Proteins of the mammalian mitotic spindle: phosphorylation/dephosphorylation of MAP-4 during mitosis

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Summary

The phosphoprotein composition of isolated CHO spindles was analyzed using the MPM-1 and MPM-2 antibodies, which are reactive with a phosphorylated epitope enriched in mitotic cells and present on the centrosome, kinetochores, midbody and fibers of the mitotic spindle. Several high molecular weight phosphorylated spindle proteins were detected on immunoblots, including species of 410×10^3 M_r, 350×10^3 M_r, a $230–240\times10^3$ M_r doublet, 210×10^3 M_r and 120×10^3 M_r. The temporal and spatial distribution of the MPM-reactive phosphoproteins was determined by examining spindle structures isolated from cells at various stages of mitosis. The susceptibility of the staining pattern to extraction with salt, a procedure known to remove most microtubule-associated proteins (MAPs), was also examined.

The phosphorylated 210×10^3 M_r species was identified as MAP-4 and localized to the spindle fibers using (1) a polyclonal antibody raised against this species, that reacted with known MAPs, and (2) established MAP-4 antibodies that reacted with the spindle 210×10^3 M_r MPM-reactive proteins. The comparative immunoblot and immunofluorescence analysis establishes a cycle of phosphorylation/dephosphorylation of MAP-4 upon entry and exit from mitosis. Regarding the other MPM-reactive proteins, comparative immunofluorescence staining and immunoblot analysis of isolated spindle samples before and after salt extraction indicate that they may be constituents of the centrosome, kinetochores or midbody, but their definitive identification awaits the production of monospecific antibodies.

Key words: mitosis, spindle apparatus, phosphorylation, microtubule-associated proteins.

Introduction

Protein phosphorylation is a common mechanism utilized to regulate a variety of cellular processes. During mitosis, phosphorylation of the nuclear lamina proteins is directly involved in nuclear envelope breakdown (Gerace and Blobel, 1980), and phosphorylation of histones (Gurley *et al.* 1978) and vimentin (Evans and Fink, 1982) may be involved in chromosome condensation and rearrangement of intermediate filaments, respectively. Protein components of the mitotic spindle are also phosphorylated during mitosis. The monoclonal antibodies MPM-1 and MPM-2, which were prepared against mitotic HeLa cell extracts and were shown to identify a set of phosphoproteins present in mitotic cells (Davis *et al.* 1983), have been used to localize MPM-reactive phosphoproteins to the spindle apparatus and microtubule-organizing centers in a variety of cells in culture (Vandré *et al.* 1984). The appearance of the MPM-reactive phosphoproteins appears to be a common phenomenon in all eukaryotic cell types examined (Vandré *et al.* 1986) including *Drosophila* (Miller *et al.* 1987), *Paramecium* (Keryer *et al.* 1987), *Aspergillus* (Engle *et al.* 1988; Doonan and Morris, 1989), and diatoms (Wordeman *et al.* 1989). While the above proteins are clearly associated with the spindle, the majority have not been characterized biochemically, nor have their functional properties been elucidated.

The presence of these phosphoproteins does appear to be tightly coupled with mitotic events, however. Phosphorylation and subsequent dephosphorylation of centrosomal components during mitosis coincides with an increase and subsequent decrease in centrosomal microtubule nucleating capacity (Vandré and Borisy, 1985). Temperature-sensitive mutant cells of *Caenorhabditis elegans* that arrest prior to mitosis show no MPM immunoreactivity, but acquire MPM-reactive material within 20 min of temperature shiftdown (Hecht *et al.* 1987), and similarly, the appearance of MPM-reactive proteins has been associated with premature chromosome condensation at the restrictive temperature in the mutant cell line ts BN-2 (Yamashita *et al.* 1985). Following microinjection of the MPM-1 and MPM-2 antibodies, cultured cells showed a significant delay in exit from mitosis (Davis and Rao, 1987) and *Rana pipiens* and *Xenopus laevis* embryos were arrested in mitosis after failing to form spindles following chromosome condensation and germinal vesicle breakdown (Davis *et al.* 1989). Finally, the dephosphorylation of MPM-reactive centrosomal components has recently been

shown to be linked with the onset of anaphase in tissue culture cells (Vandré and Borisy, 1989*a*), and the reduction of microtubule nucleating capacity *in vitro* (Centonze and Borisy, 1990). Thus, the phosphorylation state of certain centrosomal components may be involved in the modulation of centrosome function and, similarly, the phosphorylation state of other spindle components may be involved in the regulation of spindle assembly, disassembly and function during mitosis.

In addition to tubulin, isolated microtubules contain a variety of microtubule-associated proteins or MAPs (reviewed by Olmsted, 1986). The major high molecular weight MAPs associated with microtubules isolated from brain tissue have been grouped into two classes designated MAP-1 and MAP-2. MAP-1 polypeptides have also been identified in cultured cells using specific monoclonal MAP-1 antibodies (Bloom *et al.* 1984; Wiche *et al.* 1984). However, the most abundant high molecular weight MAPs isolated from the microtubules of tissue culture cells are different from those found in brain tissue. Cytoplasmic MAPs ranging between 190 and 240×10^3 M_r have been identified in HeLa (Bulinski and Borisy, 1979), mouse neuroblastoma (Olmsted and Lyon, 1981), *Drosophila* (Goldstein *et al.* 1986), and pituitary cell lines (Bloom *et al.* 1985). These cytoplasmic MAPs may all be related to a family of MAPs designated MAP-4 (Parysek *et al.* 1984).

The immunolocalization of phosphorylated spindle proteins recognized by the MPM-1 and MPM-2 antibodies suggests that some of these phosphoproteins may be related to previously identified MAPs. In an effort to catalog further the molecular components of the spindle and, more importantly, to characterize a set of phosphoproteins that may regulate spindle function we have undertaken the identification of the MPM-reactive spindle-associated phosphoproteins. We report here on the temporal and spatial distribution of these phosphoproteins in taxol-stabilized spindles isolated from Chinese hamster ovary (CHO) cells.

Materials and methods

Cell culture

Chinese hamster ovary (CHO) and HeLa human epitheloid carcinoma cell lines were grown in Ham's F-10 medium supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), penicillin (100 i.u. ml^{-1}), and streptomycin (0.1 mg ml^{-1}).

Antibodies

Preparation and characterization of monoclonal antibodies MPM-1 and MPM-2 specific for mitotic cells have been described (Davis *et al.* 1983). Polyclonal rabbit serum prepared against CHO 210K MAP (Brady and Cabral, 1985) was a gift from R. C. Brady, Department of Internal Medicine, University of Texas Medical School, Houston, TX, USA. The preparation of polyclonal rabbit serum against HeLa 210K MAP (Bulinski and Borisy, 1980) was previously reported from our laboratory. Vimentin antibody was obtained from ICN Immunobiologicals (Lisle, IL) and the antibodies to the 300×10^3 M_r intermediate filament-associated protein were a gift from R. Goldman, Department of Cell Biology and Anatomy, Northwestern Medical School, Chicago, IL.

Polyclonal antisera were raised in rabbits against the individual bands cut from polyacrylamide gels of isolated CHO spindles (see below). Briefly, isolated spindles were separated on a SDS–6% PAGE (Laemmli, 1970) preparative gel. Vertical strips were sliced from the sides of the gel and the proteins were transferred to nitrocellulose (0.22 μm, Scleicher and Schuell, Inc., Keene, NH). The nitrocellulose strips were processed for MPM-antibody staining as described below. The remaining gel was stained with Coomassie Brilliant Blue R to visualize the proteins. The antibody-stained nitrocellulose strips were aligned with the stained gel and the protein bands were excised. The gel slices containing the bands were minced, rinsed with several changes of Tris-buffered saline (TBS: 0.9% NaCl, 10 mM Tris–HCl, pH 7.4), and equilibrated overnight with TBS at 4°C to remove acetic acid and methanol used for destaining the gel. The gel pieces were homogenized with TBS in a motor-driven glass/teflon homogenizer. The suspension of polyacrylamide was emulsified with an equal volume of Freund's adjuvant and injected into rabbits. The initial immunization was performed by inoculation directly into the popliteal lymph nodes (Sigel *et al.* 1983). Subsequent booster injections were given subcutaneously or intramuscularly. To screen for the production of monospecific antibodies, rabbits were bled from the ear vein and the collected serum was tested for its recognition of P210 on Western blots of isolated spindles.

Spindle and midbody preparation

Isolated mitotic spindles were prepared from CHO cells as previously described (Vandré *et al.* 1986) with slight modification. Mitotic CHO cells were incubated with taxol (Natural Products Branch, Division of Cancer Treatment, National Cancer Institute, USA) for 2 min following release from the nocodazole block of 10, 20, 30 or 45 min. The taxol-stabilized mitotic structures were then isolated in a 2 mM Pipes (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)) buffer containing 0.5% Triton X-100 and 5 μg ml^{-1} taxol. Mitotic structures were isolated from HeLa and BHK-21 cells following a similar procedure with appropriate changes in the length of the thymidine block and release to reflect the longer generation time of these cells (24 h) in comparison to the CHO cells (14 h). In some cases the following protease inhibitors were added to the isolation buffer: 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 30 μM N_α-*p*-tosyl-L-arginine methyl ester (TAME), and 1 μg ml^{-1} leupeptin, and immediately after cell lysis the phosphatase inhibitors NaF and β-glycerophosphate were added to a final concentration of 5 mM. In the case of salt extraction, isolated spindles were first pelleted out of the standard isolation buffer using an IEC clinical centrifuge, and were then resuspended in 2.0 mM Pipes buffer containing 10 μg ml^{-1} taxol, 0.02% Triton X-100 and 0.45 M NaCl. After extraction at room temperature for 12 min, the spindles were again pelleted and resuspended in fresh standard isolation buffer.

Comparative analysis of the immunofluorescence staining patterns and immunoblots of the spindles (isolated 10–12 min following release of the nocodazole block) and midbodies (isolated 45 min following release of the nocodazole block), which were either low-salt extracted or high-salt extracted, were carried out on samples prepared from fractions of the same synchronized mitotic cell population to minimize any differences that may have been associated with cell culture and isolation conditions.

Immunofluorescence staining

Isolated mitotic structures were mixed with fixative (0.7% glutaraldehyde) while in suspension in isolation buffer and immediately adsorbed onto polylysine-coated coverslips. After 15 min the coverslips were washed in phosphate-buffered saline (PBS) and treated with two changes of $NaBH_4$ (1 mg ml^{-1}) over 30 min. Following reduction, coverslips were rinsed in PBS and incubated with 2% normal goat serum (Gibco Laboratories Inc., Grand Island, NY) in PBS for 30 min at 37°C to block non-specific binding sites. Coverslips were then incubated with the appropriate primary and secondary antibodies for 60 and 30 min, respectively. Samples were thoroughly rinsed in PBS between each antibody incubation. Coverslips were washed in PBS, rinsed in distilled water, and mounted in 10% polyvinyl alcohol containing 2 mg ml^{-1} of the anti-bleaching agent *para*phenylene diamine. Spindles that had been salt extracted were processed in a similar manner.

To ensure that the observed differences in immunofluorescence localization were not a result of fixation artifacts, separate portions of each sample were fixed for 15 min in 5 mM ethylene glycol bis-(succinic acid *N*-hydroxy succinimide ester) (EGS),

5 mM EGS followed by postfixation for 5 min in −20 °C methanol, or 3.7 % formaldehyde. As with the samples fixed in glutaraldehyde, each fixative was added to the suspension of mitotic structures immediately prior to their application to the coverslip. The EGS (Sigma Chemical Co., St Louis, MO) was prepared as a 100 mM stock in dimethyl sulfoxide (DMSO), and was added directly from the stock solution to the suspension of mitotic structures to give the appropriate final concentration. Similarly, formaldehyde (Polysciences, Inc., Warrington, PA) was added directly from a 10 % stock solution. The use of EGS or formaldehyde eliminated the need for a sodium borohydride reduction step as required for samples fixed with glutaraldehyde. Fluorescein-conjugated goat anti-mouse immunoglobulin was obtained from Organon Teknika-Cappel (Malvern, PA), and fluorescein-conjugated goat anti-rabbit immunoglobulin from ICN Immunobiologicals (Lisle, IL). Immunolocalization using CHO cell cultures was as described (Vandré *et al.* 1986).

Microscopy

Mounted coverslips were examined with a Zeiss Universal microscope equipped with epifluorescence optics using either a ×63 phase, 1.4 NA planapochromat objective, or a ×100 phase, 1.3 NA Neofluar objective. Immunofluorescence micrographs were recorded using either Tri-X or Tech-Pan film.

Polyacrylamide gel electrophoresis and immunoblotting

Samples for electrophoresis were boiled in sodium dodecyl sulfate (SDS) sample buffer containing 10 % glycerol, 5 % 2-mercaptoethanol, 2.5 % SDS, 62.5 mM Tris–HCl at pH 6.8, and analyzed on SDS/polyacrylamide slab gels (7.5 %, w/v, acrylamide) according to the method of Laemmli (1970). Following electrophoresis the gel was either stained with Coomassie Brilliant Blue R to visualize proteins, or the proteins were transferred from the gel to nitrocellulose (Schleicher and Schuell, Inc., Keene, NH) with a 1.5 μm pore size. The initial transfer was overnight at 40 V in a Hoefer Transfor apparatus using a transfer buffer that contained 0.38 M glycine, 50 mM Tris–HCl, pH 8.3, 20 % methanol (v/v), and 0.05 % SDS. The SDS was removed by a secondary transfer in the above buffer without SDS for 1.5 h. Free protein binding sites on the nitrocellulose were blocked with 10 % (v/v) fetal bovine serum in TBS. The nitrocellulose transfer was incubated with primary antibody (MPM-1) overnight, rinsed with TBS, and incubated with secondary goat anti-mouse peroxidase-conjugated antibody (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) for 2.5 h. Bound secondary antibody was visualized with 4-chloro-1-naphthol.

To test the specificity of the MPM-1 antibody reaction for phosphoproteins, appropriate samples were separated on SDS/polyacrylamide slab gels and transferred to nitrocellulose as described above. Following an incubation with 10 % fetal bovine serum to block free protein binding sites, the nitrocellulose sheet was incubated for 2 h at room temperature in 50 mM Tris–HCl, pH 8.0, containing 2 mM $MnCl_2$ and 50 i.u. ml^{-1} intestinal alkaline phosphatase (Sigma Chemical Co., St Louis, MO). A similar sheet of nitrocellulose was processed simultaneously, but in addition the buffer contained 40 mM β-glycerophosphate to block the action of the alkaline phosphatase. The alkaline phosphatase-containing buffers were aspirated and the nitrocellulose rinsed in PBS prior to incubation with the MPM-1 antibody.

Results

Phosphoprotein composition of CHO cells and isolated spindles

While the MPM-1 and MPM-2 monoclonal antibodies have been shown to react with a number of phosphoproteins present in mitotic cells (Davis *et al.* 1983), it has not been clearly established whether the specificities of these two different monoclonal antibodies are identical in any reactive cell system. In order to determine the phosphoprotein specificity of the MPM-1 and MPM-2 antibodies, we initially examined interphase and mitotic populations of CHO cells by immunoblot analysis (Fig. 1). Each antibody reacted with at least 20 different proteins in the mitotic cell sample, but only a few proteins in the interphase sample. In preparations of isolated spindles, each antibody was reactive with a subset of these mitotic phosphoproteins (Fig. 2). Since the pattern of phosphoproteins recognized by the two antibodies was indistinguishable in comparable samples, we judged the MPM-1 and MPM-2 antibodies to have identical specificity, and they were used interchangeably for immunoblot analysis. However, MPM-1, an IgM class antibody, did not give immunofluorescence localization patterns as distinct as MPM-2, an IgG antibody (data not presented) and, therefore, the MPM-2 antibody was used routinely for immunofluorescence localization.

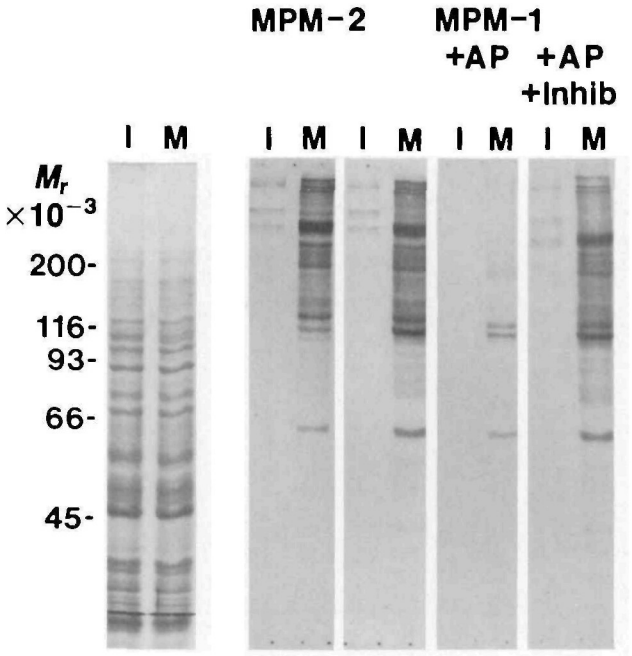

Fig. 1. Gel and immunoblot analysis of interphase and mitotic Chinese hamster ovary (CHO) cell lysates. Whole interphase (I) and mitotic (M) cells were dissolved in SDS sample buffer and the proteins were separated by SDS/polyacrylamide electrophoresis (7.5 % acrylamide). The resulting gels were either stained by Coomassie Blue (first two lanes) or transferred to nitrocellulose and probed with the MPM-1 or MPM-2 antibodies. The two antibodies gave identical immunoblots with the CHO cell lysates. Identical transfers were incubated with alkaline phosphatase (+AP) or alkaline phosphatase and β-glycerophosphate (+AP+Inhib) prior to incubation with the MPM-1 antibody. Pretreatment with alkaline phosphatase eliminated or greatly reduced the reaction with the MPM-1 antibody, indicating the specificity of the antibody for a phosphorylated epitope. Molecular weight standards used in this and subsequent figures were, myosin (200×10^3); β galactosidase (116×10^3); phosphorylase *b* (93×10^3); bovine serum albumin (66×10^3); and ovalbumin (45×10^3).

The reported specificity of the MPM antibodies for phosphoproteins (Davis *et al.* 1983) was confirmed in the CHO cell system by enzymatic removal of the phosphate groups. All of the protein bands reactive with the MPM antibodies were phosphatase sensitive (Figs 1 and 2). Although there was some variability in the sensitivity of selected protein bands to the action of the phosphatase as

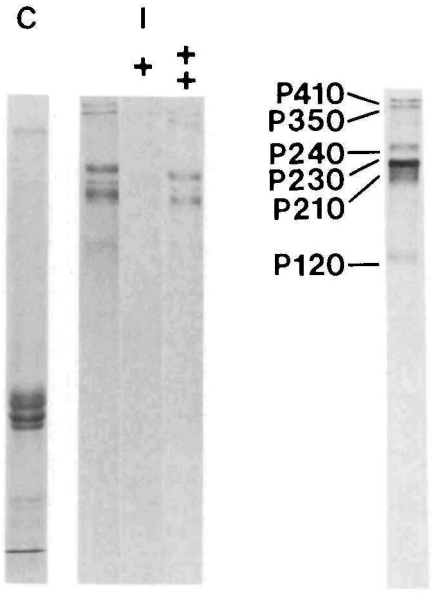

Fig. 2. Gel and immunblot analysis of isolated CHO spindles. Samples were analyzed as described for Fig. 1. Coomassie-stained proteins (C) and corresponding immunoblot (I) with the MPM-1 antibody. Transferred proteins not exposed to alkaline phosphatase (–), were compared to transfers either incubated with alkaline phosphatase alone (+) or alkaline phosphatase and β glycerophosphate (‡). The major MPM-reactive bands are indicated next to the single immunoblot lane of a CHO spindle preparation.

shown by the partial resistance of three mitotic phosphoprotein bands (Fig. 1), with longer incubation in phosphatase or higher phosphatase concentration, all of the MPM reactivity could be eliminated from the immunoblot (data not presented). When β-glycerophosphate was included during the incubation with alkaline phosphatase there was no loss in MPM reactivity (Figs 1 and 2). This confirmed that the loss of MPM reactivity was specific for the action of the phosphatase and not due to contaminating enzymatic or proteolytic activity. Similar phosphatase sensitivity was demonstrated for the MPM-2 antibody (data not presented).

The MPM antibodies recognized at least five discrete phosphoprotein bands in the prometaphase/metaphase spindle sample (Fig. 2). From the top of the transfer the MPM-reactive phosphoprotein bands consist of a single heavily stained band (P410$\times 10^3 M_r$), a single less intensely stained band (P350$\times 10^3 M_r$), a closely spaced doublet (P240 and P230$\times 10^3 M_r$, respectively), an intensely stained band (P210$\times 10^3 M_r$) with an associated region of staining distributed in a smear below the band, and the final lowest molecular weight band (P120). The region below the P210 band varied in staining intensity in different spindle preparations, and possibly reflected proteolytic degradation products. Variability in staining was also detected for other MPM-reactive bands especially the P120 band which in some preparations was absent (for example, Fig. 3). With the possible exception of P210, none of the MPM-reactive phosphoproteins corresponded to a clearly visible Coomassie-stained protein band on the polyacrylamide gel. Therefore, these phosphoproteins are minor protein components of the spindle. In contrast, a Coomassie-stained protein of approximately 300$\times 10^3 M_r$

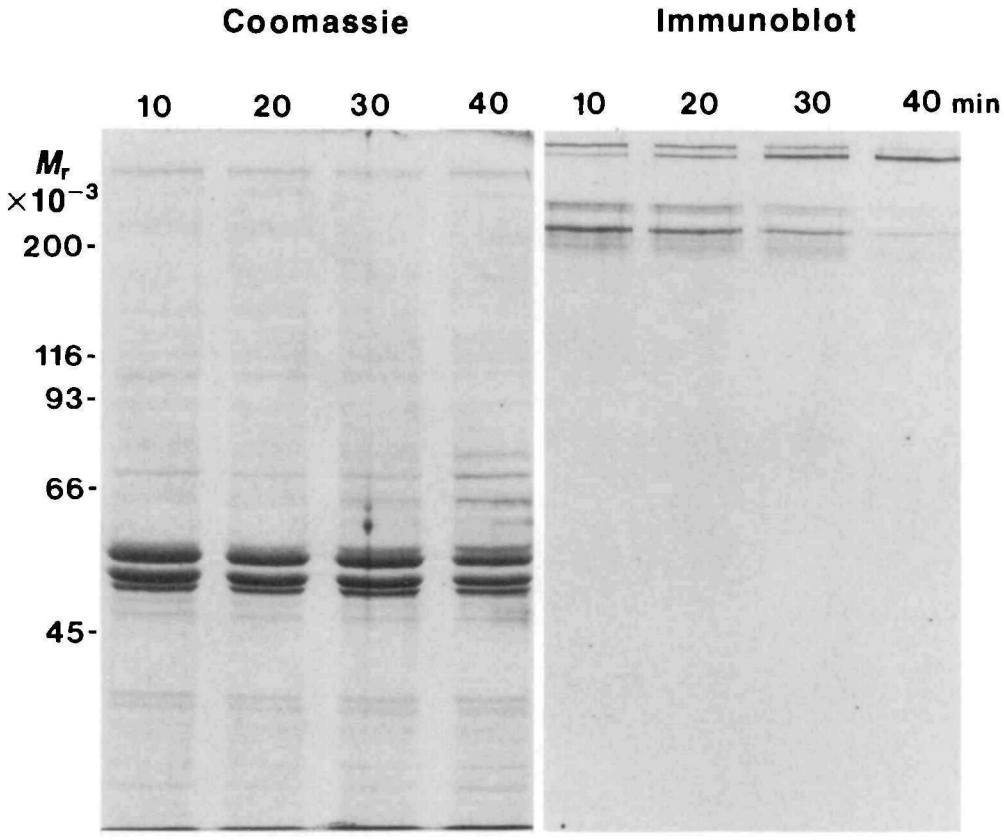

Fig. 3. Temporal distribution of MPM-reactive phosphoproteins in isolated mitotic structures. Taxol-stabilized spindle structures were prepared 10, 20, 30 and 45 min following release of the mitotic cells from a nocodazole block, corresponding to prometaphase/metaphase, metaphase/early anaphase, late anaphase and telophase structures, respectively. Polyacrylamide gels and immunoblots were prepared and the Coomassie Blue-stained protein pattern (Coomassie, lanes 10, 20, 30 and 45 min), and the pattern of MPM-1 reactivity (Immunoblot, lanes 10, 20, 30 and 45 min) were compared for each sample. In each sample the major Coomassie-stained protein bands correspond to the tubulin monomer subunits. The immunoblot showed a progressive change in the pattern of immunoreactive phosphoproteins. With the exception of a single band that increased in intensity in spindle structures isolated at later mitotic stages, the remainder of the identified bands in the prometaphase/metaphase sample decreased in staining intensity over the same period.

that was not reactive with the MPM antibodies was consistently detected in the isolated spindle preparations (Figs 2 and 3). The same set of phosphoprotein bands were detected on the immunoblots of isolated metaphase spindles regardless of whether protease or phosphatase inhibitors were included during their isolation.

Temporal distribution of mitotic phosphoproteins through mitosis

Spindles were isolated from synchronized populations of CHO cells at various times following their release from a nocodazole block. Time points of 10, 20, 30 and 45 min represented samples containing more than 80% prometaphase/metaphase, metaphase/early anaphase, anaphase and telophase spindles, respectively. The protein composition and immunoblot staining pattern of each spindle sample are compared in Fig. 3. The proteins detected by Coomassie Blue staining in each sample were similar. The most prominent bands were the α and β tubulin monomers, and in the high molecular weight region, a protein band of approximately 300×10^3 M_r. Some additional protein bands were present in the 45 min sample in comparison with the other spindle time points.

Comparison of the immunoblots (Fig. 3) with the immunofluorescence patterns (Fig. 4) of the different spindle samples revealed both a temporal and spatial change in the pattern of MPM-reactive phosphoprotein staining. In general, MPM reactivity decreased in samples prepared at progressively later times of mitosis (Fig. 3, lanes 10–45 min). However, P350 weakly stained in the prometaphase/metaphase spindle sample, demonstrated increased levels of staining in later mitotic stages. This band also decreased in staining as cells progressed into interphase (data not presented). In the telophase sample (Fig. 3 lane 45 min), P350 was the most prominent phosphoprotein band, possibly as a result of the differential stability of the protein, increased phosphorylation, and/or sensitivity of the phosphorylation site to phosphatase action. The temporal changes revealed by the immunoblot analysis correlated with changes in the spatial localization of the MPM-reactive phosphoproteins (Fig. 4). Discrete MPM-staining was associated with the spindle poles, microtubule fibers and spots near the midzone of spindles isolated 10 min after release from the nocodazole block (Fig. 4B). Although no chromosomes are present in these isolated spindles (Fig. 4A), the reactive spots represent a portion of the kinetochore that remained attached to the kinetochore fibers throughout the isolation procedure (Vandré and Borisy, unpublished results). The staining pattern of early anaphase spindles (Fig. 4D) was similar to that of metaphase spindles, with the exception that the kinetochores were no longer clearly visible, being obscured by the stronger fiber staining near the poles. In late anaphase spindles (Fig. 4E and F) the midzone was reactive, and in telophase samples (Fig. 4G and H) the staining became concentrated along the microtubule fibers on either side of the central dense matrix of the midbody. The progressive loss of centrosomal and kinetochore staining in later mitotic stages correlated with the general loss of MPM reactivity on immunoblots; in contrast, the increase in midbody staining correlated with increased P350 staining on the immunoblot. The non-MPM-reactive 300×10^3 M_r species persisted throughout the time course.

The mitotic spindle in cultured cells is known to be surrounded by a cage of intermediate filaments (Hynes and Destree, 1978). In our isolation procedure, the cage of intermediate filaments gradually breaks down after detergent lysis of the cells, releasing spindles, which are then collected by centrifugation. If the breakdown of the intermediate filament cage is not complete, they will contaminate the spindle preparations as verified directly by light microscopic examination and immunofluor-

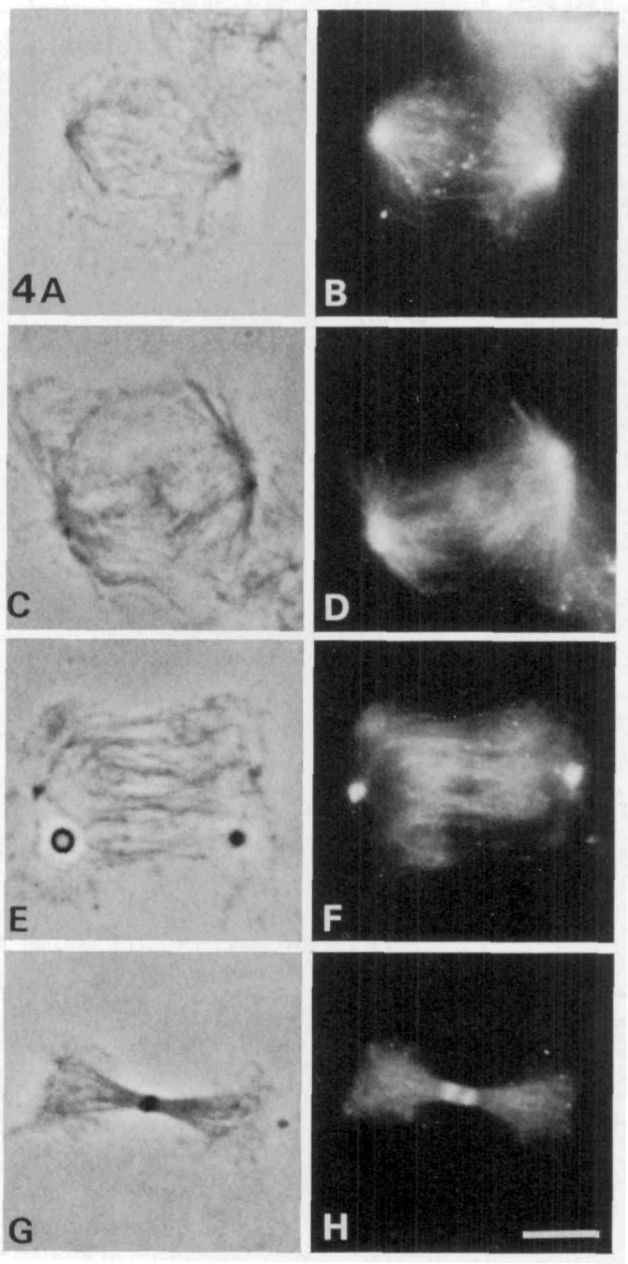

Fig. 4. Phase-contrast (A, C, E, G) and immunofluorescence staining (B, D, F, H) of mitotic structures isolated at varying times after nocodazole release. Samples correspond to those used for electrophoresis and immunoblotting shown in Fig. 3. Mitotic structures were stained with the MPM-2 monoclonal antibody. Staining was localized to the spindle poles, along spindle fibers, and at discrete spots located at the end of microtubule fibers (kinetochore remnants, see text) in the prometaphase/metaphase sample (10 min). Kinetochore reactivity was obscured by fiber staining at later stages of mitosis. Staining was localized to the midbody and along microtubule fibers associated with the midbody in the telophase sample (45 min). Bar, 5 μm.

escence with anti-vimentin antibodies (data not shown). To test further whether the proteins in the spindle preparation were bona fide components or persistent contaminants, sham isolations of spindles were performed using identical procedures but from cells not released from the nocodazole block. Under these conditions, no spindles are obtained, but residual components of the intermediate filament cage are collected. Gel electrophoretic and immunoblot analysis of the resulting pellet revealed low amounts of tubulin and MPM-2 reactivity consistent with the lack of spindles, but the $300\times10^3 M_r$ component persisted, suggesting that it was a contaminant. Immunoblots with polyclonal and monoclonal antibodies to the high molecular weight intermediate filament-associated protein (Yang *et al.* 1985) confirmed that this species is reactive (data not shown), and thus a component of the residual intermediate filament cage. Although the temporal and spatial analysis of the MPM reactivity strongly suggests that at least some of these species are components of the spindle, definitive identification of any individual species requires a monospecific antibody and a clear pattern of immunolocalization.

The MPM-reactive band P210 is a phosphorylated form of MAP-4

Rabbits were injected with the individual bands excised from polyacrylamide gels in an attempt to generate polyclonal antisera that would recognize the different phosphorylated spindle proteins independently of their phosphorylation state. So far, high titre antibodies have been obtained to the P210 band as indicated by immunoblot analysis of isolated CHO spindles (Fig. 5). Whereas the MPM antibody recognized the P210 band in mitotic CHO cells and isolated CHO spindles only (see Figs 2 and 3), the polyclonal rabbit antisera identified a protein in interphase CHO cells as well (Fig. 5). Since the reactivity of the MPM antibody is dependent upon the phosphorylation state of the antigenic epitope (Figs 1 and 2), it would not be expected to detect the corresponding protein in interphase cells if it were present in a dephosphorylated form. We infer that the polyclonal antibody, therefore, recognizes both the phosphorylated and dephosphorylated forms of P210 and that the apparent molecular weight shift is due to a mitosis-specific phosphorylation of the protein.

Interestingly, the protein detected by the polyclonal anti-P210 in the interphase cells was a doublet with a slightly higher mobility (lower apparent molecular weight) than the band detected in the mitotic cells and isolated spindles. This shift in apparent molecular size could result from a post-translational modification, such as a difference in level of phosphorylation associated with the protein as present in mitotic samples. When whole mitotic extracts were treated with alkaline phosphatase prior to electrophoresis, the MPM antibody did not stain the corresponding immunoblot; however, the apparent molecular weight of the P210 remained unchanged (data not presented). This suggests either that the modification responsible for the mobility shift is not a phosphorylation or that the protein may be phosphorylated on multiple sites during mitosis, and that loss of the phosphate recognized by the MPM antibody was not sufficient to restore the interphase mobility to the protein.

Immunofluorescence staining of CHO cells with the anti-P210 sera showed a fibrous pattern in both interphase and mitotic cells, the latter associated with spindle fibers (Fig. 6C and D). The interphase staining pattern was also

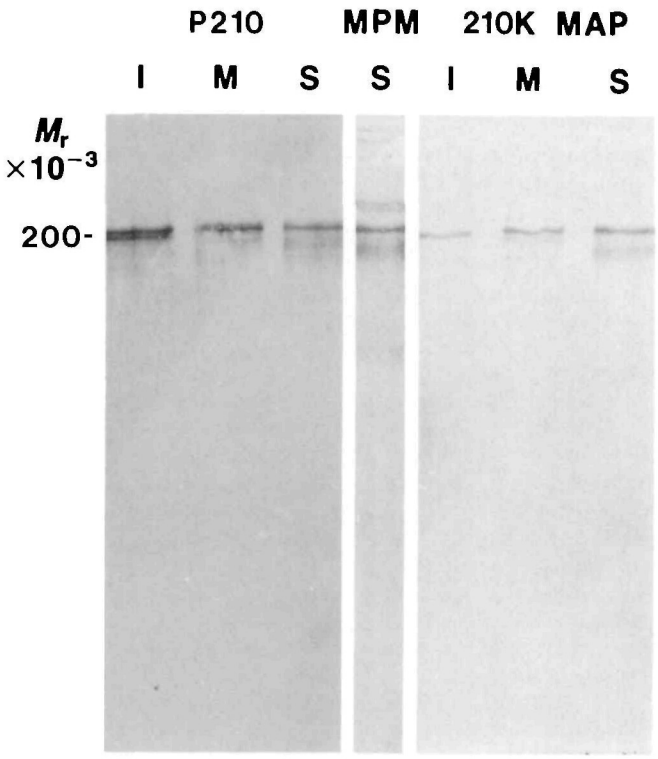

Fig. 5. Identification of MAP-4 as P210. Whole cell lysates of interphase cells (lanes I), mitotic cells (lanes M), and isolated spindles (lanes S) were subjected to SDS/polyacrylamide electrophoresis (7.5 % acrylamide) and transferred to nitrocellulose. The nitrocellulose transfers were probed with polyclonal antibodies generated against the P210 band excised from preparative polyacrylamide gels (see Materials and methods). Included is a reference lane of isolated spindles probed with the MPM-1 antibody. The P210 and MAP-4 antibodies both stained the same bands, which correspond to the position of the MPM-reactive P210 band. An apparent shift in the molecular weight of the 210K MAP is observed in the mitotic cell lysates and the isolated spindle samples.

similar to that obtained by anti-tubulin staining (Fig. 6A), and together with the spindle fiber staining, suggested that the P210 may be a microtubule-associated protein. Its molecular size suggested that it might be related to the $190–240\times10^3 M_r$ family of MAPs designated MAP-4 (Parysek *et al.* 1984), and best characterized by the 210K MAP originally identified in HeLa cells (Bulinski and Borisy, 1979). Confirmation of this relationship was demonstrated by an identical immunoblot pattern obtained using a specific CHO MAP-4 rabbit polyclonal antibody (see Materials and methods) in comparison to the immunoblots of the polyclonal anti-P210 (Fig. 5). In addition, the immunofluorescence staining pattern of the CHO MAP-4-specific antibody in interphase CHO cells (Fig. 6B) was also similar to that obtained with the polyclonal anti-P210 antibody (Fig. 6C).

Phosphorylated MAP-4 is distributed along spindle microtubules

A characteristic property of most MAPs is extractability from microtubules by increased salt concentrations. If P210, provisionally identified above as MAP-4, was indeed a microtubule-associated protein, it would be predicted to be sensitive to salt extraction. Therefore, isolated spindles and midbodies were treated with 0.45 M NaCl prior to immunoblot and immunofluorescence analysis.

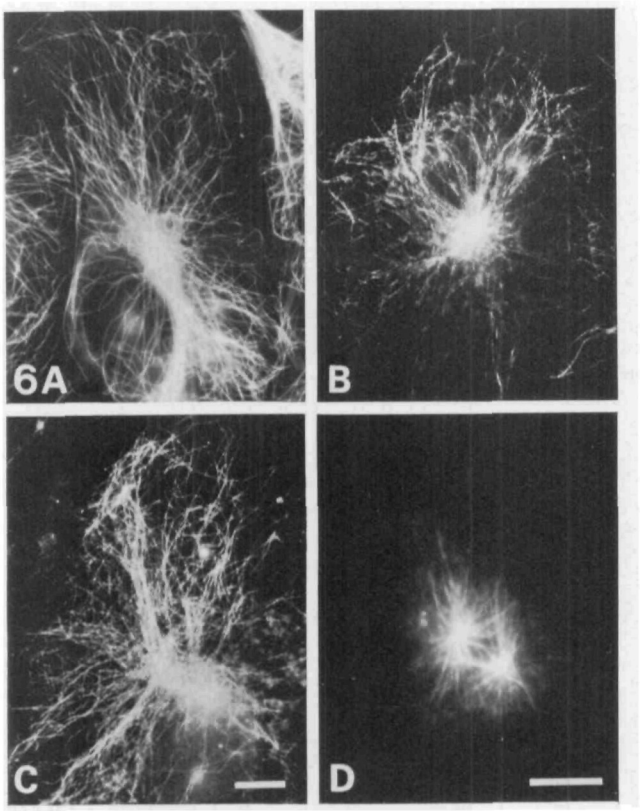

Fig. 6. Indirect immunofluorescence staining of CHO cells with P210 polyclonal antibodies show localization to microtubules of interphase and mitotic cells. The interphase array of microtubules were stained with anti-tubulin antibodies (A), and similar fibrous arrays were detected with the CHO MAP-4-specific antibody (B) and the P210 polyclonal antibodies (C). The P210 antibodies also stained the mitotic spindle microtubules as shown in the prometaphase cell presented in D. Bar, 10 μm.

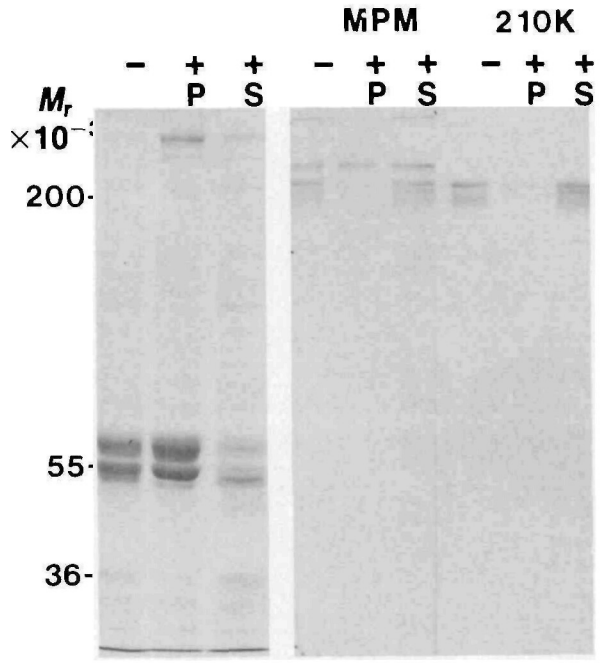

Fig. 7. Gel and immunoblot analysis of taxol-stabilized CHO spindles. Isolated mitotic structures were examined prior to (− lanes), and following 0.45 M salt extraction (+ lanes). The salt-extracted samples were first centrifuged, and both the resulting pellet fraction (lanes P) and supernatant fraction (lanes S) were examined. Coomassie-stained gels (first three lanes) indicate that the majority of the P300 band and tubulin remain in the spindle pellet, while most of the remaining bands are enriched in the supernatant fraction. Nitrocellulose transfers were probed with the MPM-1 or CHO MAP-4 antibodies. Approximately half of the P230-P240 bands remained associated with the salt-extracted spindles, while all of the P210 and corresponding MAP-4 bands were removed by the extraction. The presence of MPM-reactive bands in the salt extract pellet correlate with the residual immunofluorescence staining apparent in the similar samples examined by indirect immunofluorescence (see Fig. 8).

A polyacrylamide gel and immunoblots of the salt-extracted spindles (Fig. 7) demonstrated that most MPM-reactive proteins were substantially extracted into the 0.45 M NaCl-containing buffer. As determined by the Coomassie-stained polyacrylamide gels, the majority of the minor protein components associated with the spindle were extracted by the high salt solution. The presence of a small amount of tubulin in the salt extract reflects the amount of spindle and/or microtubule fragmentation that occurred during preparation of the salt extracts. The salt-extracted spindle pellet, on the other hand, was composed of only three major Coomassie-stained protein bands, the α and β tubulin subunits, and the intermediate filament contaminant (300×10^3 M_r), which is known to be salt insoluble (see Fig. 3). The complete extraction of the P210 band correlated with the complete extraction of MAP-4 as detected with the CHO MAP-4-specific antibody. The other MPM-reactive proteins were also extracted, but the P230-P240 doublet was only partially removed in high salt as indicated by the presence of a band in the spindle pellet as well as the supernatant.

Immunofluorescence analysis (Fig. 8) was also carried out before and after salt extraction on samples as in the immunoblotting experiments presented above. When compared with the morphology of unextracted spindles, the majority of the salt-extracted spindles had been sheared during handling into half-spindles. Microtubules in the half-spindle remained intact after salt extraction as revealed by anti-tubulin immunofluorescence staining (Fig. 8A and B). Conversion of the spindles into half-spindles was observed directly by phase-contrast microscopy when the salt extraction buffer was gently perfused across spindles on a microscope slide. This suggests that half-spindles were not generated as a result of mechanical disruption but rather were produced because an element of the spindle that was responsible for linking the half-spindles was extracted by the salt-containing buffer. Differences in the pattern of MPM staining were apparent when the unextracted and salt-extracted spindle samples were compared (Fig. 8C and D). Prior to salt extraction, discrete staining was associated with the spindle poles, microtubule fibers and spots near the midzone of metaphase spindles (Fig. 8C). This staining correlates with the presence of MPM-reactive proteins as detected by immunoblot (Figs 3 and 7). In the salt-extracted spindle sample spindle fiber staining was absent. Rather, immunoreactive material was present at a central region that corresponded to the location of the centrosome, which was surrounded by a constellation of smaller discrete spots. The number of these spots was similar to the number of chromosomes in CHO cells and

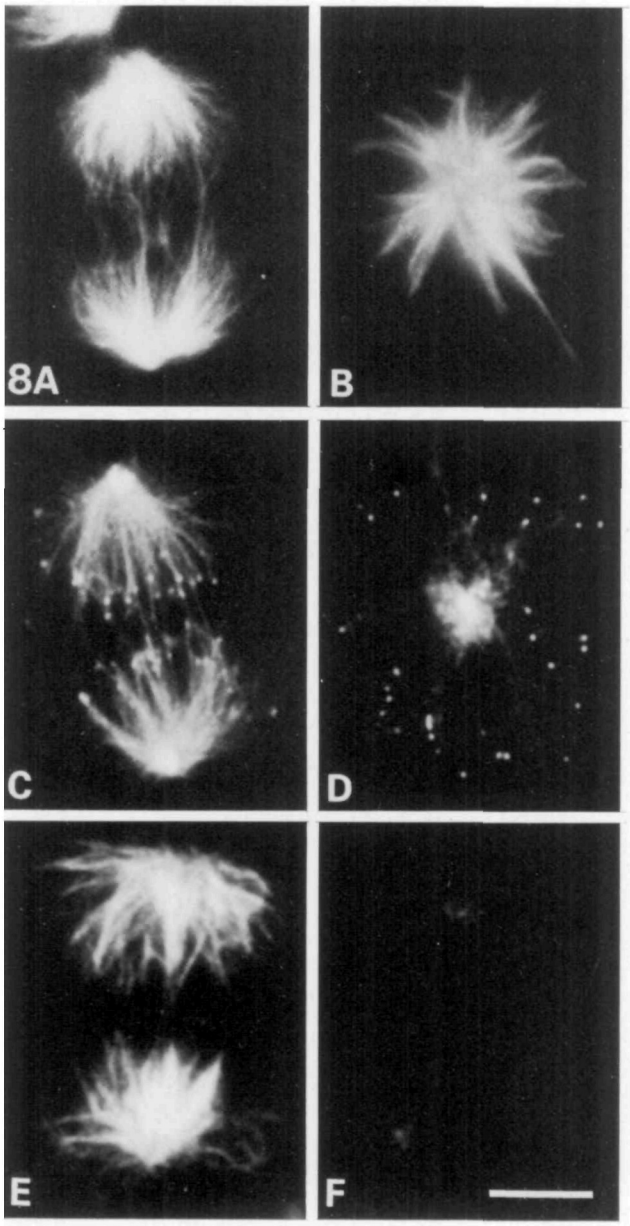

Fig. 8. Comparison of the immunofluorescence staining between 0.45 M NaCl-extracted and unextracted isolated CHO spindles. Spindles were applied to coverslips before (A, C, E) or after (B, D, F) extraction with 0.45 M NaCl. Samples were brought to 5 mM in the fixative ethylene glycol bis(succinic acid *N*-hydroxy succinimide ester) immediately prior to application to the coverslips (see Materials and methods), rinsed, and postfixed in −20°C methanol. The anti-tubulin immunofluorescence staining (A and B) demonstrate that although spindles were sheared into half-spindles when the spindle pellet was resuspended after salt extraction, the majority of the microtubules remained intact. MPM-2 staining was present at the spindle poles, along spindle fibers, and at kinetochores in unextracted samples (C). Following salt extraction (D), staining was concentrated at the centrosome, and in a constellation of surrounding spots (putatively kinetochores), but fiber staining was absent. Fiber staining was apparent in samples stained with the CHO MAP-4 antibody, but was not concentrated to either pole or kinetochores (E). Salt extraction removed all of the MAP-4 from the spindle (F). Bar, 5 μm.

the spots were located at the ends of microtubule bundles, suggesting that they were kinetochore remnants that had become detached from the chromosomes during spindle isolation. Since the immunoblots (Fig. 7) demonstrated that the MPM-reactive doublet, P230-P240, was only partially removed by the salt extraction, the immunofluorescence staining pattern suggests a localization of these proteins at the kinetochore and centrosome. Confirmation of this localization, however, requires an independent and monospecific antibody.

The absence of MPM-reactive material along spindle fibers after salt extraction suggested that one or more of the spindle proteins had been removed, as also indicated by the immunoblot (Fig. 7). The CHO MAP-4 antibody stained the spindle fibers prior to salt extraction (Fig. 8E), but all staining with this antibody was lost following salt extraction (Fig. 8F). These results, together with the immunoblot patterns, suggest that much of the spindle fiber staining observed with the MPM antibody prior to salt extraction is attributable to the association of phosphorylated MAP-4 with the spindle microtubules. The loss of MPM fiber staining following salt extraction correlates with the removal of the phosphorylated MAP-4. The possibility that other MPM-reactive proteins contribute to the spindle fiber staining cannot be excluded by these results.

MAP-4 remains associated with spindle microtubules after dephosphorylation

Reaction of the midbody samples (isolated 45 min after nocodazole release) with the same two antibodies is shown in Fig. 9. In contrast to metaphase spindles, the prominent band in midbodies recognized by MPM was the P350 species and it was largely extractable from the midbody by high salt treatment. Although phosphorylated MAP-4 was not detected on the isolated midbodies by the MPM antibody, this microtubule-associated protein was present as shown by the reaction with the CHO MAP-4 specific antibody. As with the isolated metaphase spindle samples, MAP-4 was extracted from the midbodies by salt treatment (Fig. 9).

Midbodies were also stained by the MPM antibody along microtubule fibers, but with a concentration of stained material at the central midbody core (Fig. 10A). Since by immunoblot the only MPM-reactive band in the midbody preparation is P350, this would suggest that this phosphoprotein was associated with interzonal microtubules involved in the formation of the midbody. MPM staining of midbody fibers was absent in salt-extracted samples with some residual staining associated with the central midbody core (Fig. 10B). This correlates with the loss of P350 as shown in the immunoblot of salt-extracted samples (Fig. 9). Although phosphorylated MAP-4 was not present in midbody samples, the CHO MAP-4 antibody stained microtubule fibers of the midbody (Fig. 10C). This staining was abolished by treatment with high salt (Fig. 10D). These results were consistent with the expected staining patterns of MAP-4. The combined results obtained with the MPM and MAP-4 antibodies indicate that MAP-4 is associated with microtubules throughout mitosis; however, it is converted from a phosphorylated form to a dephosphorylated form upon the completion of mitosis.

Phosphorylated MAP-4 is not unique to CHO spindles

Immunoblot analysis of HeLa cell lysates and isolated spindles (Fig. 11) demonstrated similarities with the corresponding CHO samples (compare with Fig. 1). MPM-

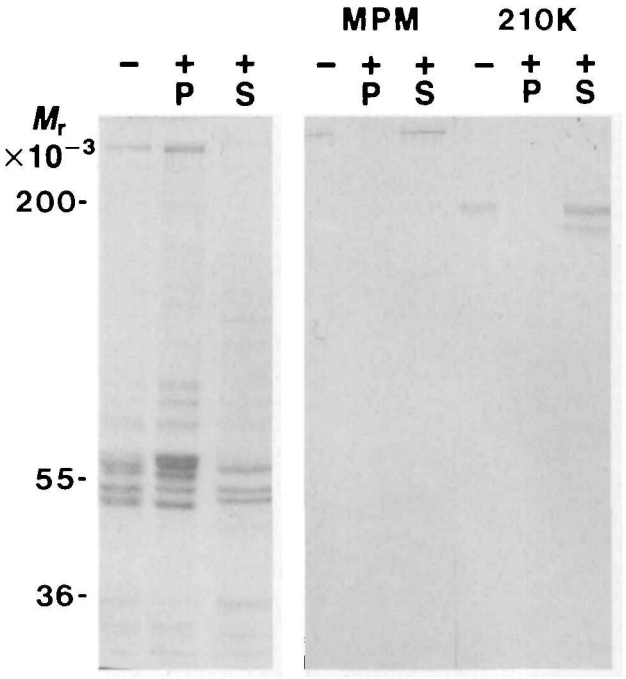

Fig. 9. Gel and immunoblot analysis of taxol-stabilized CHO midbodies. Isolated midbodies were examined prior to salt extraction (− lanes), and following salt extraction (+ lanes). The salt-extracted samples were first centrifuged and both the resulting pellet fraction (lanes P) and supernatant fraction (lanes S) were examined. As was seen with isolated spindles (Fig. 7), following salt extraction the majority of the tubulin remained in the pellet fraction as determined by Coomassie staining (first three lanes). Nitrocellulose transfers were probed with the MPM-1 and CHO MAP-4 antibodies. P350 was the primary MPM-reactive band present in the midbody samples, and was removed by salt extraction. While P210 did not stain with the MPM antibodies, MAP-4 was present on the midbodies, but in a dephosphorylated form not recognized by the MPM antibodies. The MAP-4 was also removed from the midbodies following salt extraction.

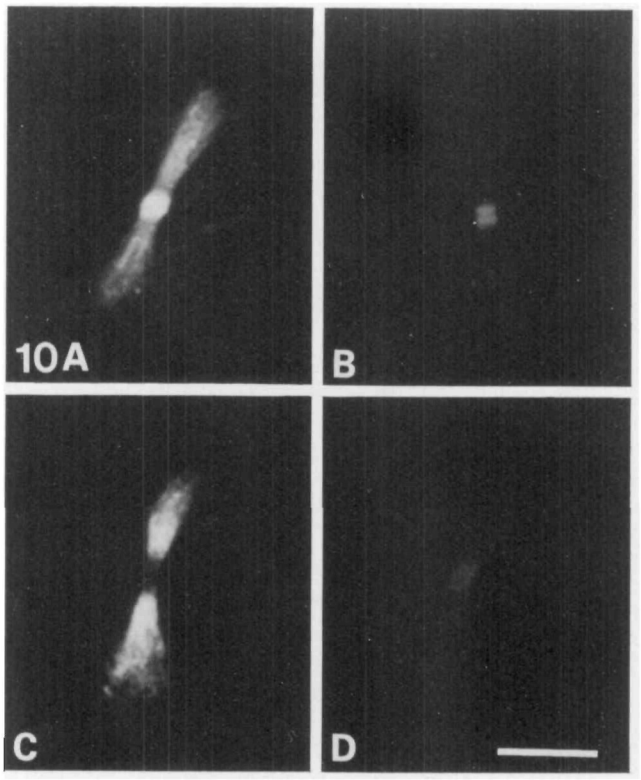

Fig. 10. Isolated taxol-stabilized CHO midbodies were applied to polylysine-coated coverslips prior to (A and C) and following (B and D) extraction for 12 min in 0.45 M NaCl. Samples were processed as described in Fig. 7, and stained for indirect immunofluorescence microscopy with the MPM-2 (A and B) and CHO 210K MAP (C and D), antibodies. In each case fibrous staining was associated with midbody microtubules prior to salt extraction, but was absent following extraction. The MPM-2 stained the midbody core (A), whereas the 210K MAP antibody did not (C). Some residual MPM-staining material remained associated with the midbody core following salt extraction (B). Bar, 5 μm.

reactive proteins that comigrated with some of the CHO proteins were detected in the HeLa spindle samples as well as an additional band of 255×10^3 M_r. In particular, a band at 210×10^3 M_r in the HeLa sample was clearly resolved into a doublet of MPM-reactive phosphoproteins. A HeLa 210K MAP (MAP-4) polyclonal antibody previously shown to identify both a 210×10^3 M_r protein and also a related 255×10^3 M_r protein in HeLa cell extracts (Bulinski and Borisy, 1980), reacted with a protein doublet in mitotic HeLa cell lysates and isolated spindles that comigrated with the HeLa 210×10^3 M_r phosphoreactive band, as well as the 255×10^3 M_r band. The MAP-4 detected in the mitotic HeLa cell lysate and isolated spindles migrated with a higher apparent molecular weight than that observed for the MAP-4 in the interphase HeLa cell lysate, as was observed in the CHO cells, suggesting that the mobility shift upon entering mitosis may be of general significance. Proteins that comigrated with other MPM-reactive bands were not detected in the HeLa spindles, but were detected in immunoblots of taxol stabilized spindles isolated from BHK-21 cells (data not presented).

Discussion

We have shown that a subset of mitotic phosphoproteins detected by the MPM antibodies in whole mitotic cells are components of the spindle. These MPM-reactive proteins, designated P410, P350, P230-P240, P210 and P120, are differentiated by their temporal and spatial distribution on spindle structures prepared from cells at various mitotic stages.

MAP-4

The best characterized of these proteins is P210, which is identical to the previously characterized 210K MAP (Bulinski and Borisy, 1979), and should be considered as a member of the MAP-4 family of proteins. Polyclonal MAP-4 antibodies prepared against CHO (Brady and Cabral, 1985) and HeLa (Bulinski and Borisy, 1980) 210K MAP, as well as a CHO spindle P210, all react with the 210×10^3 M_r band present in immunoblots of isolated spindles. There is however, an apparent difference between the 210×10^3 M_r protein (MAP-4) present in interphase and mitotic cell samples. A higher molecular weight form of MAP-4 is present in both the mitotic cell lysates and isolated spindle samples when compared with that present in interphase cells. Only this higher molecular weight mitotic form is reactive with the MPM antibodies. Since these antibodies recognize a phosphorylated epitope, the apparent shift in molecular weight could be the result of a mitosis-specific

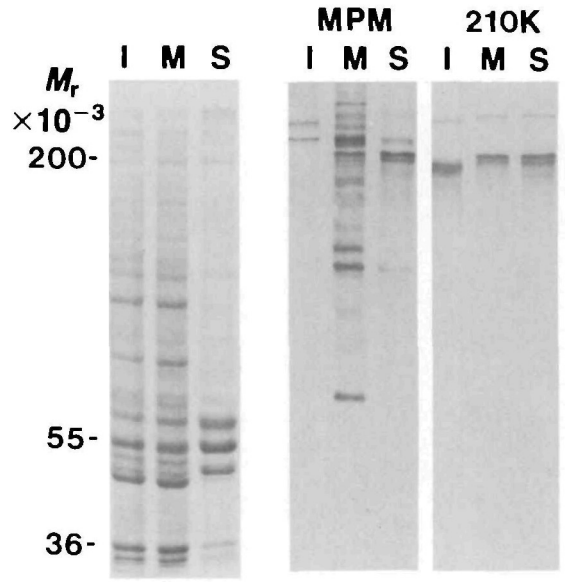

Fig. 11. Gel and immunoblots of interphase and mitotic HeLa cell lysates and isolated HeLa cell spindles. Proteins were stained by Coomassie Blue (first three lanes) and nitrocellulose transfers were probed with the MPM-1, HeLa 210K MAP antibodies. Lanes I, interphase cell lysates; lanes M, mitotic cell lysates; and lanes S, isolated spindles. The 210K MAP antibody recognizes both the 210K MAP and a related 255×10^3 M_r protein in interphase cell lysates. Both of these proteins show an apparent shift to higher molecular weight forms in the mitotic cell lysates and isolated spindles. The MPM-1 antibody identifies both 210K MAP and the 255×10^3 M_r MAP in mitotic cell samples, but does not recognize either protein in interphase samples.

hyperphosphorylation of the MAP-4. Thus, the phosphorylation state of the MAP-4 appears to be altered in a cell cycle stage-dependent manner. The relationship between the mitosis-specific phosphorylation of MAP-4 and the phosphorylation of proteins of similar molecular size, X-MAP in *Xenopus* eggs (Gard and Kirschner, 1987) or a 205×10^3 M_r component of diatom spindles (Wordeman *et al.* 1989), remains to be determined.

On the basis of the immunoblot and immunofluorescence staining patterns in unextracted *versus* salt-extracted spindles, MAP-4 (P210) was shown to be localized along metaphase spindle microtubules. Monospecific antibody staining showed MAP-4 to also be present in midbody samples; however, the lack of MPM reactivity indicated that it was present in a dephosphorylated form. It is possible that only a subset of the total number of phosphorylated residues on these proteins (those specifically associated with the MPM epitope) be dephosphorylated. Thus MAP-4 may remain phosphorylated in telophase samples, but only at sites not recognized by the MPM antibody. An analysis of the phosphorylation state of MAP-4 in both metaphase and telophase samples will resolve this possibility. With the exception of P350, the other MPM-reactive spindle-associated phosphoproteins are also not detected in telophase samples, and like P210 the protein may be present but in a dephosphorylated form not recognized by the MPM antibodies.

Mitosis-specific phosphorylation of MAP-4 (P210) may serve to alter the microtubule binding properties of the protein. It is known that phosphorylation of brain MAPs decreases their affinity for microtubules (Jameson *et al.* 1980), therefore the phosphorylation of MAP-4 may have an analogous effect. The microtubules of the mitotic spindle are more dynamic structures than their interphase counterparts (Gorbsky and Borisy, 1989). It has been established that MAP binding tends to stabilize microtubules (Sloboda and Rosenbaum, 1979). Controlling the level of MAP phosphorylation may be one method that the cell employs to modulate binding of MAPs to microtubules, thereby indirectly modulating microtubule dynamics. Interestingly, microtubule dynamics have been shown to be dramatically affected by the addition of cdc2 protein kinase to cell-free extracts of *Xenopus* eggs (Verde *et al.* 1990). It also should be noted that microtubules of the midbody are extremely stable structures and that the MAP-4 protein bound to these microtubules is no longer phosphorylated at the site recognized by the MPM antibodies.

P230-P240

The P230-P240 doublet is the only detectable MPM-reactive band associated with spindles after salt extraction. After salt extraction the immunofluorescence staining pattern shows that only the centrosomes and kinetochores continue to stain with the MPM antibodies; therefore, it is tempting to suggest that P230-P240 doublets are components of these structures. MPM-reactive bands similar to the P230-P240 bands were detected in isolated BHK-21 and HeLa spindles in addition to the isolated CHO spindles. An MPM-reactive protein of similar molecular size, 225×10^3 M_r, has been reported as a component of sea urchin egg centrospheres (Kuriyama *et al.* 1990).

Alternatively, the P230-P240 bands may be a persistant contaminant related to the phosphorylated form of non-erythrocyte spectrin (fodrin). A doublet of $220–240\times10^3$ M_r has been identified by anti-fodrin antibodies as a component of isolated centrosomes from human lymphoid cells (Klotz *et al.* 1990). As described above, the isolated taxol-stabilized CHO spindles analyzed here are known to be contaminated with intermediate filaments and associated proteins. Plectin, a 300×10^3 M_r intermediate filament associated protein, has been identified as a component of the isolated CHO spindle, and is resistant to salt extraction. In a solid-phase binding assay, plectin was shown to bind to vimentin, MAPs 1 and 2, the α spectrin from human erythrocytes, and the 240×10^3 M_r chain of brain fodrin (Herrmann and Wiche, 1987). Therefore, if P230-P240 are the α and β chains of fodrin they may be present as contaminants that co-sediment with plectin. In addition, the major MPM-reactive band present in whole mitotic cell lysates co-migrates with the P230-P240 bands present in isolated spindles raising the possibility that their presence in the spindle preparation may reflect cytoplasmic contamination. Resolution of these possibilities requires the identification or preparation of antibody reagents specific for the P230-P240 bands.

MPM epitope

The common occurrence of phosphoproteins in every spindle type examined, and their localization to the centrosome and kinetochore suggests the functional importance of these spindle components. It has been shown that the microtubule nucleation capacity of mitotic centrosomes is significantly greater than that of interphase centrosomes when assayed by the regrowth of microtubules from purified tubulin *in vitro* (Snyder and McIntosh, 1975; Kuriyama and Borisy, 1981). Preliminary evidence has indicated that the increased microtubule

nucleation capacity of the mitotic centrosome is dependent upon the presence of phosphorylated centrosomal components. This has been demonstrated by phosphatase treatment of the mitotic centrosome or pretreatment with the MPM antibody prior to the addition of the purified brain tubulin needed for the assembly of microtubules (Vandré and Borisy, 1989*b*; Centonze and Borisy, 1990), and further suggested by the activity of cdc2 kinase in egg extracts (Verde *et al.* 1990).

Addition of cdc2 kinase to an interphase *Xenopus* extract led to an increase in MPM reactivity associated with the centrosomes (Verde *et al.* 1990). This increased centrosomal phosphorylation was observed as the behavior of the extract changed from an extract showing interphase microtubule dynamics to an extract showing mitotic microtubule dynamics. This suggests that the MPM epitope may be phosphorylated through the action of the cdc2 kinase either directly or indirectly through the activation of another kinase(s) by the cdc2 kinase. The consensus recognition sequence for the cdc2 kinase has been defined as Ser/Thr-Pro-X-basic residue (Moreno and Nurse, 1990). Recent sequence analysis of MAP-4 reveals the presence of three cdc2 consensus motifs (Aizawa *et al.* 1990). It is tempting to speculate that at least one of these cdc2 consensus sequences may be phosphorylated and correspond to the MPM epitope of P210. In a similar manner, analysis of the sequence determined for human fibroblast α spectrin (Moon and McMahon, 1990) indicates the presence of a single cdc2 motif, Ser-Pro-Trp-Lys, at the C-terminal end of internal repeat 11, which is also a calmodulin binding site. Therefore, the possibility that this cdc2 motif is phosphorylated and recognized by the MPM antibody exists, and is consistent with the possibility of the P230-P240 doublet being related to non-erythroid spectrin.

Other MPM-reactive proteins

Little is known definitively regarding the other MPM-reactive proteins. P410, the highest molecular weight phosphoprotein identified by the MPM antibodies, cross-reacts with MAP-1A-specific antibody (Bloom *et al.* 1984) and the JA-2 antibody (DeMey *et al.* 1987), and is a cellular counterpart of brain MAP-1 (data not presented). The reaction of the MPM antibodies with a cellular MAP-1 is not unexpected, since brain MAP-1, but not brain MAP-2, has been shown to react with the MPM-1 antibody (Vandré *et al.* 1986). P410 is present in metaphase spindles, and is removed by salt extraction as would be expected of a typical MAP. Whereas, all of the phosphorylated spindle proteins were detectable on immunoblots of metaphase spindles, only P350 was detected on immunoblots of midbodies prepared from telophase cells. Possibly, P350 is a component of the telophase cytoskeleton that cosediments with midbodies. P350 co-migrates with brain MAP-1B on polyacrylamide gels and reacts with antibodies against MAP-1B (Tucker *et al.* 1988). The remaining MPM-reactive protein, P120, is present in both CHO and HeLa spindles, and may be related to the 125×10^3 M_r MAP previously described in HeLa cells (Bulinski and Borisy, 1979; Vandré, unpublished results).

The specific role of the individual MPM-reactive phosphoproteins in spindle function has not been determined, but it is likely that the properties of cytoskeletal structures can be modulated through the phosphorylation of relatively minor components of these structures.

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