

Threonine phosphorylation is associated with mitosis in HeLa cells

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Phosphorylation and dephosphorylation of proteins play an important role in the regulation of mitosis and meiosis. In our previous studies we have described mitosis-specific monoclonal antibody MPM-2 that recognizes a family of phosphopeptides in mitotic cells but not in interphase cells. These peptides are synthesized in S phase but modified by phosphorylation during G₂/mitosis transition. The epitope for the MPM-2 is a phosphorylated site. In this study, we attempted to determine which amino acids are phosphorylated during the G₂-mitosis (M) transition. We raised a polyclonal antibody against one of the antigens recognized by MPM-2, i.e. a protein of 55 kDa, that is present in interphase cells but modified by phosphorylation during mitosis. This antibody recognizes the p55 protein in both interphase and mitosis while it is recognized by the monoclonal antibody MPM-2 only in mitotic cells. Phosphoamino acid analysis of protein p55 from ³²P-labeled S-phase and M-phase HeLa cell extracts after immunoprecipitation with anti-p55 antibodies revealed that threonine was extensively phosphorylated in p55 during G₂-M but not in S phase, whereas serine was phosphorylated during both S and M phases. Tyrosine was not phosphorylated. Identical results were obtained when antigens recognized by MPM-2 were subjected to similar analysis. As cells completed mitosis and entered G₁ phase phosphothreonine was completely dephosphorylated whereas phosphoserine was not. These results suggest that phosphorylation of threonine might be specific to some of the mitosis-related events.

Mitosis; Threonine phosphorylation; Cell cycle; Antibody; Phosphoprotein; (HeLa cell)

1. INTRODUCTION

The event of mitosis, during which a cell divides into two daughter cells, occupies only a small fraction of the cell cycle time but the preparations for cell division may extend throughout the cell cycle. These preparations usually begin in the G₁ period, when signals are given for the initiation of a new cell cycle [1]. However, dramatic structural reorganizations occur in the cell within a brief period during the G₂-mitosis (M) transition. These

include condensation of chromatin into chromosomes, dissolution of the nuclear membrane, and organization of the mitotic spindle. These processes are rapidly reversed during telophase. The molecular mechanism that drives the cellular events in one direction during the initiation of mitosis and in the opposite direction during the completion remains to be elucidated.

Recently, spectacular progress has been made in the identification of various proteins that interact with each other to bring about the G₂-M transition (reviews [2,3]). The published studies clearly demonstrate the importance of protein phosphorylation in the regulation of mitosis and meiosis. A close correlation exists between the phosphorylation of certain proteins and the initiation of mitosis (and meiosis) on the one hand and the dephosphorylation of these proteins with the completion of mitosis on the other [4–6]. Although no currently available practical method blocks the phosphoryla-

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Abbreviations: PBS, phosphate-buffered saline (10 mM sodium phosphate, 0.15 M NaCl, pH 7.3); SDS-PAGE, SDS-polyacrylamide gel electrophoresis; MPF, maturation or mitosis promoting factor; MAP, microtubule-associated proteins

tion of these mitosis-specific proteins and thus the entry of cells into mitosis, it has been possible to use monoclonal antibodies (MPM-1 and MPM-2) that are specific to phosphoproteins in mitotic cells [7] to block the dephosphorylation of these phosphoproteins and thus prevent the transit from mitosis to the G₁ phase [8]. When MPM-1 or MPM-2 was microinjected into interphase HeLa cells or two-cell stage frog embryos, the antibody did not inhibit the entry of cells into mitosis but did inhibit the exit of cells from mitosis [8]. These antibodies do not block mitosis because they recognize the proteins only after they are phosphorylated. Antibodies bound at or near the site of phosphorylation of the protein could inhibit its dephosphorylation, which is essential for the transition from mitosis to interphase [7,8]. The objective of our study was to isolate these mitosis-specific phosphoproteins and determine which of the three amino acids – serine, threonine and tyrosine – are phosphorylated and dephosphorylated during mitosis.

2. MATERIALS AND METHODS

2.1. Isolation of protein p55

Protein p55 was isolated from the MPM-2 immunocomplex following immunoprecipitation. For immunoprecipitation with the antibody MPM-2, 200 μ l Pansorbin (*Staphylococcus aureus*) cells (Calbiochem-Behring, La Jolla, CA) were washed with buffer A (20 mM K₂HPO₄, 2 mM dithiothreitol, 5 mM adenosine triphosphate, 1% bovine serum albumin, 0.5% Triton X-100, 0.1% SDS, 100 μ l phenylmethylsulfonyl fluoride, 100 μ l protease inhibitors; pH 7.5) three times and incubated with 50 μ l rabbit anti-mouse IgG for 2 h. All incubations during immunoprecipitation were performed at 4°C with shaking. The cells were microfuged (Microfuge by Hermale, National Lab. Co., Woodbridge, NJ) and the pellet was resuspended in 100 μ l HPLC-purified MPM-2 antibody and incubated for 2 h. It was centrifuged again and the pellet was resuspended in 200 μ l mitotic HeLa cell extract, prepared as in [9], and incubated for 1 h. The suspension was centrifuged and the pellet was washed 8–10 times with buffer A. The immunocomplex was released from Pansorbin by resuspension of the pellet in 100 μ l buffer B (0.2 M glycine-HCl, 1.0 M NaCl; pH 4.0) and incubation for 1 h. After centrifugation, the supernatant was collected and boiled with 50 μ l of 3 \times sample buffer for 10 min. SDS-PAGE was performed on the immunoprecipitates obtained above. Gels were stained lightly with Coomassie blue, the protein band at 55 kDa was carefully cut out, and proteins were eluted from the gel slices using an Electro-Eluter (model 422, Bio-Rad, Richmond, CA) with the running buffer.

2.2. Production of polyclonal antibody against p55

The p55 antigen isolated as described above was concentrated

by lyophilization, resuspended in saline, and mixed with an equal volume of Freund's complete adjuvant. About 20–30 μ g protein was injected subcutaneously into a New Zealand white male rabbit at several sites on the back, as described [10]. This procedure was repeated every 2 weeks, but booster injections were mixed with Freund's incomplete adjuvant. After the fifth booster injection, the rabbit was bled at 2-week intervals and the serum tested for antibody by using double immunodiffusion against mitotic HeLa cell extracts [11]. Protein estimations were performed according to Bradford [12].

2.3. Immunoblots

HeLa cells were synchronized in S phase by the thymidine (2.5 mM) double-block method [13] and in mitosis by N₂O blockade following the reversal of a single thymidine block [14]. Cells were washed three to five times with Eagle's minimum essential medium without serum at 4°C and extracts prepared as in [9]. Approx. 100 μ g protein was loaded per slot. Polypeptides from HeLa cell extracts were separated in 8% SDS-polyacrylamide gels according to Laemmli [15], electrophoretically transferred to nitrocellulose sheets [16] and stained with MPM-2 using an indirect immunalkaline phosphatase procedure [17]. After the nitrocellulose sheet had been washed three times with Tris/saline buffer containing 0.05% Tween 20 (TBS/TW20), it was incubated for 30 min at 25°C while shaking with alkaline phosphatase-conjugated, affinity-purified goat anti-mouse IgG, heavy and light chains (Bio-Rad), diluted 1:5000 with the blocking solution. Subsequent to the sheet being washed with TBS/TW20 three times, the color was developed by adding a mixture of 5-bromo-4-chloro-3-indolyl phosphate disodium salt (BCIP) and *p*-nitro blue tetrazolium chloride (NBT) solutions in alkaline phosphatase buffer (Bio-Rad). These substrates develop a purple color on the membrane where alkaline phosphatase-conjugated antibodies are bound.

2.4. Phosphoamino acid analysis

The extracts were immunoprecipitated with MPM-2 or anti-p55 antibody, and the immunocomplexes were released from Pansorbin. The immunoprecipitates were subjected to phosphoamino acid analysis as described in [18]. Briefly, the protein antigens from the immunocomplex were precipitated by trichloroacetic acid (20%). The precipitated proteins were washed with absolute ethanol and then dissolved in 250 μ l distilled water to which 250 μ l of 12 N HCl was added. This mixture was hydrolyzed at 110°C for 2 h. The hydrolysate was diluted 10-fold with PBS, lyophilized, transferred to an Eppendorf tube, washed three times with PBS, and dried using a Speed Vac (Savant Instrument, Farmingdale, NY). To this tube were added amino acid markers, i.e. serine, threonine and tyrosine (2.5 μ g/ μ l each), along with 0.5 ml PBS and drying performed using the Speed Vac. Five microfilters of PBS were then added to the tube, the contents were mixed and centrifuged, and the supernatant was loaded on paper for chromatography [18]. After drying, the paper was exposed to X-ray film for autoradiography.

3. RESULTS AND DISCUSSION

The monoclonal antibody MPM-2 specifically

stains cells undergoing mitosis and, on immunoblots, recognizes a family of phosphopeptides in the extracts of mitotic cells but not in those of interphase cells [7]. Of these phosphopeptides, a protein of approx. 55 kDa (p55) is one of the four most prominent (fig.1). This protein was not one of the three major protein bands detectable in our earlier studies, probably because of the differences in extraction buffers used. In our initial studies [7], we extracted cells in phosphate-buffered saline (10 mM $\text{Na}_2\text{HPO}_4/0.15$ M NaCl; pH 7.3) (PBS), whereas here, we used 0.2 M NaCl buffer containing 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 2 mM EGTA, and 10 mM MgSO_4 supplemented with protease and phosphatase inhibitors [9]. Probably p55 was either not extractable with PBS or was dephosphorylated during extraction, since no phosphatase inhibitors were used in the earlier study.

Since p55 is one of the most abundant proteins recognized by MPM-2, that undergoes mitosis-specific phosphorylation, we have used this as a means to determine which amino acid residues are phosphorylated during the G_2 -M transition. From our previous studies, we know that the epitope for MPM-2 is a phosphorylated site and that the antigens recognized by MPM-2 are synthesized during S phase and phosphorylated during the G_2 -M transition [7]. Therefore, it was important to have an antibody that recognizes these proteins even in the interphase cells, i.e. when they are not phosphorylated. To achieve this objective, we raised polyclonal antibodies against p55 as described in section 2.

The polyclonal rabbit antibody generated against protein p55 (i.e. anti-p55 antibody) was used to immunoprecipitate the antigens from HeLa cells synchronized in S phase and mitosis. The polypeptides from the immunocomplexes were separated on an SDS/polyacrylamide gel and transferred to a nitrocellulose sheet, which was cut into two strips; one strip was stained with MPM-2 and the other with anti-p55 antibody (fig.2A, lanes 3,4 and 5,6, respectively). Mitotic HeLa cell extracts immunoprecipitated with MPM-2 and the nitrocellulose strip stained with MPM-2 served as controls. The 55 kDa band was the most prominent among the four or five bands recognized by MPM-2 (fig.2A, lane 2). In anti-p55 antibody immunoprecipitates, MPM-2 recognized the p55 band in mitotic cell extracts but not in S-

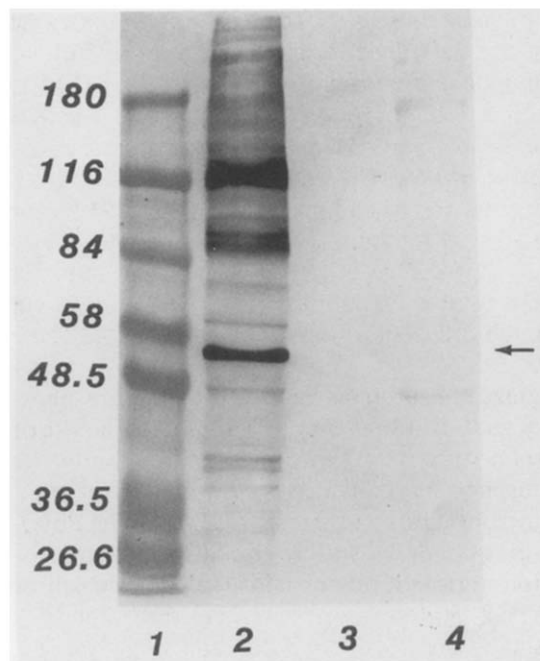


Fig.1. Mitosis-specific phosphoproteins recognized by the monoclonal antibody MPM-2. Immunoblot of mitotic and interphase HeLa cell extracts prepared and stained with MPM-2 as described in section 2. Lane 1, prestained molecular mass markers: [α_2 -macroglobulin (180 kDa), β -galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa), triosephosphate isomerase (26.6 kDa) (Sigma, St. Louis, MO)]. The protein band at 55 kDa (arrow) is one of the major bands recognized by MPM-2 in mitotic cells (lane 2) but not in S-phase cells (lane 3). Two or three faint bands were detectable in the extracts of a random population (lane 4), and could be due to the presence of late G_2 -phase cells and a small percentage (4%) of mitotic cells. The p55 band is not detectable in the random population (lane 4).

phase cell extracts (fig.2A, lanes 3,4), whereas anti-p55 antibody recognized this band in both mitotic and S-phase cell extracts (fig.2A, lanes 5,6). Although we observed some background staining of other proteins with anti-p55 (lane 6) these proteins were not recognized following phosphorylation in mitosis by MPM-2 (lane 3).

Incubation of mitotic HeLa cell extracts with either alkaline phosphatase (10 U/ml) or an equal volume of an extract of HeLa cells synchronized in G_1 phase removed the antigenicity of p55 to the MPM-2 antibody on immunoblots (fig.2B). The extent of dephosphorylation of protein p55 following incubation of ^{32}P -labeled mitotic cell

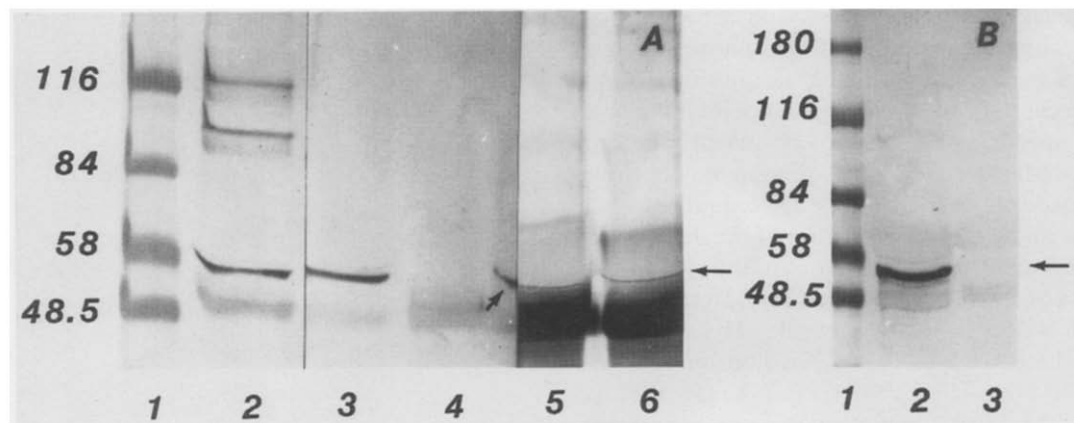


Fig.2. (A) Protein p55 in mitotic and S-phase HeLa cells. Lane 1, molecular mass markers. Lane 2, mitotic HeLa cell extracts were immunoprecipitated with MPM-2, the polypeptides separated by SDS-PAGE, and the immunoblot stained with MPM-2. Protein p55 was the most prominent among the proteins immunoprecipitated with MPM-2 (lane 2). Since MPM-2 is specific to phosphoproteins in mitotic cells, a similar preparation with interphase cells was not made. Mitotic and S-phase HeLa cell extracts were immunoprecipitated with anti-p55 antibody, peptides separated by SDS-PAGE, and transferred to nitrocellulose paper. The paper was then cut in the middle, and one strip was stained with MPM-2 (lanes 3,4) and the other with anti-p55 antibody (lanes 5,6). MPM-2 clearly identified the 55 kDa band in mitotic cells (lane 3) but not in S-phase cells (lane 4). In the section stained with the anti-p55 antibody, a dark, narrow band of 55 kDa was present in both mitotic (lane 5) and interphase (lane 6) cells (arrow). A section of lane 5 stained by MPM-2 reveals a broader segment of that band (arrow). The broad light band in lanes 3,4, and the dark broad band in lanes 5,6 represent the heavy chain of rabbit IgG. (B) Dephosphorylation of protein p55 by incubation of mitotic cell extracts with G₁-phase cell extracts. Mitotic HeLa cell extracts were incubated with an equal volume of G₁-cell extracts for 1 h at room temperature as in [28]. The mixture was then immunoprecipitated with anti-p55 antibody, the polypeptides separated by SDS-PAGE and transferred to nitrocellulose paper, and the immunoblots stained with MPM-2. The 55 kDa band seen in the mitotic cell extracts (lane 2) was removed in the mixture of mitotic and G₁-phase cell extracts (lane 3). Lane 1, molecular mass markers. G₁-phase cell extracts caused dephosphorylation of protein p55.

extracts or anti-p55 immunoprecipitates with G₁-phase cell extracts was dose-dependent (table 1): the higher the G₁/M ratio, the greater the degree of dephosphorylation. These results further support our earlier data that G₁ cell extracts contain a phosphatase activity that specifically dephosphorylates mitosis-specific phosphoproteins [4–6]. Furthermore, dephosphorylation of p55 by G₁ cell extracts could be used as an assay for the isolation of mitosis-specific phosphatases.

To determine which amino acid residues are phosphorylated and when they are phosphorylated or dephosphorylated, we performed phosphoamino acid analysis as described in [18]. HeLa cells were labeled with ³²P for 3 h either during S phase or in G₂-M transition as cells were arrested in mitosis by a nitrous oxide (90 lb/inch²) blockade. Half of the N₂O-blocked mitotic cells were washed free of label and allowed to divide to yield a ³²P-labeled G₁ population. The labeled extracts were immunoprecipitated with anti-p55 antibody or

MPM-2. Phosphoamino acid analysis of the antigens released from immunoprecipitates of ³²P-labeled mitotic cell extracts with either anti-p55 or MPM-2 antibodies revealed that serine and threonine were phosphorylated. However, phosphorylation of threonine was more extensive than that of serine (fig.3, lanes 1,2). A comparison of ³²P-labeled S phase and mitotic cell extracts by phosphoamino acid analysis after immunoprecipitation with anti-p55 antibody revealed that serine was phosphorylated in S phase as well as in G₂-M transition, whereas threonine was specifically and extensively phosphorylated only during the initiation of mitosis (fig.3, lanes 3,4). A similar analysis with G₁ cell extracts revealed total dephosphorylation of [³²P]phosphothreonine with no detectable change in ³²P-labeled phosphoserine (fig.3, lane 5). Incubation of a ³²P-labeled mitotic cell extract with an unlabeled G₁-phase cell extract in vitro caused extensive but not complete dephosphorylation of serine (fig.3, lane 6). Threonine was com-

Table 1

Dephosphorylation of mitosis-specific phosphoprotein p55 by extracts of HeLa cells in G₁ phase

Treatments	Effect of mixing with G ₁ cell extract			
	Before immunoprecipitation with anti-p55		After immunoprecipitation with anti-p55	
	³² P (cpm) associated with p55	Relative inhibition (%)	³² P (cpm) associated with p55	Relative inhibition (%)
Mitotic extract (control)	2853	0	2106	0
Mitotic extract + buffer (1:1) (control)	2898	0	2051	0
Mitotic extract + G ₁ cell extract (1:1)	1330	54.0	620	70.1
Mitotic extract + G ₁ cell extract (1:2)	532	81.5	278	86.6

HeLa cells were synchronized in G₂-phase and labeled with ³²P; mitotic cells were collected and extracts prepared as previously described. To study the effects of adding unlabeled G₁-phase cell extract on the level of p55 phosphorylation, two kinds of experiments were performed. In the first set of experiments, ³²P-labeled mitotic extract was mixed with either buffer (control) or unlabeled G₁-phase cell extract in different proportions and incubated at room temperature for 1 h. The mixtures were then processed for immunoprecipitation with anti-p55 antibody. ³²P-labeled p55 was released from the immunocomplex by a low pH-high salt buffer and precipitated with trichloroacetic acid with acid-precipitable radioactivity being determined using a liquid scintillation counter. In the second set, ³²P-labeled mitotic extracts were first immunoprecipitated with anti-p55 antibody. The immunocomplex was then treated with buffer or unlabeled G₁-phase cell extract as above. The mixture was centrifuged, and ³²P-labeled p55 was released and processed for scintillation counting as described above. Data presented are averages of three separate experiments

pletely dephosphorylated. Probably the phosphoserine residues were better protected in vivo from dephosphorylation than in vitro.

Our study clearly indicates that serine is phosphorylated throughout the cell cycle and remains phosphorylated even after completion of mitosis. In contrast, phosphorylation of threonine is highly specific to G₂-M transition and it is completely dephosphorylated as cells traverse from M to G₁. Thus threonine phosphorylation and dephosphorylation are closely associated with the initiation and completion of mitosis, respectively. There is a possibility, however slight, that phosphorylation of a specific serine residue might also be involved in the regulation of mitosis.

The significance of this observation lies not in the use of p55 protein of unknown function but in its mitosis-specific phosphorylation recognized by the MPM-2 antibody. Furthermore, the results obtained by using the p55 protein are in total conformity with those from employing all the MPM-2

antigens immunoprecipitated with MPM-2 (fig.3, lanes 1,2).

In a strain of NIH 3T3 cells in which pp60^{c-src} is overexpressed, this protein was found to be phosphorylated at threonine and, to some extent, at serine residues during mitosis [19]. Although pp60^{v-src} is known as the transforming protein of Rous sarcoma virus, the fact that its cellular homologue is modified by phosphorylation of threonine during mitosis suggests that pp60^{c-src} might be involved in some of the mitosis-specific functions. Phosphorylation of threonine and serine was also found to be associated with the maturation promoting factor (MPF) activity in amphibian and starfish oocytes [20,21]. The percentage of [³²P]phosphothreonine in labeled protein showed a better correlation with MPF activity than that of [³²P]phosphoserine [21]. In light of these studies and ours we conclude that phosphorylation of threonine may play an important regulatory role in mitosis and meiosis.

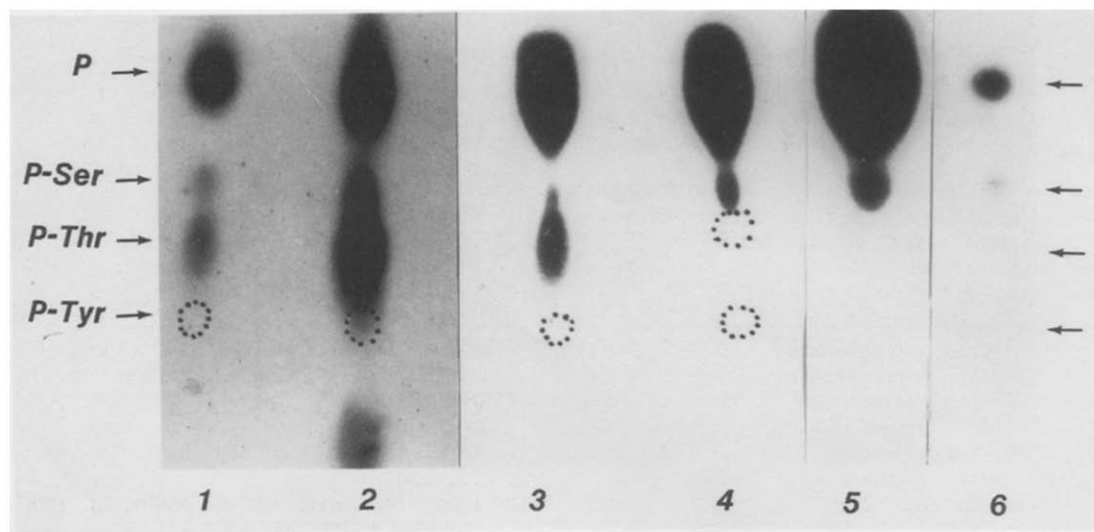


Fig. 3. Phosphorylation of threonine during G₂-mitosis and dephosphorylation during M-G₁ transition. HeLa cells were synchronized in S phase by double thymidine block. 1 h after reversal of the second thymidine block, cells were labeled with ³²P (ICN, Irvine, CA) for 3 h by incubation with H₃³²PO₄ (10 μCi/ml, spec. act., carrier-free; 1 Ci = 3.7 × 10¹⁰ Becquerels) at 37°C. After incubation, cells were trypsinized and washed thoroughly with medium, and extracts prepared as described [9]. To obtain labeled mitotic cells ³²P was added to HeLa cells at 7 h after reversal of the second TdR block. ³²P was added and dishes were incubated for 3–5 h under an N₂O (90 lb/inch²) atmosphere to block cells in mitosis [14]. ³²P-labeled mitotic cells of 98% purity were collected by selective detachment. Mitotic cells were washed free of label and half of the population was used to make extracts whereas the other half was allowed to divide and enter G₁ phase to produce G₁ cell extracts. Mitotic cell extracts were immunoprecipitated with either anti-p55 antibody (lane 1) or MPM-2 (lane 2) and subjected to phosphoamino acid analysis. Both immunoprecipitates gave identical results in that both serine and threonine were phosphorylated but tyrosine was not. Threonine was more extensively phosphorylated than serine. Phosphoamino acid analysis was performed on mitotic (lane 3) and S-phase (lane 4) cell extracts after immunoprecipitation with anti-p55 antibody. Serine and threonine were phosphorylated in mitotic cells (lane 3), but only serine was phosphorylated in S-phase cells (lane 4). The amount of [³²P]phosphoserine present in mitotic cell extracts was significantly lower than that of [³²P]phosphothreonine. Threonine was not phosphorylated in S-phase cells. Note that phosphorylation of serine seems to be greater during S-phase than during the G₂-M transition; the intensity of serine labeling is heavier in lane 4 than in lane 3. The extract of G₁ cells obtained from ³²P-labeled mitotic cells was immunoprecipitated with anti-p55 antibody and processed for phosphoamino acid analysis (lane 5). It is interesting to note that [³²P]phosphothreonine was totally dephosphorylated whereas there is a significant amount of ³²P associated with phosphoserine. ³²P-labeled mitotic cell extract was incubated with an equal volume of unlabeled G₁ cell extract for 1 h at room temperature, immunoprecipitated with anti-p55 antibody and the immunoprecipitates processed for phosphoamino acid analysis (lane 6). Incubation with G₁ cell extract caused dephosphorylation of the mitosis-specific phosphoproteins. Dephosphorylation of [³²P]phosphothreonine was complete while it was only partial in the case of [³²P]phosphoserine. In vitro mixing of extracts resulted in a greater degree of dephosphorylation of phosphoserine than in vivo (i.e. G₁ cells). P, free phosphate; P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine. The arrows and dotted circles indicate the expected positions of the three phosphoamino acids on the chromatogram.

During mitosis, a number of proteins, histone H1 [22], lamins [23], microtubule-associated proteins or MAPs, proteins associated with centrosomes, kinetochores, and midbodies, and other nonhistone proteins [24–26], are phosphorylated. In fact, the MPM-2 antibody recognizes a number of phosphopeptides, ranging in molecular mass from 55 to >210 kDa, whose individual roles or functions during mitosis have not yet been identified. Some of these could be protooncogene products. MPM-2, however, does not recognize all of

the proteins that are phosphorylated during the G₂-M transition. For example, histones [7], lamins [27], and some other nonhistone proteins in mitotic cells are not recognized by MPM-2. The antigenicity of the proteins recognized by MPM-2 depends on the site of their phosphorylation. Here, we have shown that threonine is specifically phosphorylated and dephosphorylated during mitosis in the phosphoprotein antigens recognized by MPM-2 and anti-p55. Therefore, it appears that MPM-2 is specific to some sequence or protein

conformation involving a phosphothreonine rather than a phosphoserine. Further studies are necessary to identify the amino acid sequence of the epitope recognized by the MPM-2 antibody.

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