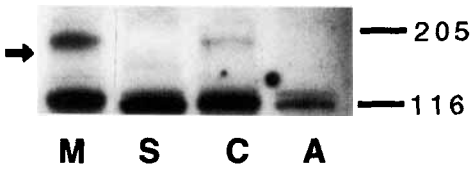

Fig. 7. Tyrosine phosphorylation of pp155 during caffeine-induced premature mitosis. BHK cells were arrested in S phase with 2.5 mM hydroxyurea and forced into premature mitosis by treatment with 5 mM caffeine, as described in Materials and Methods. A phosphotyrosine immunoblot was prepared from the following cell extracts: mitotic cells accumulated in 0.25 μg/ml nocodazole (M); cells arrested in S phase and exposed to nocodazole (S); cells arrested in S phase, exposed to nocodazole and forced into premature mitosis with 5 mM caffeine (C); and adherent cells remaining after caffeine-induced premature mitotic cells were removed by shaking (A); 100 μg of protein was added to each lane.

during arrest in S phase [Steinmann et al., 1991] and that S-phase arrest in budding yeast does not prevent activation of $p34^{cdc2}$ [Sorger and Murray, 1992; Amon et al., 1992].

At present, we cannot rule out the possibility that mitosis-specific changes in immunoreactivity are the result of changes in protein abundance rather than in tyrosine phosphorylation. Since the phosphotyrosine antibody detects changes in phosphotyrosine content, rapid changes in the synthesis and/or degradation of phosphotyrosine-containing proteins could account for changes in immunoreactivity equally well as reversible changes in tyrosine phosphorylation. Such changes in protein abundance would require particularly rapid fluctuations in synthesis and/or degradation rates, however, because most of the proteins identified in the present study were not readily detectable in any stage of the cell cycle other than mitosis, including cells that were in G_2. Rapid changes in protein stability are known to occur during mitosis, as evidenced by the mitotic cyclins A and B, but even these proteins are detectable in the S and G_2 phases of the cell cycle before undergoing rapid degradation at metaphase and anaphase, respectively [Pines and Hunter, 1989, 1990]. Although we suspect reversible tyrosine phosphorylation as the mechanism responsible for mitosis-specific immunoreactivity, a definitive answer to this question must await the development of protein-specific antibodies that can be used to examine protein abundance throughout the cell cycle.

Changes in protein tyrosine phosphorylation have previously been linked to the meiotic and mitotic stages of sea urchin and *Xenopus* oocytes and eggs. Shortly after fertilization of sea urchin eggs, a transient increase in the phosphotyrosine content of a 350 kDa protein was detected [Peaucellier et al., 1988]. In *Xenopus* oocytes, a 116 kDa protein was found to undergo a small increase in phosphotyrosine content shortly after progesterone stimulation, followed by a more dramatic increase at the time of germinal vesicle breakdown [Ferrell et al., 1991]. A 42 kDa protein was also found to be phosphorylated on tyrosine in metaphase extracts of *Xenopus* eggs [Lohka et al., 1987]. This tyrosine-phosphorylated protein has been identified as MAP kinase [Posada et al., 1991], and MAP kinases have recently been found to be activated not only during the G_0/G_1 transition, but during the G_2/M transition in Chinese hamster ovary cells [Tamemoto et al., 1992].

An earlier study using a phosphotyrosine-specific antibody to examine cell cycle phosphorylation changes in mouse 3T3 cells found numerous changes during the G_0/G_1 transition but no mitosis-specific events [Morla and Wang, 1986]. While differences in the cell types and antibodies used could play a role in the discrepancy between the above study and the present one, it is also possible that the choice of synchrony procedures was responsible. The present study focused on the mitotic phase of the cell cycle and, therefore, used S phase and mitotic synchronization techniques. The previous study released cells from quiescence, a technique that provides good synchrony at the G_0/G_1 transition but deteriorates at later cell cycle stages.

Tyrosine phosphorylation of $p34^{cdc2}$ is important for regulating mitotic onset. Dephosphorylation at tyrosine-15 activates $p34^{cdc2}$ and initiates the onset of mitosis [for reviews see Norbury and Nurse, 1992; Nishimoto et al., 1992]. Under certain circumstances, however, it appears that activation of $p34^{cdc2}$ is not sufficient to initiate mitosis. When cells of *Aspergillus nidulans* are arrested in G_2 by a temperature sensitive mutant of NimA, a protein kinase essential for mitotic onset in this organism, $p34^{cdc2}$ is present in its activated form [Osmani et al., 1991a]. In addition, when *Saccharomyces cerevisiae* cells are arrested during DNA replication, $p34^{cdc2}$ is activated but cells remain in interphase [Sorger and Murray, 1992; Amon et al., 1992]. These examples raise the possibility that higher eukaryotes also contain multiple pathways that regulate mitotic onset. The proteins identified in the present study could be

tyrosine kinases and/or substrates of tyrosine kinases that are activated during mitosis, such as *c-src* [Chakalaparampil and Shalloway, 1988]. The future purification and cloning of these proteins should help provide an understanding of their respective roles in mitosis.

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REFERENCES

Amon A, Surana U, Muroff I, Nasmyth K (1992): Regulation of $p34^{CDC28}$ tyrosine phosphorylation is not required for entry into mitosis in *S. cerevisiae*. Nature 355:368–371.

Cantley LC, Auger KR, Carpenter C, Duckworth B, Graziani A, Kapeller R, Soltoff S (1991): Oncogenes and signal transduction. Cell 64:281–302.

Chackalaparampil I, Shalloway D (1988): Altered phosphorylation and activation of $pp60^{c\text{-}src}$ during fibroblast mitosis. Cell 52:801–810.

Dunphy WG, Brizuela L, Beach D, Newport J (1988): The Xenopus cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. Cell 54:423–431.

Featherstone C, Russell P (1991): Fission yeast $p107^{wee1}$ mitotic inhibitor is a tyrosine/serine kinase. Nature 349: 808–811.

Ferrell Jr. JE, Wu M, Gerhart JC, Martin GS (1991): Cell cycle tyrosine phosphorylation of $p34^{cdc2}$ and a microtubule-associated protein kinase homolog in *Xenopus* oocytes and eggs. Molec Cell Biol 11:1965–1971.

Gautier J, Norbury C, Lohka M, Nurse P, Maller J (1988): Purified maturation-promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle control gene cdc2+. Cell 54:433–439.

Gautier J, Minshull J, Lohka M, Glotzer M, Hunt T, Maller JL (1990): Cyclin is a component of maturation-promoting factor from *Xenopus*. Cell 60:487–494.

Gautier J, Solomon MJ, Booher RN, Bazan JF, Kirschner MW (1991): cdc25 is a specific tyrosine phosphatase that directly activates $p34^{cdc2}$. Cell 67:197–211.

Hamlin JL, Pardee AB (1976): S phase synchrony in monolayer CHO cultures. Exp Cell Res 100:265–275.

Heintz N, Sive HL, Roeder RG (1983): Regulation of human histone gene expression: Kinetics of accumulation and changes in the rate of synthesis and in the half-lives of individual histone mRNAs during HeLa cell cycle. Molec Cell Biol 3:539–550.

Hunter T, Sefton BM (1980): Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc Natl Acad Sci USA 77:1311–1315.

Hunter T (1989): Oncogene products in the cytoplasm: the protein kinases. In Weinberg RA (ed): "Oncogenes and the Molecular Origins of Cancer." Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp 147–173.

Labbé J-C, Capony J-P, Caput D, Cavadore J-C, Derancourt J, Kaghad M, Lelias J-M, Picard A, Doree M (1989): MPF from starfish oocytes at first meiotic metaphase is a heterodimer containing one molecule of cdc2 and one molecule of cyclin B. EMBO J 8:3053–3058.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lee MS, Ogg S, Xu M, Parker LL, Donoghue DJ, Maller JL, Piwnica-Worms H (1992): cdc25+ encodes a protein phosphatase that dephosphorylates $p34^{cdc2}$. Molec Biol Cell 3:73–84.

Levine L, Gjika HB, Van Vunakis H (1989): Antibodies and radioimmunoassays for phosphoserine, phosphothreonine and phosphotyrosine. J Immunol Methods 124:239–249.

Lohka MJ, Kyes JL, Maller JL (1987): Metaphase protein phosphorylation in *Xenopus laevis* eggs. Molec Cell Biol 7:760–768.

Lundgren K, Walworth N, Booher R, Dembski M, Kirschner M, Beach D (1991): mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. Cell 64:1111–1122.

Millar JBA, McGowan CH, Lenaers G, Jones R, Russell P (1991): $p80^{cdc25}$ mitotic inducer is the tyrosine phosphatase that activates $p34^{cdc2}$ kinase in fission yeast. EMBO J 10:4301–4309.

Morla AO, Wang JYJ (1986): Protein tyrosine phosphorylation in the cell cycle of BALB/c 3T3 fibroblasts. Proc Natl Acad Sci USA 83:8191–8195.

Nishimoto T, Uzawa S, Schlegel R (1992): Mitotic checkpoints. Curr Opin Cell Biol 4:174–179.

Norbury C, Nurse P (1992): Animal cell cycles and their control. Annu Rev Biochem 61:441–470.

Osmani AH, McGuire SL, Osmani SA (1991a): Parallel activation of the NIMA and $p34^{cdc2}$ cell cycle-regulated protein kinases is required to initiate mitosis in *A. nidulans*. Cell 67:283–291.

Osmani AH, O'Donnell K, Pu RT, Osmani SA (1991b): Activation of the nimA protein kinase plays a unique role during mitosis that cannot be bypassed by absence of the bimE checkpoint. EMBO J 10:2669–2679.

Parker LL, Atherton-Fessler S, Piwnica-Worms H (1992): $p107^{wee1}$ is a dual-specificity kinase that phosphorylates $p34^{cdc2}$ on tyrosine 15. Proc Natl Acad Sci USA 89:2917–2921.

Peaucellier G, Veno PA, Kinsey WH (1988): Protein tyrosine phosphorylation in response to fertilization. J Biol Chem 263:13806–13811.

Peterson GL (1977): A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83:346–356.

Pines J, Hunter T (1989): Isolation of a human cyclin cDNA: Evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with $p34^{cdc2}$. Cell 58:833–846.

Pines J, Hunter T (1990): Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. Nature 346:760–763.

Posada J, Sanghera J, Pelech S, Aebersold R, Cooper JA (1991): Tyrosine phosphorylation and activation of homolo-

gous protein kinases during oocyte maturation and mitogenic activation of fibroblasts. Molec Cell Biol 11:2517–2528.

Riabowol K, Draetta G, Brizuela L, Vandre D, Beach D (1989): The cdc2 kinase is a nuclear protein that is essential for mitosis in mammalian cells. Cell 57:393–401.

Russell P, Nurse P (1987): Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog. Cell 49:559–567.

Schimke RT, Kung AL, Rush DF, Sherwood SW (1991): Differences in mitotic control among mammalian cells. Cold Spring Harb Symp Quant Biol 56:417–425.

Schlegel R, Pardee AB (1986): Caffeine-induced uncoupling of mitosis from the completion of DNA replication in mammalian cells. Science 232:1264–1266.

Schlegel R, Belinsky GS, Harris MO (1990): Premature mitosis induced in mammalian cells by the protein kinase inhibitors 2-aminopurine and 6-dimethylaminopurine. Cell Growth Diff 1:171–178.

Schlegel R, Craig RW (1991): Mitosis: Normal control mechanisms and consequences of aberrant regulation in mammalian cells. In Inouye M, Campisi J, Cunningham D, Riley M (eds): "Perspectives on Cellular Regulation: From Bacteria to Cancer." New York: Wiley-Liss, pp 235–249.

Shibuya EK, Boulton TG, Cobb MH, Ruderman JV (1992): Activation of p42 MAP kinase and the release of oocytes from cell cycle arrest. EMBO J 11:3963–3975.

Sorger PK, Murray AW (1992): S-phase feedback control in budding yeast independent of tyrosine phosphorylation of $p34^{cdc28}$. Nature 355:365–368.

Steinmann KE, Belinsky GS, Lee D, Schlegel R (1991): Chemically induced premature mitosis: Differential response in rodent and human cells and the relationship to cyclin B synthesis and $p34^{cdc2}$/cyclin B complex formation. Proc Natl Acad Sci USA 88:6843–6847.

Strausfeld U, Labbé JC, Fesquet D, Cavadore JC, Picard A, Sadhu K, Russell P, Doree M (1991): Dephosphorylation and activation of a $p34^{cdc2}$/cyclin B complex in vitro by human CDC25 protein. Nature 351:242–245.

Tam SW, Schlegel R (1992): Staurosporine overrides checkpoints for mitotic onset in BHK cells. Cell Growth Diff 3:811–817.

Tamemoto H, Kadowaki T, Tobe K, Ueki K, Izumi T, Chatani Y, Kohno M, Kasuga M, Yazaki Y, Akanuma Y (1992): Biphasic activation of two mitogen-activated protein kinases during the cell cycle in mammalian cells. J Biol Chem 267:20293–20297.

Th'ng JPH, Wright PS, Hamaguchi J, Lee MG, Norbury CJ, Nurse P, Bradbury EM (1990): The FT210 cell line is a mouse G2 phase mutant with a temperature-sensitive CDC2 gene product. Cell 63:313–324.

Tobey RA, Anderson EC, Petersen DF (1967): Properties of mitotic cells prepared by mechanically shaking monolayer cultures of Chinese hamster cells. J Cell Physiol 70:63–68.

Ullrich A, Schlessinger J (1990): Signal transduction by receptors with tyrosine kinase activity. Cell 61:203–212.

Yamashita K, Yasuda H, Pines J, Yasumoto K, Nishitani H, Ohtsubo M, Hunter T, Sugimura T, Nishimoto T (1990): Okadaic acid, a potent inhibitor of type 1 and type 2A protein phosphatases, activates cdc2/H1 kinase and transiently induces a premature mitosis-like state in BHK21 cells. EMBO J 9:4331–4338.