**MPM-2 antibody-reactive phosphorylations can be created in detergent-extracted cells by kinetochore-bound and soluble kinases**

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**INTRODUCTION**

Phosphorylation is a ubiquitous mechanism for regulating cell cycle processes and has been implicated in all stages of mitosis. Antibodies that specifically recognize the phosphorylated forms of proteins are valuable tools for studying the regulation of mitotic events. One such monoclonal antibody, termed MPM-2, was raised against mitotic HeLa extracts (Davis et al., 1983). The MPM-2 antibody labels phosphorylated proteins found at kinetochores, chromosome arms, midbody and spindle poles of mitotic cells. In cells extracted without phosphatase inhibitors, labeling of the MPM-2 antibodies at kinetochores is greatly diminished. However, in cytoskeletons this epitope can be regenerated through the action of kinases stably bound at the kinetochore. Various kinase inhibitors were tested in order to characterize the endogenous kinase responsible for these phosphorylations. We found that the MPM-2 epitope will not rephosphorylate in the presence of the broad specificity kinase inhibitors K-252a, staurosporine and 2-aminopurine. Several other inhibitors had no effect on the rephosphorylation indicating that the endogenous MPM-2 kinase at kinetochores is not p34cdc2, casein kinase II, MAP kinase, protein kinase A or protein kinase C. The addition of N-ethylmaleimide inactivated the endogenous kinetochore kinase; this allowed testing of several purified kinases in the kinetochore rephosphorylation assay. Active p34cdc2-cyclin B, casein kinase II and MAP kinase could not generate the MPM-2 phosphoepitope. However, bacterially expressed NIMA from *Aspergillus* and ultracentrifuged mitotic HeLa cell extract were able to catalyze the rephosphorylation of the MPM-2 epitope at kinetochores. Furthermore, fractionation of mitotic HeLa cell extract showed that kinases that create the MPM-2 epitope at kinetochores and chromosome arms are distinct. Our results suggest that multiple kinases (either soluble or kinetochore-bound), including a homolog of mammalian NIMA, can create the MPM-2 phosphoepitope. The kinetochore-bound kinase that catalyzes the formation of the MPM-2 phosphoepitope may play an important role in key events such as mitotic kinetochore assembly and sister chromatid separation at anaphase.

**SUMMARY**

The MPM-2 antibody labels mitosis-specific and cell cycle-regulated phosphoproteins. The major phosphorylations of mitotic chromosomes recognized by the MPM-2 antibody are DNA topoisomerase II (topoII) α and β. In immunofluorescence studies of PtK1 cytoskeletons, prepared by detergent lysis in the presence of potent phosphatase inhibitors, the MPM-2 antibody labels phosphoproteins found at kinetochores, chromosome arms, midbody and spindle poles of mitotic cells. In cells extracted without phosphatase inhibitors, labeling of the MPM-2 antibodies at kinetochores is greatly diminished. However, in cytoskeletons this epitope can be regenerated through the action of kinases stably bound at the kinetochore. Various kinase inhibitors were tested in order to characterize the endogenous kinase responsible for these phosphorylations. We found that the MPM-2 epitope will not rephosphorylate in the presence of the broad specificity kinase inhibitors K-252a, staurosporine and 2-aminopurine. Several other inhibitors had no effect on the rephosphorylation indicating that the endogenous MPM-2 kinase at kinetochores is not p34cdc2, casein kinase II, MAP kinase, protein kinase A or protein kinase C. The addition of N-ethylmaleimide inactivated the endogenous kinetochore kinase; this allowed testing of several purified kinases in the kinetochore rephosphorylation assay. Active p34cdc2-cyclin B, casein kinase II and MAP kinase could not generate the MPM-2 phosphoepitope. However, bacterially expressed NIMA from *Aspergillus* and ultracentrifuged mitotic HeLa cell extract were able to catalyze the rephosphorylation of the MPM-2 epitope at kinetochores. Furthermore, fractionation of mitotic HeLa cell extract showed that kinases that create the MPM-2 epitope at kinetochores and chromosome arms are distinct. Our results suggest that multiple kinases (either soluble or kinetochore-bound), including a homolog of mammalian NIMA, can create the MPM-2 phosphoepitope. The kinetochore-bound kinase that catalyzes the formation of the MPM-2 phosphoepitope may play an important role in key events such as mitotic kinetochore assembly and sister chromatid separation at anaphase.

**Key words:** Rephosphorylation, Kinetochore, MPM-2, Mitosis, Chromosome, NIMA
also inhibit ubiquitin-mediated destruction of cyclin B in *Xenopus* egg extracts, by binding components of the ubiquitin-ligase complex termed the cyclosome or anaphase promoting complex (King et al., 1995).

Other important proteins recognized by PMP-2 include NIMA (Ye et al., 1995), certain microtubule-associated proteins (Tombes et al., 1991; Vandere et al., 1991; Kuang and Ashorn, 1993), *Xenopus* CDC25 (Kuang et al., 1994), *Xenopus* wee1-like kinase (Mueller et al., 1995) and other mitotic phosphoproteins still unidentified (Matsumoto-Taniura et al., 1996). Controversy exists over the exact nature of the PMP-2 epitope. It appears to recognize phosphothreonine in the context of surrounding amino acids (Zhao et al., 1989; Westendorf et al., 1994; Taagepera et al., 1993, 1994; Ding et al., 1997).

Several kinases appear to create PMP-2-reactive phosphorylations. MAP kinase kinase (MEK) is responsible for creating the PMP-2 reactive phosphorylations on MAP kinase (Taagepera et al., 1994). Westendorf (1994) used p34cdc2 kinase to generate the PMP-2 phosphoepitope on peptides in vitro, and p34cdc2 kinase can also create an PMP-2 reactive phosphorylation on NIMA protein kinase (Ye et al., 1995). Kuang and Ashorn (1993) have reported two kinase activities that can generate the PMP-2 phosphoepitope on unphosphorylated substrates present in a *Xenopus* extract. One of them was found to be MAP kinase. Recent data show that Plx1, a *Xenopus* polo-like kinase, acts as an PMP-2 kinase by phosphorylating CDC25C (Kumagai and Dunphy, 1996). However, little is still known about other kinases that create the PMP-2 phosphoepitope in vivo.

In PtK1 cells extracted with detergent in the presence of phosphatase inhibitors, the PMP-2 antibody detects phosphorylated epitopes present at the kinetochores of mitotic chromosomes. This labeling is bright in prophase, prometaphase, and metaphase but decreases in anaphase and telophase (Vandere et al., 1984; Taagepera et al., 1995). If cells are extracted in the absence of phosphatase inhibitors, labeling of PMP-2 phosphoepitopes diminishes, particularly at kinetochores (Taagepera et al., 1995). We have developed a protocol to reprophosphorylate the dephosphorylated PMP-2 epitope using the endogenous kinase bound at the kinetochores. We went on to develop methods to test if purified kinases can create the PMP-2 epitope on chromosomes. Active p34cdc2-cyclin B, casein kinase II and MAP kinase could not generate the PMP-2 phosphoepitope. However, purified NIMA or soluble HeLa mitotic cell extract were able to catalyze the reprophosphorylation of the PMP-2 epitope at kinetochores. Fractionation of the mitotic extract showed that kinetochores and chromosome arms can be separately reprophosphorylated by two different chromatographic column fractions. Our results suggest that multiple kinases (either soluble or kinetochore-bound), including the mammalian homolog of NIMA, can create the PMP-2 phosphoepitope. Kinetochore kinases that catalyze the formation of the PMP-2 phosphoepitope may play an important role in such key events as mitotic kinetochore assembly and sister chromatid separation at anaphase.

**MATERIALS AND METHODS**

**Materials**

PtK1 *Potoroo tridactylis* kidney cells (ATCC CCL35) and HeLa S3 cells (ATCC CCL 2.2) were obtained from American Type Culture Collection (Rockville, MD). Cell culture reagents as well as tyr-phostin, RCAM-lysozyme (5-amino-(N-2-5-diarylbenzoyl)-N’-2-hydroxybenzyl-salicylic acid (lavendustin A) and calphostin C were from Gibco-BRL (Gaithersburg, MD). CHAPS detergent, ATP, DTT, nocardazole, vinblastine, heparin, pepstatin A, leupeptin, N-ethylmaleimide (NEM), ethyleneglycol-bis-(B-aminoethyl)-N,N,N’,N’-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical CO (St Louis, MO). Taxol was a generous gift from Dr Matthew Sufness, National Cancer Institute, NIH. Olomoucine was from Promega (Madison, WI). Protease inhibitor Pefablock was purchased from Boehringer Mannheim Corp. (Indianapolis, IN). p133pp1 beads and the PMP-2 antibody were provided by Upstate Biotechnology Inc. (Lake Placid, NY). 4’,5’,7-tri-hydroxyisoflavone (genistein), 2-aminopurine, K-252a, staurosporine, microcystin LR and protein phosphatase 1 were from Calbiochem Novabiochem (San Diego, CA). Centricon-100, Centriprep-50 and Centriprep-10 concentrations were purchased from Amicon Inc. (Beverly, MA). For immunoreactions, Cy3-conjugated goat anti-mouse, fluorescein-conjugated goat anti-human and horseradish peroxidase-conjugated anti-mouse secondary antibodies were from Jackson Immunoresearch Lab. (West Grove, PA). The DNA stain Yo-pro-1 (Molecular Probes, Inc., Eugene OR) and Vectashield mounting medium (Vector Labs, Inc., Burlingame, CA) were also used. Immunoblots were developed with Supersignal ChemiLuminescence-HRP Substrate System (Pierce, Rockford, IL). Okadaic acid was purchased from LC Laboratories (Woburn, MA). Rabbit polyclonal anti-polo-like kinase (PLK) antibodies and recombinant Protein G-Sepharose were from Zymed Lab. Inc. (San Francisco, CA). Purified topoII was from Topogen (Columbus, OH). For the FPLC fractionation, the 5×5 MonoQ column was obtained from Pharmacia Biotech (Piscataway, NJ).

**Cell culture and preparation of cell extracts**

PtK1 cells were grown in MEM medium supplemented with 10% (v/v) fetal bovine serum, 20 mM Hepes, 0.1 mM non-essential amino acids, 1 mM pyruvate, 60 μg/ml penicillin and 100 μg/ml streptomycin. Cells were plated on 18 × 18 mm glass cover slips and grown to approximately 70% confluence in a humidified incubator with 5% CO2. In experiments in which nocardazole (16 μM), vinblastine (10 μM) and taxol (5 μM) were used, PtK1 cells were incubated for 3 hours in the presence of microtubule poisons before the reprophosphorylation assay was performed. HeLa S3 cells were grown in a 1 litre spinner flask at a density of 5×105 cells/ml in DMEM containing 5% (v/v) bovine calf serum, 20 μg/ml gentamicin, 20 mM Hepes, 0.1 mM non-essential amino acids and pluronic F68 0.05% (w/v). Cells were blocked in mitosis by adding 5 μM vinblastine 18 hours before harvesting. This procedure resulted in a mitotic index of 80% as judged by fluorescence labeling of DNA. To prepare S-phase cell extract, HeLa cells were blocked with 1 μg/ml aphidicolin for 18 hours before harvesting. Cells were centrifuged at 300 g, rinsed twice in MBS (10 mM MOPS, pH 7.4, 150 mM NaCl), then rinsed once in mitotic extraction buffer (MEB): 50 mM Tris-HCl, pH 7.3, 50 mM KCl, 10 mM MgCl2, 10 mM EGTA, 1 mM diithiothreitol (DTT). Cells were resuspended in MEB (5 ml per 1 ml pelleted cells), incubated 20 minutes on ice and then lysed with a Dounce homogenizer (pestle A, about 100 strokes). Homogenate was spun down 10 minutes at 1,000 g. The pellet was discarded, and supernatant centrifuged for 1 hour at 140,000 g. The high speed supernatant, termed S2, was collected, flash frozen and stored at −70°C.

Partial purification of the mitotic HeLa cell extract was accomplished by precipitation with ammonium sulphate, ultrafiltration through a 100 kDa cut-off membrane and chromatography with a MonoQ column.

**Chromosome isolation**

HeLa cells were grown in suspension at the same conditions described above. Cells were collected for 16-18 hours in 0.15 μg/ml colcemid. Cells were collected by centrifugation at 300 g for 5 minutes, and...
pellet rinsed twice in 50 ml swelling buffer (10 mM Hepes, 40 mM KCl, 5 mM EGTA, 4 mM MgSO₄, pH 7.4, in the presence of protease inhibitors as described above). Pelleted cells were checked for mitotic index, always above 75% as judged by fluorescence labeling of DNA. Swollen and aggregates were washed by repetitive pipetting in 10 ml extraction/lysis buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 4 mM MgSO₄, pH 6.9, in the presence of 1 mM DTT, 1% CHAPS detergent and protease inhibitors). When indicated, 200 nM microcystin was used. The suspension was centrifuged at 64 g for 5 minutes, and supernatant containing chromosomes was saved. In order to get the majority of chromosomes free in the supernatants, lysis and centrifugation steps were repeated 4 times. The collected supernatants were then centrifuged for 7 minutes at 200 g to pellet the few remaining interphase nuclei and large pieces of cell debris. The supernatant was centrifuged again for 10 minutes at 1,600 g to pellet chromosomes. The pellet was washed three times in extraction lysis buffer, and resuspended in 7 ml of the same buffer. One aliquot of the chromosome suspension was centrifuged for 10 minutes containing 10 mM NEM for 20 minutes at room temperature to inactivate chromosome kinases, and further processed as described below. A 40/80% glycerol step-gradient was used to purify chromosomes. The suspension above was layered on top of the gradient and centrifuged at 2,700 g for 30 minutes. Most other organelles and soluble molecules remain above or within the 40% glycerol during centrifugation of the chromosomes through glycerol step-gradient. Chromosomes were collected from the 40/80% interphase and within the 80% glycerol. The suspension was washed twice in buffer, flash frozen and stored at −70°C.

Purification of NIMA protein kinase from Escherichia coli

BL21(DE3)pLYS S E. coli cells containing pET-21a-NIMA (Ye et al., 1995) were cultured in NYZ medium with 34 µg/ml chloramphenicol and 200 µg/ml carbenicillin at 37°C. Cells were harvested by centrifugation when the OD600 nm of the culture reached 0.4-0.6. Bacteria were washed by centrifugation and resuspended in fresh NYZ medium. Expression of recombinant NIMA was induced by addition of IPTG to a final concentration of 0.4 mM and shaking the culture at 21°C for 3.5 hours. The induced cells were then harvested by centrifugation and frozen in liquid nitrogen. Soluble cell extract was prepared in binding buffer from the His-Bind™ buffer kit (Novagen) with addition of protease inhibitors (10 µg/ml leupeptin, 10 µg/ml soybean chymotrypsin/trypsin protease inhibitor, 50 µg/ml PMSF and 10 µg/ml aprotinin). NIMA protein kinase was purified using the His-Bind™ protein purification system (Novagen). After elution, fractions containing NIMA were determined by kinase assay and western blot. The purified NIMA protein was washed, concentrated by centrifugation in a Centricon concentrator (molecular mass cut-off: 30,000), and stored in phosphate buffer (pH 7.2 with 2.5 KCl, 10 mM MgSO₄, 0.1 mM EGTA, 20% glycerol and protease inhibitors).

Immunodepletion of mitotic HeLa cell extract

To investigate if it was possible to deplete the kinase activity from the mitotic HeLa cell extract, immunoprecipitation was performed by using either p133 nucleic or anti-PLK antibodies. GST-p133 nucleic beads were used to precipitate p34cdc2 out of mitotic HeLa cell extract. The mitotic extract diluted 1:10 in TM was incubated for 2 hours on a roller in the presence of 6.2 mg/ml GST-p133 nucleic. Immunocomplexes were pelleted, rinsed and stored at −70°C. Supernatant was used in the rephosphorylation assay to test for the presence of MPM-2 kinase activity.

Immunoprecipitation of PLK from mitotic HeLa cell extract was performed as follows. 50 µl Protein G bead slurries were washed three times in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, pH 8.0) and prebound to 0.2 µg/µl anti-PLK antibodies for 2 hours while rocking at 4°C. Beads were then washed three times in TM, and added to 500 µl of mitotic HeLa cell extract diluted 1:10 in TM in the presence of 0.5% NP-40, for 2 hours on a roller at 4°C. Immunocomplexes were pelleted, rinsed in TM and stored at −70°C. Supernatants were used in the rephosphorylation assay to test for MPM-2 kinase activity. The pellet and the immunodepleted supernatant were analyzed by electrophoresis and immunoblotting to verify the complete depletion of PLK. Immunoprecipitation by using beads alone was carried out as control.

Kinetochore rephosphorylation assay

Throughout the rephosphorylation assay, buffers were maintained at 37°C. PtK1 cells were rinsed briefly in 50 mM Tris-HCl, 5 mM MgSO₄, pH 7.4 (TM buffer) and extracted for 4 minutes in TM buffer containing 1% CHAPS detergent. The coverslips were then rinsed and incubated in TM buffer for 7 minutes to allow dephosphorylation of kinetochores by endogenous phosphatases. The kinetochore rephosphorylation reaction was then performed by inverting coverslips onto 50 µl drops of TM containing 670 nM microcystin LR, 1.7 mM ATP, and 1.7 mM DTT. The coverslips were allowed to rephosphorylate for 20 minutes at 37°C in a humidified chamber. To test whether the disassembly of microtubules would affect the expression of the MPM-2 phosphoepitope, the endogenous rephosphorylation assay was also carried out on PtK1 cells treated with vinblastine for 3 hours before lysis.

Experiments to understand which protein phosphatase was responsible for kinetochore dephosphorylation were also carried out. PtK1 cells were extracted for 4 minutes in TM containing 1% CHAPS, in the presence of either microcystin LR or okadaic acid (1, 10, 100 and 1,000 nM), and immediately fixed as described below.

In order to test kinase inhibitors, the assay was conducted very similarly to the rephosphorylation assay, with the inhibitors being included directly in the rephosphorylation mixture. The reagents tested were: heparin (1 µM), ethylenglycol-bis-(B-aminoethyl)-N,N′,N′,N′-tetraacetic acid (EGTA, 10 mM), ethylenediaminetetraacetic acid (EDTA, 5 mM); 4',5,7-trihydroxyisoflavone (genistein, 200 µM), 2-amino-panarine (10 mM), K-252a (20, 50, 100 µM), staurosporine (100, 1,000 nM); tyrphostin (200 µM), RCAM-lysozyme (10 µg/ml), (5-amino-[N'2,3-dihydroxybenzyl]-N'-2-hydroxybenzyl-salicilic acid (lavendustin A, 5 µM), calphostin C (1 µM) and olomoucine (100 µM).

The rephosphorylation assay was further modified to test the ability of externally applied kinases to catalyze the rephosphorylation of kinetochores. Immediately before the rephosphorylation step, the coverslips were incubated in 5 mM NEM for 10 minutes to inactivate the kinase activity bound at the kinetochore. The coverslips were then rinsed 3 times in TM buffer, and inverted on drops of purified activated MAP kinase (0.2 ng/µl) (a gift from W. Weber, University of Virginia), casein kinase II (3 and 6 ng/µl) (a gift from D. Litchfield, University of Manitoba; New England Biolabs), p34cdc2-cyclin B (0.02 and 0.2 U/µl) (New England Biolabs), NIMA (41.5 ng/µl) or mitotic HeLa cell extract. Rephosphorylation buffers included 670 nM microcystin LR, 1.7 mM ATP, and 1.7 mM DTT. The coverslips were allowed to rephosphorylate for 20 minutes at 37°C in a humidified chamber. In some cases coverslips were treated with protein phosphatase 1 (PP1, 0.1 U/µl) before the rephosphorylation step, and then washed with TM containing microcystin and DTT. Phosphatase treatment reduced the background labelling of the kinetochores and thus increased the sensitivity of the rephosphorylation assay.

Cell fixation and immunofluorescence

Coverslips were briefly rinsed in TM buffer and then fixed in freshly prepared 1% formaldehyde in 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 4 mM MgSO₄, pH 6.9 for 12 minutes. Coverslips were then rinsed in MBS, pH 7.4, containing 0.05% Tween-20 (MBST). MPM-2 mouse ascites fluid was used at a 1/30,000 dilution in MBST with 5% (v/v) normal goat serum (NGS). When applied, anti-kinetochore antibodies (A CA, a gift from J. B. Rattner, University of California) were used at 1/2,000 dilution in MBST with 5% NGS. Coverslips were inverted onto 40 µl drops of the antibody solution and incubated at 37°C for 20 minutes. Afterwards, the coverslips were rinsed in 2 changes of MBST. The coverslips were then incubated at 37°C for 20 minutes on 40 µl drops of Cy3-conjugated goat anti-
mouse IgG, 2.5 μg/ml in 5% NGS/MBST. When ACA was used as primary antibodies, coverslips were incubated with fluorescein-conjugated goat anti-human IgG, 7.5 μg/ml in 5% NGS/MBST. The coverslips were then rinsed with 2 changes of MBST, and stained for 1 minute with the DNA stain Yo-Pro-1 at 0.2 μM in water or DAPI at 5 μg/ml. The coverslips were then rinsed in distilled water and mounted on slides with Vectashield mounting medium supplemented with 10 mM MgSO4. Images were taken using a Nikon inverted microscope configured for epifluorescence with a planapochromat x60 oil immersion 1.4 NA objective. An image intensifier and videorate CCD camera (Dage-MTI, Michigan City, IN) with Image-1 software (Universal Imaging, Media, PA) were used to digitize the images. Except where indicated in the figure legends, images in each figure were taken under identical exposure conditions and processed identically to allow comparison of the degree of labeling. Images were printed on a Kodak XLS 8600 PS dye sublimation printer.

**Kinase assay, electrophoresis and immunoblot analysis**

In order to compare the patterns of MPM-2 reactive proteins on chromosomes in vivo and after rephosphorylation, chromosomes extracted in the absence of phosphatase inhibitors were allowed to rephosphorylate in vitro. Isolated chromosomes (equivalent to approximately 3×10^3 cells), prepared in the absence of microcystin, were incubated at 37°C for 30 minutes in the presence of a phosphorylation buffer containing 1 mM ATP, 1 mM DTT, 200 nM microcystin and protease inhibitors (Pefablock, leupeptin, pepstatin A; each at 5 μg/ml). When indicated, NIMA (83 ng/μl) or the PLK-immunodepleted extract (2 μl) was incubated with the isolated chromosomes previously treated with NEM to inactivate endogenous chromosome kinases. For controls, equal amounts of isolated chromosomes, prepared with or without microcystin, were incubated in the identical buffer without added kinases or cell extract.

NIMA kinase, mitotic HeLa cell extract and FPLC fractions from HeLa extract were tested for their ability to create the MPM-2 epitope on topoII. Purified topoII (15 ng/μl) was incubated with NIMA (83 ng/μl), mitotic HeLa cell extract or the FPLC fractions at 37°C for 30 minutes in the presence of a phosphorylation buffer containing 1 mM ATP, 1 mM DTT, 200 nM microcystin and protease inhibitors. To test NIMA kinase activity, NIMA was incubated for 20 minutes with 100 μg β-casein and topoII in the presence of 25 μCi [32P]ATP.

After the phosphorylation reactions, 2x sodium dodecyl sulfate (SDS) sample buffer was added (1:1, v/v) to samples and heated to 70°C for 5 minutes. Protein samples were then analyzed by electrophoresis on 5-20% gradient SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Autoradiography was performed at this point for the NIMA kinase assay. For western analysis, blots were blocked overnight in 5% BSA/TBST (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween-20) at 4°C. MPM-2 antibody was used to immunostain blots at 1/20,000 dilution of ascites fluid in TBST for 2 hours on a rocker at room temperature. After rinsing 3 times for 5 minutes, blots were incubated with 30 ng/ml antimouse horseradish peroxidase-conjugated secondary antibody in TBST for 90 minutes on a rocker at room temperature. After rinsing as above, immunoblots were developed with Supersignal Chemiluminescence-HRP Substrate System.

**RESULTS**

**Dephosphorylation and endogenous rephosphorylation of the MPM-2 phosphoepitope**

Mitotic PtK1 cells that were detergent extracted in the presence of the phosphatase inhibitor microcystin and labeled with MPM-2 displayed strong kinetochore labeling (Fig. 1A,B). If the cells were extracted without microcystin and allowed to incubate in TM buffer, the bright kinetochore labeling disappeared completely (Fig. 1C,D). When the extracted, dephos-

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**Fig. 1.** Immunofluorescence labeling of the MPM-2 phosphoepitope in prometaphase PtK1 cells. Cells were extracted in the presence of the phosphatase inhibitor microcystin (MC) (A,B), or in the absence of microcystin (C,D). The MPM-2 phosphoepitope at kinetochores is lost when cells are extracted in the absence of phosphatase inhibitor (C,D). Kinetochore-bound MPM-2 kinases can recreate the MPM-2 phosphoepitope at kinetochores in the presence of ATP and microcystin either in untreated (E,F) or in vincristine-treated (G,H) cells. Spindle poles (arrows) are resistant to dephosphorylation (D). Cells were fixed, labeled by indirect immunofluorescence with the MPM-2 antibody and counterstained with the DNA dye Yo-Pro. Images B, D and F were taken under identical conditions. Image H was obtained in a separate experiment. Bar, 5 μm.
phorylated cells were incubated with ATP, the reducing agent DTT, and the phosphatase inhibitor microcystin. MPM-2 labeling of the kinetochores was restored (Fig. 1E,F). Cells treated with the microtubule-disrupting agent vinblastine before extraction showed also robust kinetochore rephosphorylation (Fig. 1G,H). The spindle poles were relatively insensitive to dephosphorylation (Fig. 1D, arrows). Moreover, they did not exhibit any noticeable increase in MPM-2 labeling after the dephosphorylation step. Chromosome arms were weakly labeled by the MPM-2 antibody (Fig. 1B,D,F,H), with no remarkable difference after extraction in the presence or absence of microcystin. In order to identify the protein phosphatase involved in the dephosphorylation of the MPM-2 epitope, okadaic acid, which inhibits type 2A protein phosphatases more effectively than type 1 protein phosphatases, and microcystin, which inhibits both types, were used. Results, shown in Table 1, indicate that microcystin was more effective than okadaic acid in inhibiting the dephosphorylation of the MPM-2 epitope at kinetochores. The MPM-2 epitope was preserved only in the presence of 1 μM okadaic acid, while 10 nM microcystin was sufficient to achieve strong MPM-2 labeling.

In most cases, the pattern of rephosphorylation of the MPM-2 epitope at kinetochores was similar to the in vivo labeling seen after extraction in the presence of microcystin (Fig. 2A,B). Interestingly, in late anaphase cells the MPM-2 labeling after rephosphorylation showed higher intensity compared to anaphase cells labeled before dephosphorylation (Fig. 2A,B). Although MPM-2 labeling at kinetochores was evident after only 5 minutes of rephosphorylation, robust rephosphorylation was achieved in 20 minutes at 37°C.

The immunofluorescence data were consistent with the pattern shown by the western blot analysis on isolated chromosomes (Fig. 3). Chromosomes isolated in the presence of microcystin showed distinct bands (Fig. 3, w/MC) that are lost when chromosomes are extracted in the absence of microcystin.
Characterization of kinases responsible for the rephosphorylation of the MPM-2 phosphoepitope

The kinetochore rephosphorylation assay described above was further modified, allowing us to test exogenously applied kinases for their ability to create the MPM-2 phosphoepitope. As noted,

(Fig. 3, w/oMC). When in vitro rephosphorylation of dephosphorylated chromosomes (i.e. chromosomes extracted in the absence of phosphatase inhibitors) was carried out, some of the MPM-2 reactive bands reappeared (Fig. 3, rephos).

The kinetochore-bound MPM-2 kinase was inhibited only by kinase inhibitors of broad specificity

In order to characterize the endogenous kinase that could create the MPM-2 epitope at kinetochores, kinase inhibitors and other chemicals were included in the rephosphorylation procedure described above. Microcystin was included in the rephosphorylation mixture to ensure that endogenous phosphatases did not interfere with the assay. The results of these experiments are indicated in Table 2. At low concentrations, staurosporine, K-252a, calphostin C, heparin, and olomoucine all failed to inhibit rephosphorylation. Collectively at these concentrations these compounds should inhibit cyclin dependent kinases, calcium-calmodulin dependent protein kinases I and II, myosin light chain kinase, casein kinase II, protein kinases A, C and G, and MAP kinase. Thus, it is unlikely that any of these kinases are the endogenous kinase responsible for rephosphorylation of the MPM-2 phosphoepitope. Additionally, the specific tyrosine kinase inhibitors genistein, lavendustin, RCAM-lysozyme, and tyrphostin all failed to inhibit the rephosphorylation supporting previous data that MPM-2 phosphoepitope contains phosphothreonine (Zhao et al., 1989; Taagepera et al., 1994; Westendorf et al., 1994; Ding et al., 1997). The rephosphorylation reaction was blocked under conditions of general kinase inhibition. These conditions included the addition of 2-aminopurine, and high concentrations of staurosporine (1 μM) and K-252a (100 μM). At these concentrations staurosporine and K-252a lose their specificity and inhibit many kinases. Brief pretreatment of the extracted cells with 5 mM NEM and 5 mM DTNB, two sulfhydryl oxidizing agents, also inhibited rephosphorylation, even after washing away the reagents. These results suggested that NEM and DTNB could permanently inactivate the kinetochore-bound kinase. Furthermore, the kinase was not able to use GTP as an alternative for ATP. Inclusion of 5 mM EDTA, but not 10 mM EGTA, inhibited rephosphorylation, indicating that kinase activity at kinetochores requires magnesium but not calcium.

Characterization of kinases responsible for the rephosphorylation of the MPM-2 phosphoepitope

The kinetochore rephosphorylation assay described above was further modified, allowing us to test exogenously applied kinases for their ability to create the MPM-2 phosphoepitope. As noted,
extract, spindle poles did not undergo dephosphorylation or rephosphorylation, as shown in Fig. 4. Interestingly, PtK1 cells rephosphorylated in the presence of mitotic HeLa cell extract showed brighter MPM-2 labeling at chromosome arms (Fig. 4, HeLa extract + ATP) with respect to control cells (Fig. 4, buffer + ATP).

Typically, mitotic HeLa cell extracts (prepared under the conditions described in Materials and Methods) could be diluted 1,000-fold and still catalyze kinetochore rephosphorylation. Full strength extracts from S-phase cells showed some activity in rephosphorylating kinetochores but this was lost if the S-phase extracts were diluted only 10-fold (not shown).

Fig. 4. Mitotic HeLa cell extract can catalyze the rephosphorylation of kinetochores. PtK1 cells were detergent extracted without phosphatase inhibitors and incubated with NEM to inactivate endogenous kinases. Cells were then incubated with mitotic HeLa cell extract or in buffer in the presence of ATP. Kinetochores are rephosphorylated by soluble kinases present in the mitotic HeLa cell extract. As shown in previous figures, spindle poles are always intensely labeled both in treated and in control cells. Some increase in MPM-2 labeling at chromosome arms occurs in treated cells with respect to controls. Bar, 5 μm.
This evidence suggests that the MPM-2 kinase is less active or is present at lower concentrations in cells blocked in S-phase. PtK₁ cells blocked in a prometaphase-like state by microtubule drugs, either by microtubule-stabilizing agents such as taxol or microtubule-disrupting agents such as nocodazole and vinblastine, could also be rephosphorylated with mitotic cell extract at the same extent as untreated cells (data not shown).

To determine whether known kinases were able to regenerate the MPM-2 epitope on kinetochores, purified activated MAP kinase, p34cdc2-cyclin B, casein kinase II and NIMA were tested on dephosphorylated cellular substrates. MAP kinase, p34cdc2-cyclin B and casein kinase II failed to rephosphorylate the dephosphorylated MPM-2 epitope. NIMA was able to create the MPM-2 epitope at kinetochores when incubated with ATP, as shown in Fig. 5. However, the extent of the rephosphorylation by NIMA at kinetochores was less than the MPM-2 signal after rephosphorylation by the endogenous kinase or by M-phase extract. Control cells, either incubated with ATP without NIMA or with NIMA without ATP did not show any MPM-2 labeling at kinetochores. It has been shown that hyperphosphorylated NIMA is reactive to the MPM-2 antibody (Ye et al., 1995). For this reason we wished to determine whether the added NIMA became bound to kinetochores during the assay. After incubation with NIMA, cytoskeletons were washed and subsequently incubated with ATP. Kinetochores were not able to be rephosphorylated under these conditions, suggesting that NIMA does not bind to kinetochores. These data indicate that NIMA kinase activity creates the MPM-2 epitope at kinetochores, without representing an MPM-2 antigen bound to kinetochores. Since it was less effective than mitotic extract even after extended incubation, it may be only partly responsible for the rephosphorylation at kinetochores. Alternatively, the *Aspergillus* NIMA kinase expressed in bacteria may not efficiently recognize the PtK₁ cell substrate protein. In vitro rephosphorylation on isolated chromosomes by NIMA did not show any specific patterns of regenerated MPM-2 epitopes (see Fig. 10A).

Mitotic extract depleted of cyclin-dependent kinases (CDKs) with p13{sub}1-bound agarose beads did not have reduced MPM-2 kinase activity. Mitotic HeLa cell extract was also immunodepleted with antibodies to PLK. The anti-PLK antibodies detected a band at approx. 68 kDa corresponding to PLK, as well as a cross reactive protein of unknown identity at around 46 kDa (Fig. 6A). The immunoprecipitation with this antibody out of mitotic HeLa cell extract left an undetectable amount of PLK in the supernatant (Fig. 6A). When the kin-...
Kinetochores and topoII rephosphorylation

Phosphorylation of the MPM-2 epitope on topoII

Since it is known that topoII is a major protein recognized by the MPM-2 antibody on mouse chromosomes (Taagepera et al., 1993), we wanted to investigate if topoII could be rephosphorylated by the mitotic HeLa cell extract. Western blot analysis showed that the MPM-2 reactivity of topoII increased after incubation with the mitotic cell extract and ATP (Fig. 9A). This suggests that mitotic HeLa cell extract contained soluble kinases able to phosphorylate topoII and generate the MPM-2 epitope. In order to characterize topoII kinases in the mitotic cell extract and understand if the same kinase was also able to rephosphorylate kinetochores or chromosome arms in our exogenous rephosphorylation assay, several fractions from MonoQ chromatography of mitotic HeLa cell extract were incubated with ATP to rephosphorylate topoII. The products were run on a PAGE gel and blotted with MPM-2 antibody (Fig. 9B). Only one fraction was able to rephosphorylate the topoII (fraction 4, 0.5-0.6 M NaCl eluate), and this was the same fraction that catalyzed the appearance of MPM-2 labeling on chromosome arms in the kinetochore rephosphorylation assay. The flowthrough (fraction 1), which was able to rephosphorylate kinetochores, did not rephosphorylate topoII in solution.

In order to identify if topoII is a target protein rephosphorylated by NIMA, we incubated NIMA with topoII and the rephosphorylation mixture. Western blotting with MPM-2 ab (Fig. 10B) showed that NIMA did not rephosphorylate topoII in the MPM-2 site. A kinase assay using [32P]ATP (Fig. 10C) showed that NIMA was active and able to rephosphorylate β-casein as substrate, but unable to rephosphorylate topoII.

Fig. 7. Anti-PLK antibodies immunodeplete MPM-2 kinase activity. Mitotic HeLa cell extract was incubated with protein-beads alone or immunoprecipitated with beads and anti-PLK antibody. Supernatants were tested for MPM-2 kinase activity. The incubation with the PLK immunodepleted sample shows reduced MPM-2 kinase activity at kinetochores (G, arrowhead), while incubation with control beads maintains full kinase activity (H). Rephosphorylation with buffer and ATP alone is also shown (I). (A,B,C) DNA was counterstained with DAPI; (D,E,F) anti-kinetochore antibodies (ACA). Bar, 5 μm.
Phosphoepitope antibodies are useful tools to study groups of proteins that share consensus phosphorylation domains, providing more specificity in contrast to the traditional radioactive incorporation of phosphate. The MPM-2 phosphoepitope antibody labels mitosis-specific and cell cycle-regulated phosphoproteins localized at chromosome arms, spindle poles, midbody and kinetochores (Davis et al., 1983; Vandre et al., 1984; Engle et al., 1988; Zhao et al., 1989; Hirano and Mitchison, 1991; Vandre et al., 1991; Taagepera et al., 1993, 1995). Kinetochores play a fundamental role in regulating the checkpoint system that monitors the metaphase-anaphase transition at mitosis (reviewed by Gorbsky, 1995). The signaling pathway prevents chromosome segregation at anaphase until all the chromosomes have reached the metaphase plate. Rieder and colleagues (1994, 1995) recently demonstrated that a single unattached kinetochore can inhibit anaphase onset, and destruction of the last unattached kinetochore can relieve this block, allowing the cell to complete mitosis. Still largely unknown is the signaling pathway that activates the kinetochore checkpoint. Tension alters the phosphorylation status of kinetochore proteins, such as the cell cycle-regulated dephosphorylation of proteins recognized by the 3F3/2 antibody (Li and Nicklas, 1995; Nicklas et al., 1995). Work done in our laboratory with 3F3/2 antibody has shown that 3F3/2 phosphoepitope is an integral part of the metaphase-anaphase checkpoint (Campbell and Gorbsky, 1995). PtK1 kinetochores are brightly labeled with 3F3/2 antibody in prophase and prometaphase, but this epitope needed to be dephosphorylated for anaphase onset to occur. The fact that MPM-2 labeling also undergoes a dramatic change as the cell progresses through mitosis suggests that phosphorylation and dephosphorylation of the MPM-2 phosphoepitope may also play an important role in regulation of mitosis or meiosis.

We have developed a unique protocol for creating the MPM-2 phosphoepitope by dephosphorylating the dephosphorylated epitope present at the kinetochores of mitotic PtK1 cells. Since MPM-2 only recognizes phosphorylated proteins, the absence of kinetochore labeling when cells are extracted without phosphatase inhibitors would suggest that the MPM-2 phosphoepitope has been either dephosphorylated by endogenous phosphatases or detached from kinetochores. The reappearance of kinetochore labeling in rephosphorylation conditions suggests that protein(s) on which the MPM-2 phosphorylation occur are retained at kinetochores. Furthermore, this rephosphorylation occurs in cells that have been detergent extracted and washed several times, suggesting that endogenous MPM-2 kinases must also be bound at the kinetochore. Western blots indeed confirm these data, showing that dephosphorylated isolated chromosomes can regenerate the MPM-2 patterns of phosphoprotein after rephosphorylation in vitro.

The rephosphorylation and appearance of the MPM-2 epitope provides some clues about the regulation of kinetochore-bound kinases. Kinetochore kinases are bound and able to rephosphorylate kinetochores from prophase through anaphase. How the kinases are turned off in late anaphase is unknown. The immunofluorescence data and the western blot analysis demonstrate the striking similarity between the MPM-2 labeling in vivo and after rephosphorylation, supporting the idea that the endogenous kinase that is phosphorylating the MPM-2 epitope also creates MPM-2 reactive phosphorylations in vivo.

Mitotic HeLa cell extract contains highly active kinases that catalyze the phosphorylation of the MPM-2 epitope at kineto-
We do not know yet if the kinetochore-bound kinases and the soluble kinases present in the mitotic HeLa cell are similar molecules. One explanation is that the soluble and insoluble activities represent two distinct pools of the same kinase, one cytoplasmic and the other bound at kinetochores. The soluble kinetochore kinases are cell cycle-regulated, since the highest MPM-2 kinase activity at kinetochores was recovered in cell extracts from HeLa cells blocked in mitosis. On the other hand we find low levels of the kinase activity in S-phase cells. This suggests that MPM-2 kinases need further processing or production after S phase to be fully active in mitosis.

Microcystin-LR, a cyclic heptapeptide isolated from freshwater cyanobacteria, is a potent inhibitor of both PP1 and PP2a phosphatases (MacKintosh et al., 1990). In our experiments, microcystin was used to preserve the MPM-2 epitope from dephosphorylation, and we show that it effectively inhibited the dephosphorylation of MPM-2 epitope at very low concentrations. Okadaic acid was not equally effective in preventing kinetochore dephosphorylation. PP1 is located in chromosomes in mitotic cells (Fernandez et al., 1992), and our recent immunolabeling data using an anti-PP1 antibody shows that this phosphatase is concentrated at kinetochores (D. L. Brautigan and G. J. Gorbsky, unpublished data). Together, these results suggest that PP1 is likely the phosphatase responsible for kinetochore dephosphorylation on cytoskeletons of PtK1 cells.

Ultrastructural immunolabeling studies indicate that MPM-2 labeling is concentrated in the inner and the outer dense plaque of kinetochores (Taagepera et al., 1995). Our experiments in the presence of microtubule-stabilizing and -disrupting agents indicate that dephosphorylation of the MPM-2 epitope occurs in cell with disrupted microtubules. This kinetochore-bound kinases do not require interaction with microtubules for catalyzing expression of the MPM-2 phosphoepitope.

MPM-2 also labels midbody and spindle poles, as reported also by Vandere et al. (1984). Epitopes present at the spindle poles and midbody do not dephosphorylate when cells are detergent extracted without phosphatase inhibitors. Furthermore, these structures do not show any difference in MPM-2 labeling when dephosphorylation occurs. These results suggest that phosphorylations of midbody and spindle poles may be associated with different proteins compared to the phosphorylations at the kinetochore, or that they are less accessible to endogenous phosphatases. Chromosome arms also show MPM-2 labeling (Taagepera et al., 1993, 1995). We report here that the intensity of labeling at chromosome arms increases with respect to the control cells when mitotic HeLa cell extract is used in the exogenous dephosphorylation assay. In fact, the dephosphorylation of the cellular substrate achieved using PPI treatment in this assay allows a better dephosphorylation at chromosome arms by kinases present in the incubation reaction.

The dephosphorylation protocol functions as a sensitive assay for learning about the kinases that phosphorylate the MPM-2 phosphoepitope. The results of this study indicate that the kinase is a magnesium-dependent serine-threonine kinase, and argue against the possibility that the phosphorylations are caused by purified MAP kinase, casein kinase II or CDC2 kinase. Additionally, this study suggests that calcium-calmodulin dependant protein kinases I and II, myosin light chain kinase, casein kinase II, or protein kinases A or C are unlikely to be responsible for the kinetochore dephosphorylation. Another possibility is that some of these kinases require accessory proteins or belong to a complex to be fully active and able to dephosphorylate kinetochores.

Interestingly, we found that anti-PLK antibodies can partially deplete MPM-2 activity from the mitotic HeLa cell extract. Recent data show that Ptx1, a Xenopus polo-like kinase, directly phosphorylates CDC25C (Kumagai and Dunphy, 1996). Since CDC25C is an MPM-2 antigen, we suspect that PLK plays a role in the phosphorylation of the MPM-2 epitope at kinetochores.

NIMA is a protein kinase isolated from Aspergillus nidulans required for mitotic progression (Osmani et al., 1991a). NIMA kinase activity is low in G1 but increases in S and G2 to reach
a maximal level in mitosis (Osmani et al., 1991b). The protein is then degraded before exit from mitosis (Pu and Osmani, 1995). NIMA can be phosphorylated and activated by p34<sup>cdc2</sup> (Ye et al., 1995). When hyperphosphorylated, NIMA shows some MPM-2 reactivity (Ye et al., 1995). NIMA induces premature mitosis in HeLa cells (Lu and Hunter, 1995), and NIMA-related kinases or Neks have been isolated in mammals (Letwin et al., 1992; Schultz and Nigg, 1993; Lu and Hunter, 1995). We show here that NIMA from Aspergillus can act as an MPM-2 kinase at kinetochores in PtK<sub>1</sub> cytoskeletons, although we do not know yet if a mammalian NIMA homolog is involved in the kinetochore phosphorylation. Our experiments testing the ability of NIMA to bind to kinetochores show that it is washed away after a quick rinse in buffer. This MPM-2 labeling at kinetochores is not due to NIMA binding to the kinetochores, but rather to phosphorylation of an endogenous kinetochore protein by the added NIMA kinase. Substrates of NIMA kinase are still unknown. So far we have no evidence that topOII is a NIMA substrate.

We show here that mitotic HeLa cell extract contains soluble MPM-2 kinases. The kinases that create the epitope at kinetochores and chromosome arms are distinct and can be separated by ion exchange chromatography. Further fractionation is ongoing. Nevertheless, we show here that one FPLC fraction, namely the flowthrough, can rephosphorylate kinetochores on lysed PtK<sub>1</sub> cells but is unable to phosphorylate topOII in solution. This indicates that the MPM-2 kinase at kinetochores is not a topOII kinase. This kinase could be a NIMA-related kinase in mammals. In contrast, the eluted fraction could rephosphorylate chromosome arms in cytoskeletons and topOII in solution, but is not able to rephosphorylate kinetochores. Literature data show that casein kinase II is a topOII kinase in vitro (Cardenas et al., 1993; Wells and Hickson, 1995), and in yeast it is associated with topOII (Cardenas et al., 1993). Other data report that protein kinase C (Wells et al., 1995), p34<sup>cdc2</sup> kinase and MAP kinase (Wells and Hickson, 1995) are able to phospha tyrilate topOII in vitro. In our exogenous rephosphorylation assay casein kinase II, p34<sup>cdc2</sup> or MAP kinase are unable to regenerate the MPM-2 phosphoepitope on kinetochores or chromosome arms. Our study with kinase inhibitors would also exclude a role for protein kinase C in rephosphorylating the MPM-2 epitope.

On the whole, the pattern of MPM-2 phosphorylation we described, together with other literature data, strongly suggest that the kinetochore complex is transiently activated/phosphorylated in a cell cycle-regulated manner. The identification and characterization of kinetochore-associated kinases and phosphatases should give us insight into the signal transduction pathway that controls mitotic chromosome assembly and segregation.

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