Anaphase onset and dephosphorylation of mitotic phosphoproteins occur concomitantly

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Summary

The cyclical phosphorylation and dephosphorylation of the centrosome during mitosis was analyzed by immunofluorescence methods using the MPM-2 antibody, which reacts with a subset of mitotic phosphoproteins. Quantification of MPM-reactivity indicated that centrosomal phosphorylation attained a maximal level just prior to anaphase onset. This level was maintained in metaphase cells blocked from further mitotic progression with the microtubule depolymerizing agent nocodazole. However, when nocodazole was added to cells that had just initiated anaphase, the level of centrosomal phosphorylation decreased rapidly as in untreated anaphase cells. We conclude that the onset of dephosphorylation of the centrosome coincided with the onset of anaphase and continued in the absence of chromosome movement. Dephosphorylation of MPM-2 reactive phosphoproteins may be taken as a biochemical indicator of anaphase onset.

Key words: mitosis, centrosome, phosphorylation, anaphase onset.

Introduction

Morphologically, several established landmark events are associated with entry into mitosis. These include chromosome condensation, nuclear envelope breakdown, and spindle formation. The transition from mitosis back to interphase is initiated upon anaphase onset, morphologically identified by the splitting of the sister chromatids. Segregation of the chromosomes is achieved by both movement of the chromosomes to the spindle poles (anaphase A) and separation of the poles (anaphase B). In addition to chromosome movement, reversal of the morphological landmark events associated with entry into mitosis also occurs as anaphase progresses towards completion and cytokinesis. Specifically, spindle breakdown, nuclear envelope re-formation, chromosome decondensation and re-establishment of the interphase microtubule array are all events associated with exit from mitosis. Clearly, coordinated control of these events must involve both specific temporal and spatial regulation.

Increasingly, evidence has accumulated indicating an important functional role for specific protein phosphorylation events in regulating cell cycle progression. A general increase in the levels of protein phosphorylation has been shown to occur prior to and during M-phase in a variety of meiotic and mitotic cell systems (Westwood *et al.* 1985; Domon *et al.* 1986; Lohka *et al.* 1987; Karsenti *et al.* 1987). In many of these different cell systems the increase in phosphorylation has been associated with the activity of maturation and/or mitotic promoting factor (MPF) (Maller *et al.* 1977; Capony *et al.* 1986). MPF appears to be a phosphoprotein, and its activity is regulated through changes in its phosphorylation state (Cyert and Kirschner, 1988). Additional evidence suggests that purified MPF is a kinase, and this kinase activity is thought to be involved in the generation of the signal promoting further progression towards the M-phase state (Lohka *et al.* 1988). Other protein kinases involved in regulating the entry of cells into mitosis have also been identified. The best characterized of these proteins are the cdc28 and cdc2 protein products identified in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, respectively (Reed *et al.* 1985; Simanis and Nurse, 1986). The *Xenopus* homolog of the cdc2 protein has recently been identified as a component of purified MPF (Dunphy *et al.* 1988; Gautier *et al.* 1988). Another protein kinase capable of inducing mitosis and maintaining chromosomes in a condensed state is the *nim*A gene product identified in *Aspergillus* (Osmani *et al.* 1988). The cyclins A and B, initially identified as proteins that accumulated during the cell cycle and were then abruptly destroyed during mitosis (Evans *et al.* 1983), are also possible regulatory molecules. It is possible that the cyclins regulate the activity of MPF by activation of the MPF moiety or, alternatively, are acted upon by MPF and, therefore, are involved in the propagation of the mitosis-induction signal (Murray, 1987).

In addition to the different protein kinase activities

associated with entry into mitosis, a number of proteins specifically phosphorylated at mitosis have been identified. These proteins include the nuclear lamin proteins (Gerace and Blobel, 1980), histones (Ajiro and Nishimoto, 1985), and intermediate filament proteins (Evans and Fink, 1982). The functional importance of these phosphorylation events has been carefully documented for the nuclear lamina proteins. Phosphorylation of these proteins is required for disassembly of the nuclear envelope, and subsequent dephosphorylation is necessary for nuclear envelope reconstruction during exit from mitosis (Burke and Gerace, 1986; Newport and Spann, 1987). In general, progression from interphase into mitosis is characterized by specific phosphorylation events, and progression from mitosis back to interphase is characterized by a series of dephosphorylation events.

A subset of phosphoproteins associated with mitotic cells has been identified by monoclonal antibodies prepared against mitotic cell lysates, designated MPM-1 and MPM-2 (Davis *et al.* 1983). MPM antibody identification of these proteins was abolished if nitrocellulose transfers of mitotic proteins were first treated with alkaline phosphatase. Thus, the MPM antibodies recognized a specific phosphorylated epitope(s) present on the reactive mitotic proteins. These antibodies have been shown to be localized at mitotic microtubule-organizing centers in a variety of different cell types (Vandré *et al.* 1984, 1986). Although the identification of all the MPM-reactive phosphoproteins and the functional consequences of this phosphorylation have not been determined, it is known that the appearance and disappearance of these phosphoproteins correlates with entry and exit from mitosis. Brain MAP-1 is one phosphoprotein recognized by the MPM antibodies (Vandré *et al.* 1986), and it is known that changes in the phosphorylation state of other MAPs affects microtubule stability (Jameson and Caplow, 1981). Our analysis of isolated spindles has shown that a cellular protein related to brain MAP-1, as well as the 210K ($K=10^3 M_r$) MAP identified by Bulinski and Borisy (1980), is also specifically reactive with the MPM antibody during mitosis (Vandré and Borisy, unpublished observations).

Microtubules are the main structural components of the spindle, and changes in the distribution, nucleation and dynamics of microtubules are characteristic markers of the different stages of spindle assembly and disassembly. Regulation of the mitotic microtubule-organizing centers could provide the temporal regulation needed for spindle formation. For example, the microtubule nucleation capacity of the centrosome increases at least fivefold during prophase and prometaphase in comparison to interphase centrosomes, as determined by the addition of exogenous tubulin dimer to lysed cell models (Kuriyama and Borisy, 1981). The enhanced nucleating activity correlates with increased amounts of pericentriolar material surrounding the mitotic centrosome (Rieder and Borisy, 1982), and with the appearance of phosphorylated centrosomal protein components reactive with the MPM antibodies (Vandré *et al.* 1984, 1986). By telophase, centrosomal MPM-reactivity has returned to interphase levels, suggesting that dephosphorylation of microtubule-organizing center components occurs during anaphase. The phosphorylation and dephosphorylation of the centrosome correlates with the increase and decrease in microtubule nucleating capacity (Vandré and Borisy, 1985), and suggests that the post-translational modification of centrosomal components may play a functional role in the assembly and subsequent disassembly of the spindle through mitosis. In this study, we determined the precise timing and physiological dependence of dephosphorylation upon the trigger for anaphase onset.

Materials and methods

Cell culture and video microscopy

LLC-PK cells, derived from porcine kidney, were maintained in monolayer culture. Cells were grown in DME medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), penicillin (100 units ml^{-1}), streptomycin (0.1 mg ml^{-1}), and 20 mM-Hepes buffer. Cells were subcultured onto round glass coverslips at least 18 h prior to microscopic examination. Coverslips containing monolayer cultures were placed in a Sykes-Moore chamber (Bellco Glass, Vineland, NJ), and held in place using the 2.5 mm working-distance silicone gasket. The chamber was left as an open system, since a second coverslip was not placed on top of the gasket. Fresh DME medium (1 ml) was added to the chamber and covered with a minimal amount of mineral oil (E. R. Squibb and Sons, Princeton, NJ). The temperature of the medium within the chamber was monitored using a BAT-8 digital thermometer and an IT-23 temperature probe (Sensortek, Clifton, NJ) inserted into the chamber through a 23 gauge needle that penetrated the sealing gasket. The temperature was maintained at 37°C by constant illumination with an infrared incubator lamp (Opti-Quip Inc., Highland Mills, NY). The Sykes-Moore chamber had been modified to allow the temperature probe from the infrared lamp to be screwed into the side of the chamber in the position of one of the needle access ports. The cells were monitored under phase-contrast at low levels of illumination with a Nikon Diaphot inverted microscope and a ×40 planachromat objective. Video recordings were made on a Sony model SL-HF900 Beta VCR from the images obtained with a Venus model DV2 camera (Venus Scientific Inc., Farmingdale, NY). Photographs of selected recordings of live cells were taken from the screen of a high-resolution black and white monitor (Sierra Scientific Corp., Mountain View, CA) following image processing of the recorded Beta tapes using the Quantex 9200 image analysis system (Quantex Corp., Sunnyvale, CA).

Nocodazole treatment of metaphase and anaphase cells

A prophase cell was located on each coverslip and its position marked on the underside of the coverslip using a diamond scribe objective. The cell was monitored until nuclear envelope breakdown (NEB), which was clearly visible in the LLC-PK cells viewed by phase-contrast. Cells were allowed to progress through mitosis until late metaphase (15–17 min after NEB) or until anaphase onset, at which time the medium was aspirated off and fresh DME, prewarmed to 37°C, containing 1 μg ml^{-1} nocodazole was added. The nocodazole-containing medium was covered with a minimal layer of mineral oil, and the cultures were exposed to the nocodazole for various periods of time. After the appropriate incubation period the nocodazole-containing medium was aspirated off and the cells were either rinsed

with three changes of fresh DME without nocodazole and further incubated, or lysed in PHEM buffer (60 mM-Pipes, 25 mM-Hepes, 10 mM-EGTA, 2 mM-$MgCl_2$) pH 6.9, containing 0.5% Triton X-100 for 90 s. Following aspiration of the lysis buffer, the cells were fixed in PHEM buffer containing 0.7% glutaraldehyde. Following a 15-min fixation, the coverslips were rinsed with phosphate-buffered saline (PBS), pH 7.4, and removed from the Sykes-Moore chamber for immunofluorescence staining.

Immunofluorescence staining

Coverslips containing fixed cells were rinsed thoroughly with phosphate-buffered saline (PBS), and then treated with two changes of $NaBH_4$ (1 mg ml^{-1} in Tris-buffered saline, pH 7.4) over 30 min. Following the reduction, the $NaBH_4$ was rinsed with PBS and the coverslips were treated with 2% normal goat serum (Gibco) for 30 min at 37°C to block nonspecific binding sites, and were processed for double-label immunofluorescence using the MPM-2 mouse monoclonal antibody (Davis *et al.* 1983) and rabbit anti-tyrosyl-tubulin antibody (Gunderson *et al.* 1984). Rhodamine-conjugated goat anti-mouse immunoglobulin was obtained from Organon Technika-Cappel (Malvern, PA) and fluorescein-conjugated goat anti-rabbit immunoglobulin was obtained from ICN Immunobiologicals (Lisle, IL). To ensure complete saturation of all available antigenic sites by the MPM-2 antibody, the antibody was applied at a 1:50 dilution for 30 min at 37°C, a 10-fold higher concentration than normally used for routine immunolabelling. Coverslips were rinsed with PBS following each antibody incubation, given a final rinse in distilled water, and mounted in 10% polvinyl alcohol containing 2 mg ml^{-1} of the anti-bleaching agent *para* phenylene diamine as described (Sammak *et al.* 1987).

LLC-PK cells to be treated with alkaline phosphatase were subcultured onto glass coverslips 48 h prior to being processed. Cells were detergent-lysed as described above. Lysed cells were rinsed twice with PHEM buffer, followed by a third rinse in PHEM buffer containing 1 μg ml^{-1} taxol (Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD) to stabilize microtubules. Intestinal alkaline phosphatase was added to the final rinse solution at a concentration of 100 units ml^{-1}. Following a 60 min incubation at room temperature, the cells were rinsed and fixed in PHEM buffer containing 0.7% glutaraldehyde. Coverslips were processed for immunofluorescence staining as described above, with the exception that the MPM-2 antibody was used at a 1:500 dilution.

Microscopy and quantification of immunofluorescence staining

Mounted coverslips were examined with a Zeiss Universal microscope equipped with epifluorescence optics using a ×63 phase, 1.4 NA, planapochromat objective. Immunofluorescence micrographs were recorded using Kodak Tech-Pan 2415 film. The film was developed in Kodak HC-110 Developer (Dilution D) for 8 min at 68°C, under which conditions the film gave a nearly linear response over a dynamic range of approximately 3 O.D. units. All immunofluorescence photomicrographs were printed from their respective negatives, and each was obtained from a 15-s exposure, giving exposures well within the dynamic range of the film. The prints were made using Kodak Ektamatic SC polycontrast paper and developed in a Kodak Ektamatic Processor model 214-K. The linear response of the SC polycontrast was approximately 1.3 O.D. units, or less than half the dynamic range of the Tech-Pan 2415 film. The exposure times and polycontrast filter combination established to print the immunofluorescence micrograph of metaphase centrosomes stained with the MPM-2 antibody were used for the printing of all subsequent micrographs of MPM-2 staining in each figure presented.

Quantification of the level of MPM-2 staining associated with the centrosome was obtained indirectly by measuring the density of the silver grains present on Tech-Pan negatives of equivalent exposure that corresponded to the region of centrosomal immunofluorescence staining. Each negative was transferred to the stage of a Zeiss IM-35 microscope, and examined under uniform illumination through a 0.6 O.D. neutral density filter using a ×2.5 plan objective. The negative was imaged with a Dage SIT camera (Dage-MIT, Inc., Michigan City, IN) and processed with a Quantex 9200 image processor (Quantex Corp., Sunnyvale, CA). Under the illumination conditions used, the linear dynamic range of the SIT camera was approximately 1.3 O.D. units. The MPM-2 staining level associated with each centrosomal region on the negative was measured by defining the largest inscribed rectangular area that could be identified within the centrosomal image as displayed on a monitor following image processing. A radiance value was assigned by the Quantex to each pixel within the defined area, and the total radiance was calculated by summing all such values contained within the inscribed region. Thus the MPM-reactivity of the centrosome was assayed as a radiance value that was dependent upon both the area and the density of silver grains on the negative in the centrosomal region. In each case a background area the same size as the inscribed region and adjacent to the centrosomal region was subtracted from the centrosomal intensity. Although indirect, the method gave a relative quantitative value for the level of MPM-reactivity at the centrosome, and permitted the direct comparison of different samples stained with the MPM-2 antibody that were prepared and analyzed in an identical fashion.

Results

Phosphatase sensitivity of MPM-2 immunostaining

The MPM-1 and MPM-2 antibodies identified a subset of cellular proteins present in immunoblots of mitotic cell lysates (Davis *et al.* 1983), and MPM-1 antibody reactivity with these proteins was abolished if the nitrocellulose transfers of mitotic proteins were first treated with alkaline phosphatase. We have also determined that the MPM-1 and MPM-2 antibodies recognize identical sets of phosphoproteins in immunoblots of CHO cell lysates or isolated spindles (data not presented). Thus, the two MPM antibodies appear to recognize the same phosphorylated epitope(s) present on the reactive mitotic proteins. The MPM-2 antibody has been localized to mitotic microtubule-organizing centers by indirect immunofluorescence staining (Vandré *et al.* 1984); however, only a small subset of the mitotic phosphoproteins recognized by the MPM-2 antibody in whole mitotic cell lysates were associated with isolated spindles (Vandré *et al.* 1986). As with other cell types, centrosomes were the most intensely stained structures within mitotic LLC-PK cells stained by the MPM-2 antibody, and MPM-reactive material remained associated with the centrosome and kinetochore following detergent extraction of the cells prior to fixation (Fig. 1A–C). To determine if the MPM-2-reactive components were (also phosphoproteins) sensitive to phosphatase treatment while associated with

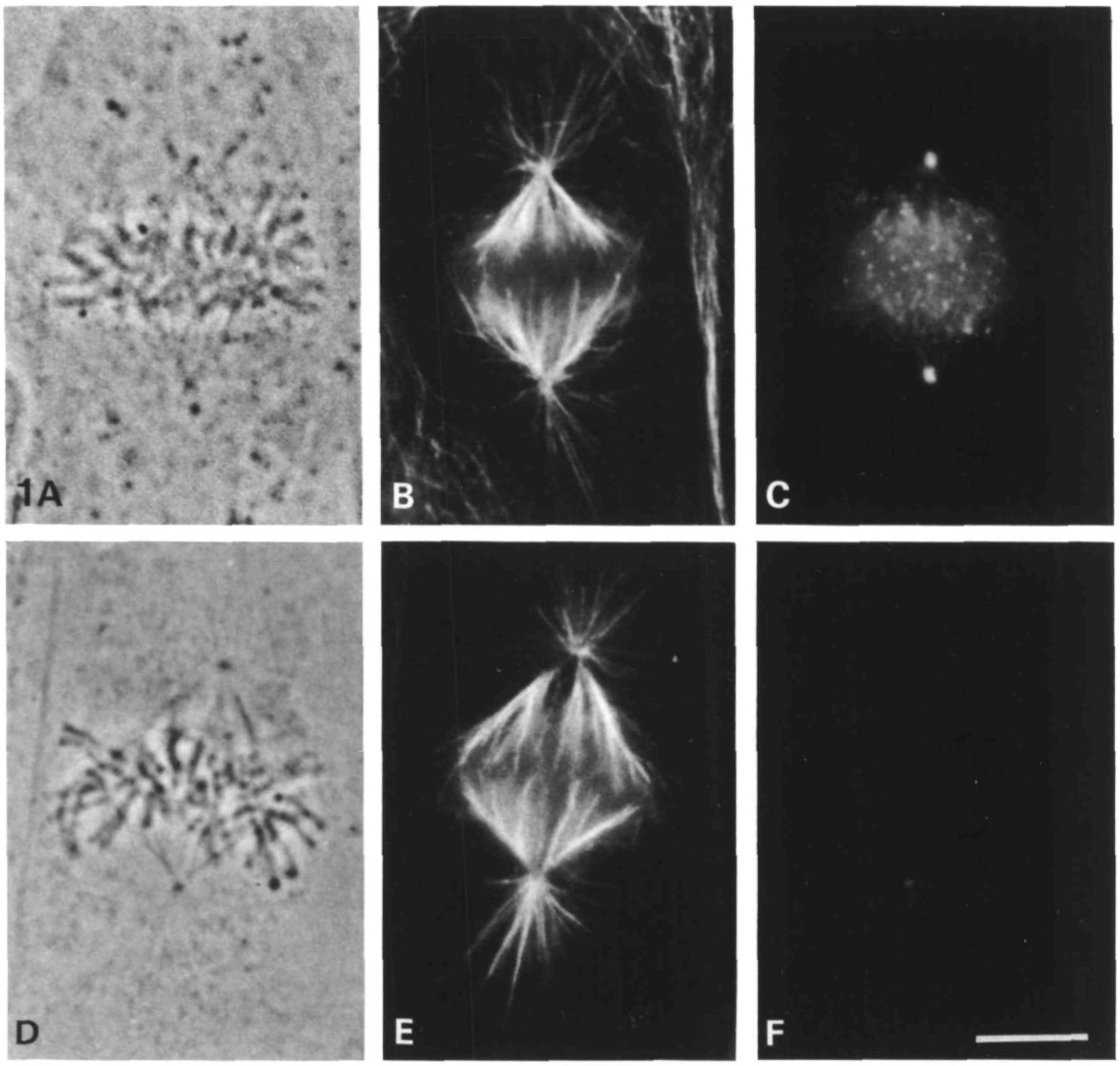

Fig. 1. Phase-contrast and double-label immunofluorescence staining of LLC-PK cells demonstrating the phosphatase sensitivity of the MPM-2 staining. Cells were detergent-lysed, rinsed, and subsequently treated with buffer either lacking (A–C), or containing alkaline phosphatase (D–F), prior to fixation and processing for immunofluorescence microscopy (see Materials and methods). Spindle integrity was maintained during treatment prior to fixation as shown by the phase-contrast (A and D) and anti-tubulin immunofluorescence (B and E). MPM-2 staining of kinetochores and centrosomes was apparent in the control cells (C), but was abolished by preincubation in buffer containing alkaline phosphatase (F). Bar, 5 μm.

intact cellular structures, detergent-lysed LLC-PK cells were treated with alkaline phosphatase prior to fixation and antibody incubation (Fig. 1D–F). The majority of the MPM staining associated with the centrosome and all of the kinetochore staining was eliminated by alkaline phosphatase pretreatment (compare Fig. 1C and F). Even in the presence of intact spindle microtubules (stabilized by including taxol in the buffer or phosphatase incubation steps following detergent lysis of the cells) (Fig. 1D and E), the MPM-reactive phosphorylated residues were accessible to the action of the phosphatase (Fig. 1F). The loss of MPM-reactivity was specifically associated with phosphatase activity, since inclusion of the phosphatase inhibitor β-glycerol phosphate during the incubation with alkaline phosphatase prevented any reduction in MPM staining (data not presented).

Cyclical phosphorylation of centrosomes during mitosis

The appearance of MPM-immunoreactive material upon entry of cells into mitosis, and the subsequent loss of MPM-reactivity upon exit from mitosis (Vandré *et al.* 1984), indicated a specific cycle of phosphorylation and dephosphorylation during mitotic progression. It was apparent, even in cells fixed prior to detergent extraction, that centrosomes were reactive with the MPM-2 antibody, and that this centrosomal staining reflected the same overall cyclical changes in staining intensity as seen in the cell as a whole. The magnitude of the changes in staining intensity, especially those associated with the centrosome, could not be determined simply from immunofluorescence micrographs. Many factors contribute to this technical problem, including: the level of immunoreactive material present in the cytoplasm of mitotic cells

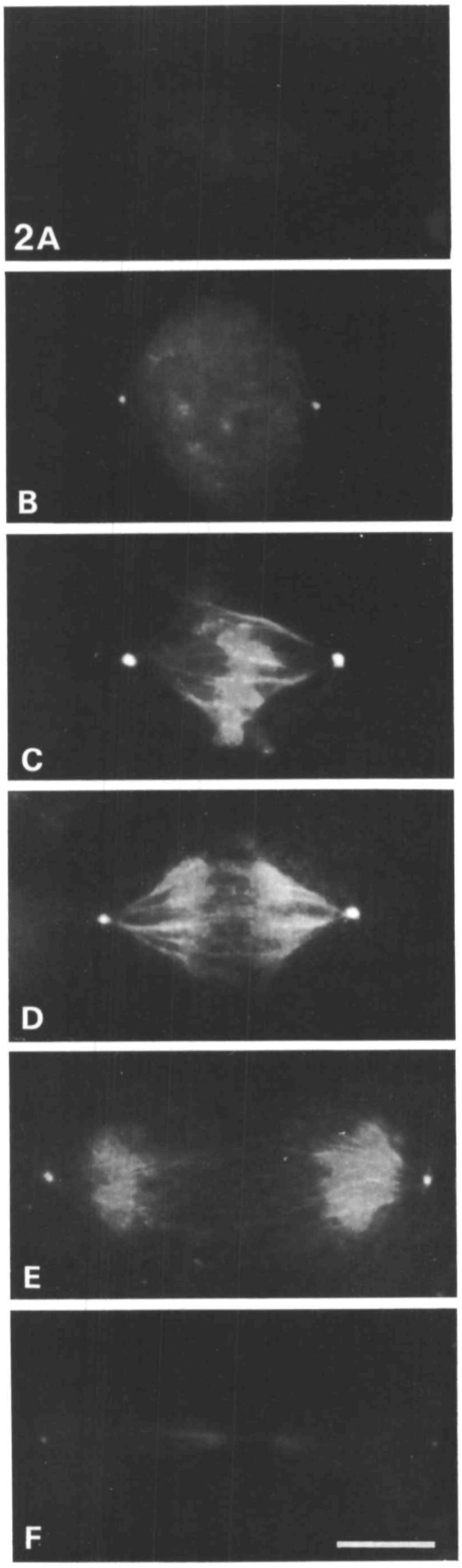

Fig. 2. Indirect immunofluorescence staining of LLC-PK cells with the MPM-2 monoclonal antibody. A cyclical pattern of centrosomal phosphorylation was observed through mitosis that was characterized by increased staining from interphase through metaphase (A–C), and decreased staining from early anaphase through telophase (D–F). Comparison of the centrosomal staining intensity provides a qualitative measurement of the change in the level of MPM-reactive phosphoproteins associated with the centrosome since standardized conditions were used to print each panel (see Materials and methods). Bar, 5 μm.

that was not specifically associated with the spindle apparatus and/or centrosome as determined by its extractability by detergent lysis of the cells prior to fixation; lack of uniformity of staining between experimental samples (coverslips) prepared at different times; varied film exposure times used during photographic documentation; linear response of the film used; and variation in the conditions used to print individual negatives (print exposure time, paper contrast, and print development time). Also, when reproduced in printed form, an additional loss of contrast occurs.

In an effort to better represent and quantify the degree of, and changes in, the level of MPM staining during mitosis, we examined MPM staining localized to the centrosome in detergent-extracted LLC-PK cells. Cells used in this comparison were grown on the same coverslips, processed for immunofluorescence microscopy under conditions of antibody excess, and all photographic and printing procedures were standardized (see Materials and methods for details). Under the conditions described above, interphase centrosomal staining by the MPM-2 antibody could not be observed (Fig. 2A), but prophase centrosomes were clearly stained (Fig. 2B). Maximal staining of centrosomes was obtained at metaphase and early anaphase (Fig. 2C and D), and by late anaphase a clear reduction in the amount of MPM staining associated with the centrosome was apparent (Fig. 2E). Staining was nearly absent in telophase cells (Fig. 2F).

As apparent from the immunofluorescence micrographs, both the intensity of the centrosomal staining and the size of the stained region varied during mitosis. Both of these parameters, therefore, needed to be considered in any quantification of the changes in MPM-2 staining associated with the centrosome. In order to quantify the staining intensity, we chose to examine photographic negatives of the immunofluorescence staining, which would allow for direct comparison of photomicrographs and image analysis obtained from the same negative, rather than the immunofluorescence directly. A Quantex image analysis system was used to determine the density of silver grains corresponding to the region of centrosomal fluorescence on the negatives (see Materials and methods for details). Data collected from the image analysis consisted of the average radiance of each pixel within the inscribed region of the centrosome, the area of the inscribed region, and the absolute radiance within the inscribed region. These parameters reflect not only the increase in staining intensity of the centrosome (average radiance), but also the increase in apparent size of the

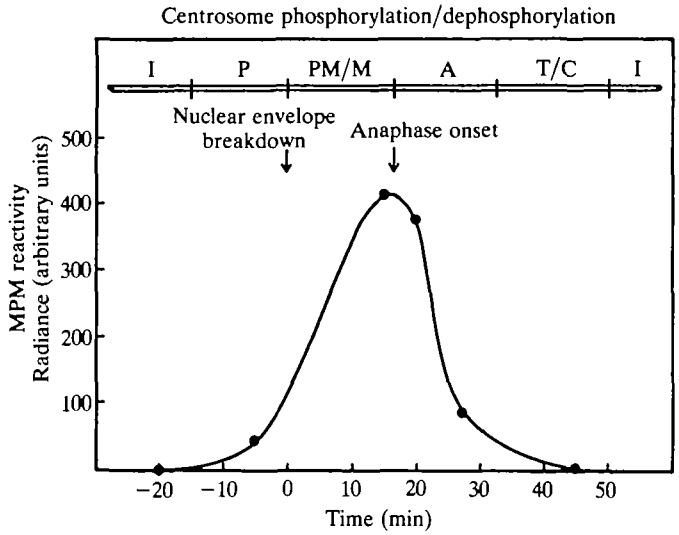

Fig. 3. Quantitation of the MPM-2 staining (phosphorylation) associated with the centrosome through mitosis. The intensity of MPM-2 staining was quantitated on regions of the photographic negatives used in Fig. 1 by measuring the silver grain density that corresponded to the centrosomal staining (see Materials and methods). Centrosomal phosphorylation increased prior to nuclear envelope breakdown, attained its maximal level at metaphase, and dephosphorylation occurred during anaphase. Reproducibility of measurement for both centrosomes in the mitotic cells were less than the diameter of the data points illustrated. Mitotic stages are indicated in the time line (I, interphase; P, prophase; PM/M, prometaphase/metaphase; A, anaphase; T/C, telophase/cytokinesis).

centrosomal structures labeled by the antibody (area of the inscribed region). Taken together, these two parameters account for the absolute radiance measurement.

The absolute radiance values, represented as the radiance of MPM-2 staining associated with the centrosome, were obtained from image analysis of the same negatives used to print the micrographs presented in Fig. 2, and are shown in Fig. 3. While both the immunofluorescence micrographs (Fig. 2) and the quantification of the staining (Fig. 3) represented the rapid increase and decrease in MPM-reactivity through mitosis, the quantitative analysis provided a measurement of the magnitude of these changes. Also, subtle differences in staining intensity of the centrosomes, not easily observed in the micrographs alone, were readily indicated in the image analysis data. It should be noted that the quantitative measurements presented in Fig. 3, although limited to those cells presented in Fig. 2, reflected the pattern of changes observed in the immunofluorescence staining seen in a population of cells. Owing to this limited sample size, the radiance values presented in Fig. 3 cannot be considered as absolute values, but should be considered as representative values of the population. It was clear from this quantification, however, that MPM-reactivity of the centrosome increased prior to nuclear envelope breakdown, at which time it was approximately one-third of that of the maximal reactivity observed at metaphase, which in turn was, at a minimum, more than eightfold greater than interphase levels. Early anaphase centrosomes stained less intensely than metaphase centrosomes, suggesting that the decrease in MPM-reactivity was initiated near the onset of anaphase (see below). The level of staining decreased rapidly during anaphase, returning to interphase levels. Similar results were obtained if each of the parameters contributing to the total radiance – i.e. the average staining intensity or size of the stained centrosomal structure – were analyzed individually (data not presented). The image analysis, therefore, provides a means of quantifying and comparing the intensity of immunofluorescence staining represented in a set of samples processed in an identical manner.

The dephosphorylation of centrosomes is dependent upon anaphase onset, but independent of chromosome segregation

Although an increase and decrease in MPM staining associated with the centrosome correlated with progression through mitosis, it was not a logical necessity that the changes in centrosomal phosphorylation were coupled to mitotic progression. For example, phosphorylation could be associated with entry into mitosis, but not required for the maintenance of the mitotic state. Once metaphase had been established, dephosphorylation could occur without affecting further progress through mitosis. Alternatively, the dephosphorylation that occurs during anaphase could either be coupled with anaphase onset and re-establishment of the interphase state in general, or be specifically associated with the process of chromosome segregation. To explore these possibilities, we examined the phosphorylation state of centrosomes in individual mitotic LLC-PK cells at specific times following NEB. Treatment of mitotic cells with nocodazole was used to block mitotic progression, and the resulting changes in centrosomal phosphorylation were assayed using the MPM-2 antibody.

Under the culture conditions employed, the progress of the LLC-PK cells through mitosis was highly reproducible. In 20 untreated cells examined continuously from prophase through mitosis, the average time from nuclear envelope breakdown (NEB) to anaphase onset was 16.4±2.4 min. This reproducibility permitted us to time prospectively the application of nocodazole relative to the moment of anaphase onset. Individual cells were monitored from NEB, allowed to progress through mitosis, and, at varying times, medium containing $1\ \mu g\ ml^{-1}$ nocodazole was added to block further progress. By knowing the time after NEB, we could compare the response of cells to which drug was applied just prior to and just after anaphase onset. For example, a cell to which drug was applied 16 min after NEB was likely to have entered anaphase within 1 min had the drug not been applied. Its response could be compared with that of a cell 1 min after it had been seen to enter anaphase.

Metaphase or anaphase cells were treated with nocodazole for 20 min, at which time the drug was removed, and cells were further incubated in fresh medium. The typical response of cells to this treatment is shown diagrammatically in Fig. 4. Upon drug addition the metaphase spindle disassembled rapidly. Phase-contrast microscopy indicated this disassembly by the loss of the

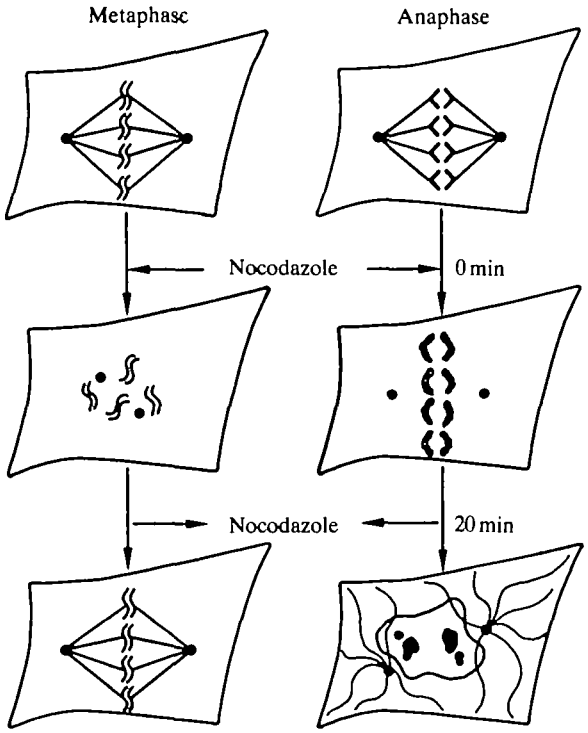

Fig. 4. Diagrammatic representation of the effects of nocodazole on further mitotic progression in treated metaphase or anaphase cells. Nocodazole addition resulted in the rapid depolymerization of the metaphase spindle. While chromosomes condensed during further incubation, their alignment along the metaphase plate was lost. Removal of drug after a 20 min incubation resulted in the reformation of the spindle. When nocodazole was added to cells immediately after the onset of anaphase, chromosome motion stopped. Unlike metaphase cells, chromosome decondensation was apparent during further incubation, and a spindle was not reformed after removal of the drug. The anaphase cell continued to progress towards interphase both during and after the nocodazole block, as shown by the formation of an interphase nucleus shortly after drug removal.

less phase-dense spindle region that excluded cytoplasmic organelles (data not presented). While the cells were held in the nocodazole block, chromosome alignment along the metaphase plate was lost. The chromosomes remained condensed throughout drug treatment, but assumed a random arrangement in the center of the cell. Following drug removal the spindle reassembled and chromosome alignment along the metaphase plate was re-established within 10 min (Fig. 5A). The cell culture was immediately fixed for examination by double-label indirect immunofluorescence staining with anti-tubulin and the MPM-2 antibodies (Fig. 5B and C). Anti-tubulin staining showed that the spindle microtubules had reformed following removal of the nocodazole, and that the phosphorylation state of the centrosome (MPM-2 staining) was elevated as in normal metaphase cells. Metaphase cells treated with nocodazole for periods up to 45 min reproducibly reassembled their spindle and entered anaphase following removal of the nocodazole (data not presented). In general, anaphase onset occurred 15–25 min after drug release, a time slightly longer than the normal time from NEB to anaphase onset in untreated cells.

The behavior of anaphase LLC-PK cells treated with nocodazole was dramatically different from their metaphase counterparts (Figs 4, 5D–F). Chromosome movement stopped within 1 min when nocodazole was added to cells that had just entered anaphase. Anaphase cells were also held in the nocodazole block for 20 min, during which time further chromosome separation was not apparent, though chromosome decondensation occurred (data not presented). A spindle did not re-form when the drug was removed following the 20-min block. Instead, chromosome decondensation continued and a nuclear envelope was formed around both sets of chromosomes. At the time the cell was fixed, an interphase nucleus had formed containing twice the number of nucleoli found in normal interphase cells (Fig. 5D). An interphase array of microtubules was present when the cell was examined by tubulin immunofluorescence staining (Fig. 5E), and no apparent MPM-2 staining was associated with the centrosomes or cytoplasm in this cell (Fig. 5F). Therefore, dephosphorylation of the MPM-reactive centrosomal components occurred in the absence of chromosome movement in anaphase cells blocked with nocodazole. In anaphase cells treated in a similar manner but fixed prior to detergent extraction, staining of other MPM-reactive material, typically observed in the cytoplasm of metaphase cells, was also absent. These results suggested that once anaphase onset was triggered, cells were committed to progress towards the interphase state. Reversal of the mitotic state, chromosome decondensation, nuclear envelope formation, and dephosphorylation of MPM-reactive phosphoproteins all continued in the absence of chromosome segregation. The interphase state was also reached in anaphase cells held continually in the presence of nocodazole (data not presented). Chromosome movements stopped and interphase progression continued in cells treated with nocodazole at later stages of anaphase as well. Cytokinesis was also initiated and completed in late anaphase cells blocked with nocodazole. However, both the initiation and completion of cytokinesis were dependent upon the extent of chromosome separation prior to the addition of the nocodazole (data not presented).

The MPM-2 staining of the centrosomes in metaphase and anaphase cells examined after 20 min in nocodazole followed by drug release differed significantly. Metaphase centrosomes showed significant MPM staining, but staining of anaphase centrosomes was absent. While these results suggested that metaphase centrosomes remained phosphorylated throughout the nocodazole block and that dephosphorylation occurred only after anaphase onset, it was also possible that the metaphase centrosomes were dephosphorylated during the treatment with nocodazole when the spindle was disassembled and became rephosphorylated upon drug release and spindle re-formation. To examine this possibility, metaphase cells were treated with medium containing 1 $\mu g\,ml^{-1}$ nocodazole for various periods of time, and then fixed while in the presence of the nocodazole. Metaphase cells exposed to the nocodazole for 5, 10, 20, 45 or 60 min

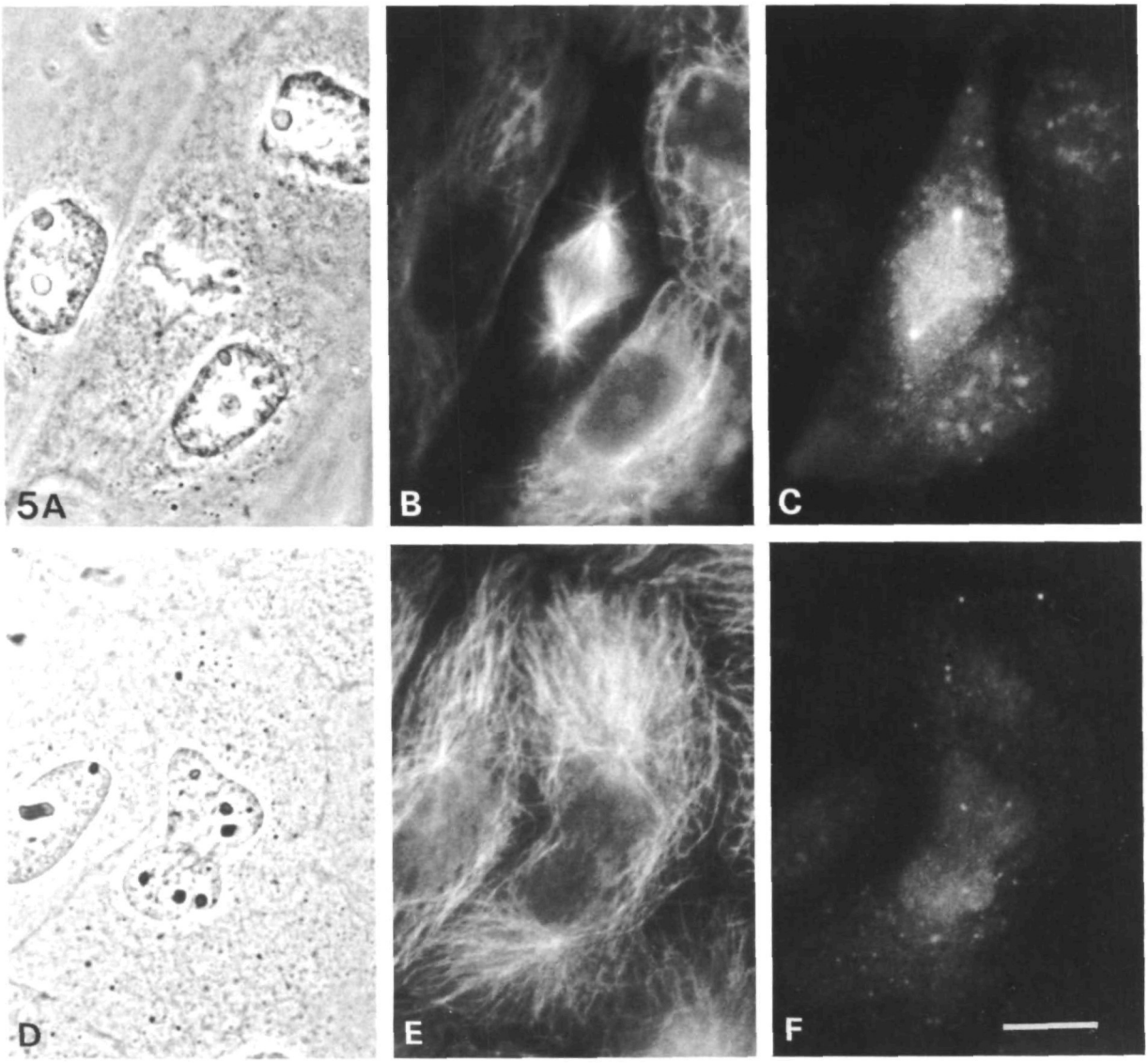

Fig. 5. Phase-contrast and double-label immunofluorescence staining with anti-tubulin and MPM-2 antibodies of cells following incubation and subsequent release from a nocodazole block. Individual cells were monitored until late metaphase (16 min after NEB) or anaphase onset. Following a 20-min incubation in nocodazole (1 $\mu g\,ml^{-1}$), cells were released from the block, incubated for an additional 7 min, and fixed. In the treated metaphase cell (A–C), realignment of the chromosomes along the metaphase plate (A), re-formation of the spindle microtubules (B), and elevated levels of MPM-2 centrosomal staining (C) were observed. The anaphase cell (D–F) progressed towards interphase while in nocodazole as shown by the formation of an interphase nucleus (D), the presence of an interphase microtubule array (E), and the lack of MPM-2 centrosomal staining (F) after removal of the nocodazole. Bar, 10 μm.

showed no apparent difference in the level of MPM staining (see below). Nocodazole was added to a culture of LLC-PK cells and mitotic cells were accumulated during an additional 18-h incubation. These cells (Fig. 6A) were examined by indirect anti-tubulin immunofluorescence staining, which showed that microtubules were not present (Fig. 6B). However, the MPM staining of the centrosomes was similar to that of untreated metaphase cells (Fig. 6C). Although these cells were blocked in a prometaphase-like stage of mitosis, centrosomes remained phosphorylated for an extended period, equivalent to 25 times the time necessary to complete a normal mitosis. It was not apparent that the level of MPM staining increased to levels greater than that associated with typical metaphase centrosomes, suggesting that the level of phosphorylation reached during normal metaphase was its maximal level. Whether the MPM-reactive phosphoproteins were saturated with phosphate and the phosphate remained stable throughout the mitotic block, or whether the phosphate was continually turning over, was not determined.

Centrosome dephosphorylation is initiated immediately after anaphase onset

It was clear that the overall dephosphorylation of the centrosome occurred during anaphase, but to determine more precisely when centrosomal dephosphorylation was

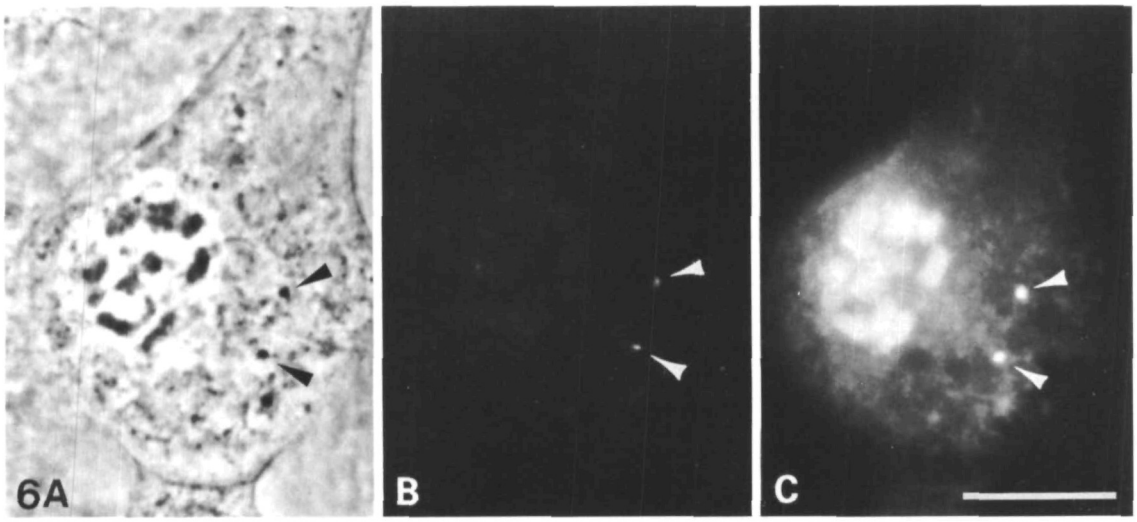

Fig. 6. Centrosomes remain phosphorylated in cells blocked at metaphase for extended periods. Phase-contrast (A), anti-tubulin staining (B) and MPM-2 staining (C) of an LLC-PK cell blocked from further mitotic progression with nocodazole ($1\,\mu g\,ml^{-1}$) for 18 h. The position of the centrosomes is indicated by arrowheads in each panel. The centrosomes were located in the cytoplasm separate from the group of condensed chromosomes (A). Microtubules were not apparent, and the anti-tubulin antibody was only localized to the centrioles (B). The levels of MPM-2 centrosomal staining was similar to that found in untreated metaphase cells (C). Bar, 10 μm.

initiated, individual mitotic LLC-PK cells were monitored until anaphase onset and then treated with nocodazole for 5, 10, 20 or 30 min before being fixed and processed for immunofluorescence staining with anti-tubulin and MPM-2 antibodies (Fig. 7). After 5 min, the chromosomes were highly condensed and had not moved in relation to their position prior to nocodazole treatment (Fig. 7, 5 min). Unlike metaphase cells, the kinetochore fibers showed significant resistance to the microtubule depolymerizing effects of nocodazole. Centrosomal MPM staining was intense. By 10 min, chromosome decondensation was apparent, and centrosomal phosphorylation diminished. Although the kinetochore fibers remained focused at their minus ends approximating the position of the spindle pole, in many cells at least one of the centrosomes became detached and was located in the cytoplasm (Fig. 7, 10 min). After 20 min the early stages of nuclear envelope reconstruction were apparent, and a typical interphase nucleus had formed by 30 min, with the exception that the reconstructed nucleus was larger and showed twice the number of nucleoli present in normal interphase cells (Fig. 7, 30 min). Remnants of kinetochore fibers were retained, but their minus ends were no longer focused at a single point. Re-formation of the interphase microtubule array was not observed in these cells since they remained in the presence of nocodazole until the time of fixation. Centrosomal staining by the MPM-2 antibody was further diminished by 20 min (Fig. 7, 20 min), and undetectable by 30 min (Fig. 7, 30 min). By contrast, a cell that was in metaphase at the time of nocodazole addition, and incubated in parallel for 30 min (Fig. 7, 30 min, M), showed condensed chromosomes and a high level of MPM staining at the centrosome.

Quantitative determination of centrosome reactivity of individually monitored metaphase and anaphase cells treated with nocodazole for 5–30 min is shown in Fig. 8. Centrosomal staining remained at the same elevated level in metaphase cells regardless of the length of time the cells were incubated in nocodazole-containing medium. In contrast, rapid decrease in the level of MPM staining was observed in anaphase cells. Loss of reactivity was initiated immediately upon the onset of anaphase and continued with a half-time of approximately 7 min.

The results presented above focused on the staining of the centrosome by the MPM-2 antibody through mitosis, which correlated with the phosphorylation state of the centrosome. It should also be noted that the kinetochores were also stained by the MPM antibody. Like centrosomes, the kinetochore staining was sensitive to pretreatment of detergent-lysed cells with alkaline phosphatase (Fig. 1). In the other experiments, although the level of MPM staining at the kinetochore was not quantified, the immunofluorescence micrographs showed that the kinetochores exhibited staining characteristics similar to those observed for the centrosomes. For example, MPM staining of the kinetochore was readily apparent in the metaphase cell held in nocodazole for 30 min (Fig. 6, 30 min, M), and could still be observed in the anaphase cell treated for 5 min (Fig. 6, 5 min). MPM-associated kinetochore staining was not observed, however, in anaphase cells treated with nocodazole for more than 5 min (Fig. 6, 10 min–30 min). Therefore, dephosphorylation of the MPM-reactive kinetochore components was similar to that shown for centrosomes, in that it was both dependent upon and initiated at anaphase onset.

Discussion

We examined the effects of nocodazole treatment on the

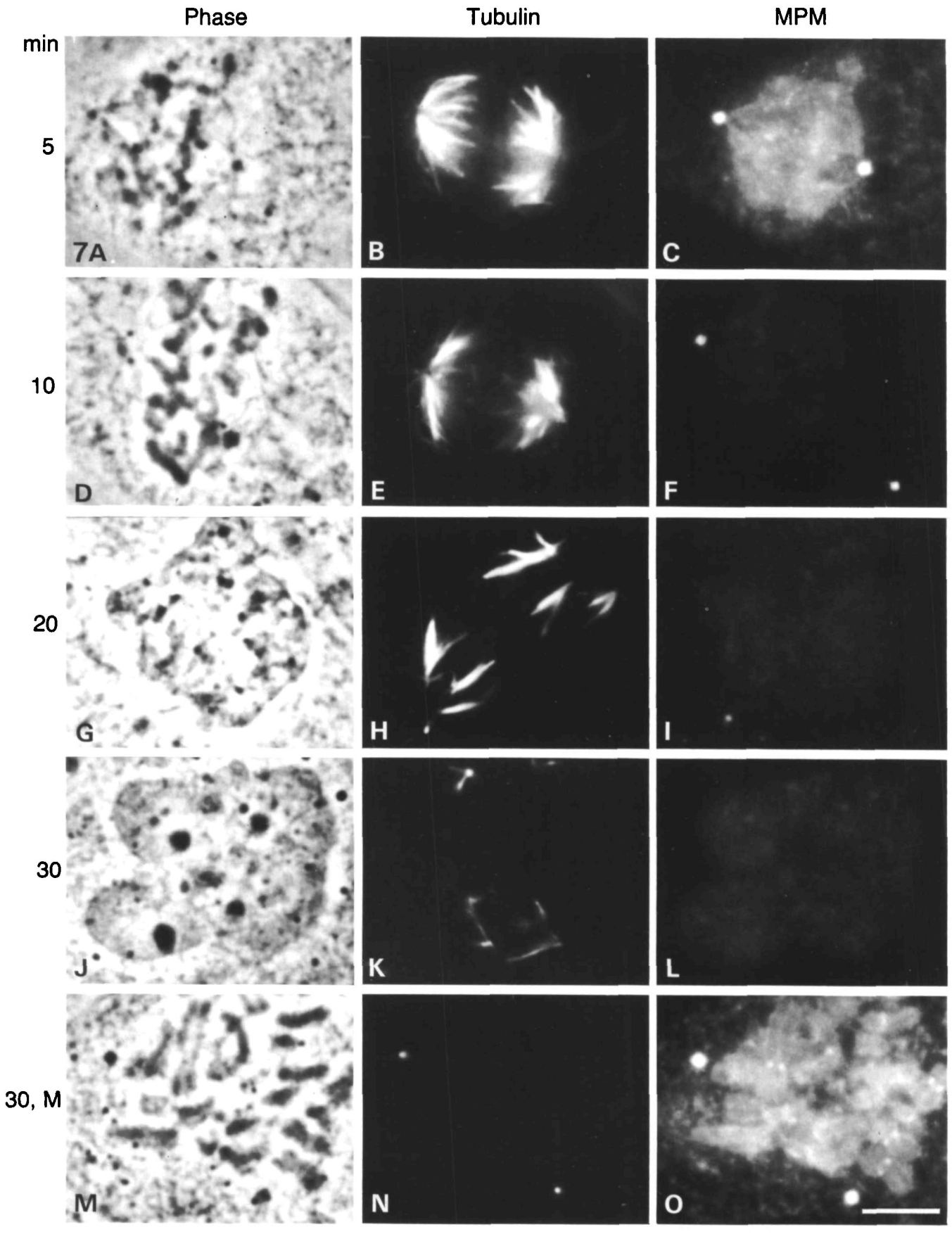
Phase
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Fig. 7. Centrosomal dephosphorylation occurs in anaphase cells in the absence of chromosome segregation. Individual LLC-PK cells were monitored until the onset of anaphase, at which time they were treated with nocodazole (1 $\mu g\,ml^{-1}$). Cells were fixed either 5 (A–C), 10 (D–F), 20 (G–I), or 30 (J–O) min later. In the cells treated at anaphase onset (A–L), chromosome decondensation and nuclear envelope reformation was apparent by phase-contrast (A,D,G,J) after 10, 20 and 30 min, anti-tubulin staining (B,E,H,K) showed the presence of kinetochore fiber remnants, and dephosphorylation of the centrosomes was shown by the decrease in MPM-2 staining (C,F,I,L). A metaphase cell held in nocodazole for 30 min is included for comparison (M–O). The chromosomes remained condensed (M), spindle microtubules were absent (N), and centrosomal phosphorylation remained elevated (O) in the metaphase cell. Bar, 5 μm.

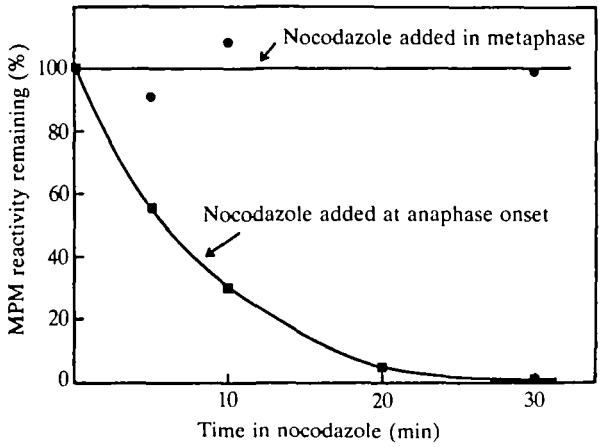

Fig. 8. Quantitation of the MPM-2 staining (phosphorylation) associated with the centrosome in metaphase and anaphase cells treated with nocodazole. Photographic negatives were analyzed as described in Materials and methods, and each time point represents the average radiance determined for a minimum of four centrosomes. Values did not vary more than two diameters of any one data point. While centrosomes remained phosphorylated in metaphase cells regardless of the length of time in nocodazole, dephosphorylation of centrosomal components occurred after anaphase onset with a half-time of approximately 7 min.

progression of both late metaphase and early anaphase cells, with specific regard to the phosphorylation state of the centrosome as determined by its immunoreactivity with the MPM-2 antibody. Individual metaphase cells timed from NEB to within one minute of anaphase onset showed no change in the overall level of MPM staining when blocked from further mitotic progression. However, rapid dephosphorylation occurred in cells that had initiated anaphase, and coincided with anaphase onset. Once anaphase had been initiated, centrosomal dephosphorylation was not dependent upon further chromosome separation.

The overall decrease in phosphorylation may result from either the dephosphorylation or the dissociation of centrosomal phosphoproteins following anaphase onset, or both. The detergent extraction of cells prior to their fixation could exacerbate any dissociation process, should it exist. However, comparison of immunofluorescence micrographs between cells fixed prior to extraction or following detergent extraction indicates that the level of centrosomal staining decreases in both cases (Vandré *et al.* 1984). In addition, the same MPM-reactive phosphoproteins have been identified on immunoblots of isolated CHO cell spindles from either metaphase or anaphase cells, and some of these phosphoproteins remain associated with the centrosomes of isolated spindles even following extraction with 0.45 M-NaCl (Vandré and Borisy, unpublished data). Therefore, the decrease in centrosomal phosphorylation cannot be due solely to dissociation of phosphoprotein constituents. Rather, dephosphorylation of MPM-reactive centrosomal components may be taken as a specific molecular indicator of anaphase onset, and this dephosphorylation may represent a state-specific biochemical alteration in the centrosome, reflecting its decreased capacity to initiate microtubule growth. Our results are consistent with the conclusions of Mullins and Snyder (1981) and Snyder *et al.* (1982) that anaphase onset corresponds to an irreversible commitment point for progression from a mitotic state towards an interphase state even in the absence of chromosome segregation.

The observed increase in centrosomal staining during mitosis results both from an increase in the intensity of the immunofluorescence staining and from an increase in the size of the stained structure. These increases could result from the phosphorylation of existing centrosomal proteins during mitosis that were present as structural components throughout the cell cycle, the recruitment by or association of cytoplasmic proteins with the centrosome and subsequent phosphorylation during mitosis, accumulation of pre-existing mitotic phosphoproteins, or a combination of the above. The increase in amount of pericentriolar material during prophase, and the behavior of antigens such as the prophase-originating polar antigen (POPA) (Sager *et al.* 1986), suggest that material does accumulate around the centrosome during entry into mitosis. The preparation of antibodies that recognize the dephosphorylated forms of the individual centrosomal components recognized by the MPM-2 antibody will be required to determine whether these components are pre-existing structural components of the centrosome, whether they accumulate at the centrosome during mitosis, and if they are related to pericentriolar components, POPA, or other centrosomal components. The subsequent decrease in MPM-2 staining following anaphase onset could result from the reversal of any one or a combination of the above. Therefore, we cannot exclude the possibility that individual centrosomal components dissociate during anaphase either before or after their dephosphorylation. In any case the control of the association/dissociation appears to be related to the phosphorylation state of the components, and the end result is a change in the phosphorylation state of the centrosome from a high level to a low level of phosphorylation.

While microtubule nucleation capacity of the centrosome increases upon entry into mitosis (Kuriyama and Borisy, 1981), as does the level of MPM-reactive phosphoproteins, dephosphorylation coincides with the decrease in centrosomal nucleation capacity occurring during anaphase. Preliminary results have indicated that the dephosphorylation of centrosomes in detergent-lysed mitotic cells by alkaline phosphatase significantly decreases the number of microtubules that are regrown by the centrosome upon the addition of exogenous porcine brain tubulin (Vandré and Borisy, 1989; Centonze, Vandré and Borisy, unpublished observations). Therefore, in the lysed cell system the microtubule nucleation capacity of the centrosome can be directly modulated by the phosphorylation state of certain centrosomal components, supporting the possible role for centrosomal phosphorylation *in vivo*.

These results suggest the involvement of phosphorylation in the regulation of mitotic processes, but a defined protein kinase/phosphoprotein phosphatase-dependent biochemical pathway, responsible for the phosphorylation/dephosphorylation of the MPM-reactive spindle components, remains to be elucidated. The examination of M-phase phosphorylation in *Xenopus* eggs has shown that there is a general increase in phosphate incorporation into most phosphoproteins, as well as specific labeling of certain M-phase proteins (Karsenti *et al.* 1987). The overall level of phosphate turnover was also shown to be higher during M-phase than that observed during interphase. These results indicate that in the *Xenopus* egg M-phase is characterized not only by an increase in both interphase kinase and phosphatase activities, but also by the activation of new kinase activities. The rapid loss of phosphate from M-phase-specific phosphoproteins at the end of mitosis was attributed to a rapid switching off of this new kinase activity in the presence of continued phosphatase activity. Whether overall phosphate incorporation is regulated in a similar manner in somatic cells during mitosis is unknown. However, the phosphorylation of MPM-reactive centrosomal components may reflect the activity of a new M-phase specific kinase, and the dephosphorylation initiated upon anaphase the inactivation of this particular kinase coupled with phosphatase activity. Since the level of MPM-staining does not increase in metaphase cells blocked with nocodazole, the phosphates are either stable until anaphase onset, or there is continual turnover of existing phosphates. The latter possibility seems more likely, since continued turnover of ^{32}P has been shown to occur in mitotic phosphoproteins of HeLa cells blocked in mitosis with N_2O, colcemid, or taxol (Sahasrabudde *et al.* 1984). It is unlikely that the rapid dephosphorylation of MPM-reactive phosphoproteins at anaphase could result from a non-enzymatic or spontaneous dephosphorylation reaction as has been suggested (Wolniak, 1988); rather, the dephosphorylation probably reflects the continued activity, stimulated activity, and/or specific activation of a phosphoprotein phosphatase.

The inactivation of a specific mitotic protein kinase may be involved in the sequence of events leading to the dephosphorylation of MPM-reactive phosphoproteins at anaphase onset, but phosphorylation of other proteins continues during anaphase. Reactivation of diatom spindle elongation *in vitro* is associated with the phosphorylation of a 205K ($K=10^3 M_r$) protein (Wordeman and Cande, 1987), and a Ca^{2+}/calmodulin-dependent protein kinase and an endogenous 62K substrate protein have been identified in isolated sea-urchin spindles (Dinsmore and Sloboda, 1988). It was shown that phosphorylation of the 205K protein correlates with microtubule sliding at the midzone of isolated diatom spindles, and that phosphorylation of the 62K protein results in the depolymerization of a subset of sea-urchin spindle microtubules. These latter results suggest that activation of the Ca^{2+}/calmodulin kinase upon anaphase onset is involved in regulating the depolymerization of spindle microtubules during anaphase. Anaphase onset has also been closely linked to a transient rise in intracellular Ca^{2+} (Poenie *et al.* 1986; Ratan *et al.* 1986). Thus, a spindle-associated Ca^{2+}/calmodulin-dependent kinase, such as the one identified in the sea-urchin system, could be regulated by changes in the localized Ca^{2+} concentration within the spindle. Could transient changes in the Ca^{2+} concentration also regulate spindle-associated phosphatase activity? It is possible to speculate that a Ca^{2+}/calmodulin-dependent phosphatase related to calcineurin and/or protein phosphatase-2B (Cohen, 1982) could also be associated with the spindle. An association of calcineurin with dendritic microtubules has previously been demonstrated (Wood *et al.* 1980). Regardless of whether changes in Ca^{2+} concentration specifically trigger anaphase or result from another triggering signal, both the phosphorylation and dephosphorylation of spindle-associated proteins during anaphase could result from the regulation of both kinase and phosphatase activities by intracellular Ca^{2+} concentration.

In addition to the cyclical changes in centrosomal phosphorylation, important differences were also observed between the sensitivity of metaphase and anaphase spindle microtubules to the depolymerizing effects of nocodazole. The majority of spindle microtubules were sensitive to nocodazole when added at the time of anaphase onset but, unlike metaphase cells, the kinetochore fibers of anaphase cells demonstrated a marked resistance to depolymerization. With time in nocodazole, a shortening of the kinetochore fibers similar to that occurring in normal anaphase A, and changes in the position of the spindle poles were observed, however. Chromosomes did not segregate to the poles; but rather, the spindle poles, no longer fixed in position by astral and/or interzonal microtubules, moved towards the chromosomes. In a similar manner, Spurck *et al.* (1986) reported that spindle poles moved towards the chromosomes in metaphase cells exposed to nocodazole. The change in the kinetochore stability at anaphase may be related to the activity of the kinetochore in its anaphase motion (Gorbsky *et al.* 1987; Koshland *et al.* 1988).

We have not established whether anaphase progression is dependent upon the dephosphorylation of centrosomal components specifically, but it is clear that dephosphorylation of the centrosomal components is dependent upon

anaphase onset. Dephosphorylation of the MPM-reactive phosphoproteins, in general, however, has been shown to be necessary for exit from mitosis. Injection of the MPM-2 antibody into cleaving *Rana pipiens* embryos inhibits cell division, and HeLa cells loaded with the antibody by red cell ghost fusion are delayed in mitosis (Davis and Rao, 1987). These results were attributed to the inhibition of phosphoprotein dephosphorylation through the binding of the MPM antibody. Continued efforts towards the identification and biochemical characterization of the MPM-reactive phosphoproteins associated with specific spindle structures, therefore, are required to define the exact role that these phosphoproteins play in the regulation of spindle assembly, function and disassembly. Coupled with the identification of the kinase(s) and phosphatase(s) present in mitotic cells responsible for the phosphorylation/dephosphorylation of these proteins, a clearer understanding of the overall relationship between these processes and other regulatory events involved in controlling the proper sequence of mitotic progression such as Ca^{2+} concentration and the activity of MPF and other mitotic kinases will become apparent.

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