

Cell Cycle-Dependent Phosphorylation of Centrosomes: Localization of Phosphopeptide Specific Antibodies to the Centrosome

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ABSTRACT The microtubule nucleation capacity of the centrosome increases dramatically as cells progress from interphase into mitosis. The increase in nucleation capacity of the centrosome correlates with the cell cycle-dependent localization of the mitotic protein monoclonal-2 (MPM-2) phosphopeptide-specific antibody to the mitotic centrosome. Therefore, the phosphorylation state of centrosomal components may regulate the microtubule nucleation capacity of this organelle during mitosis. Neither the identity of the MPM-2 kinase(s) nor all of the MPM-2-reactive phosphoproteins associated with the centrosome have been fully elucidated. Only recently have the characteristics of the MPM-2 epitope site been defined, and we used this information to prepare polyclonal antibodies against synthetic phosphopeptides containing potential MPM-2 epitopes derived from the sequences of two MPM-2-reactive proteins, topoisomerase II, and microtubule associated protein 1B (MAP1B). We demonstrate that these phosphopeptide-specific antibodies also localize to the centrosome in a cell cycle-dependent fashion. Thus, polyclonal antibodies have been generated against defined phosphopeptides that reiterate many of the immunofluorescence staining properties exhibited by the MPM-2 antibody. These new phosphopeptide-specific antibodies will provide additional probes to examine the phosphorylation of centrosomal components and the functional consequences of their phosphorylation during mitosis. *Microsc. Res. Tech.* 49:458–466, 2000. © 2000 Wiley-Liss, Inc.

INTRODUCTION

Centrosomes serve as the primary nucleation site for cytoplasmic microtubules in most eukaryotic cells. The functional activity of the centrosome is regulated not only as a result of cellular differentiation, but also throughout the cell cycle. The microtubule nucleating capacity of the centrosome resides within the components of the pericentriolar material, which surrounds the paired centrioles located at the core of the centrosomal complex. Electron microscopic studies revealed that the pericentriolar material expanded as cells progress into mitosis (Gould and Borisy, 1978; Rieder and Borisy, 1982). This correlated with a nearly 10-fold increase in the microtubule nucleating capacity of the mitotic centrosome in comparison to the interphase centrosome (Kuriyama and Borisy, 1981).

In the past few years, components of the pericentriolar material involved in microtubule nucleation have begun to be identified. These factors include γ -tubulin (Oakley and Oakley, 1989; Stearns et al., 1991), components of the γ -tubulin ring complex (Moritz et al., 1995; Zheng et al., 1995), pericentrin (DICTENBERG et al., 1998), and centrin (Salisbury, 1995). Many other proteins that may be transiently associated with the centrosome have also been identified, including various kinases, phosphatases, and motor molecules (for a recent review, see Brinkley and Goepfert, 1998). The centromatrix, a structural framework that may be involved in both the spatial and temporal association of centrosomal components has also recently been de-

scribed by Palazzo and co-workers (Schackenberg et al., 1998).

Phosphorylation of either structural and/or transient components of the centrosome may have significant regulatory activities, potentially affecting cell cycle-dependent processes including centrosomal replication, recruitment of centrosomal components, and microtubule nucleation. Disruption of any one of these processes could result in the assembly of a defective spindle, leading ultimately to aberrant chromosomal segregation. Recent work has demonstrated the presence of abnormal numbers of centrosomes in cells lacking the tumor suppressor gene p53 (Fukasawa et al., 1996), and a variety of human tumors (Lingle et al., 1998; Pihan et al., 1998; Wang et al., 1998). This suggests that aneuploidy in tumor cells may be related to defects in centrosomal and spindle function.

Evidence for the cell cycle-dependent association of phosphorylated proteins with the centrosome has come from the use of antibodies that recognize phosphoepitopes enriched in mitotic cells. The monoclonal protein monoclonal antibody (MPM-2) is the best-characterized of these reagents, and is a monoclonal antibody generated against a mitotic extract obtained from HeLa cells (Davis et al., 1983). The MPM-2 antibody

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was shown to recognize a number of phosphoproteins present in mitotic cell extracts. Subsequent immunofluorescence staining localized the MPM-2 antibody to microtubule-organizing centers present in mitotic cells, including kinetochores, centrosomes, and midbodies (Vandré et al., 1984, 1986). Ultrastructural localization of the MPM-2 antibody following silver enhancement of ultrasmall immunogold-labeled secondary antibodies showed that the MPM-2 antigens accumulated in the pericentriolar material of mitotic centrosomes (Vandré and Burry, 1992). A second phosphoepitope-specific monoclonal antibody raised against *Xenopus* extracts, designated 3F3 (Cyert et al., 1988), has also been shown to label centrosomes and kinetochores in mitotic cells (Gorbsky and Ricketts, 1993; Bousbaa et al., 1997).

The presence of the MPM-2 epitope at the centrosome correlated with entry of cells into mitosis, and the level of phosphorylation reached a maximum at metaphase (Vandré and Borisy, 1989). Exit from mitosis was also shown to be associated with dephosphorylation of the MPM-2-reactive centrosomal antigens in this same study. In vitro analysis of microtubule nucleation by centrosomes using *Xenopus* extracts have shown that increased nucleation activity correlated with increased MPM-2 staining (Verde et al., 1990), as well as the accumulation of pericentriolar material and phosphorylation (Ohta et al., 1993). Additional data supporting a functional role for MPM-2 phosphoepitopes in centrosomal microtubule nucleation was provided by analysis of a lysed Chinese hamster ovary (CHO) cell model system (Centonze and Borisy, 1990). It was demonstrated in this study that preincubation of the MPM-2 antibody with lysed mitotic cells inhibited the capacity of the mitotic centrosomes to nucleate microtubules following incubation with exogenous brain tubulin. Similar results have been obtained using yeast spindle pole bodies (Masuda et al., 1992). However, the presence of MPM-2-reactive centrosomal components alone does not appear to be sufficient to nucleate microtubules. Disruption of γ -tubulin in *Aspergillus nidulans* inhibited the assembly of spindle microtubules in the mutant cells, even though staining for the MPM-2 antigen remained high (Martin et al., 1997). While the presence of MPM-2-reactive phosphoproteins may not be sufficient for microtubule nucleation by the centrosome, results using lysed cell models and isolated centrosomes indicate that, like γ -tubulin, the MPM-2 epitope may be required for microtubule nucleation (Centonze and Borisy, 1990; Masuda et al., 1992; Félix et al., 1994). For example, it is possible that phosphorylation of centrosomal components during mitosis facilitates the assembly and organization of γ -tubulin rings within the pericentriolar material.

The first MPM-2-reactive proteins to be identified were phosphoproteins associated with isolated mitotic spindles and included the microtubule-associated proteins MAP4 and MAP1B (Vandré et al., 1991; Tombes et al., 1991). A number of additional proteins have been identified that are reactive with the MPM-2 antibody in a cell cycle-dependent manner, including DNA topoisomerase II (Taagepera et al., 1993), mitogen-activated protein kinase (Taagepera et al., 1994), cdc25 (Kuang et al., 1994), the *Aspergillus* serine/threonine

protein kinase termed NIMA for Never In Mitosis A (Ye et al., 1995), the tyrosine kinases myt1 and wee1 (Mueller et al., 1995), and RNA polymerase II (Lavoie et al., 1999). Screening of proteins translated from pools of *Xenopus* cDNA plasmids by immunoblot with the MPM-2 antibody after in vitro phosphorylation led to the identification of several additional MPM-2-reactive proteins, including C-INCENP, selected Hox genes, and several other novel gene products (Stukenberg et al., 1997). Expression cloning has also led to the identification of an additional set of unique MPM-2-reactive proteins designated MPP1 through MPP11 (Westendorf et al., 1994; Matsumoto-Taniura et al., 1996).

The capacity of the MPM-2 antibody to recognize a wide variety of phosphoproteins suggested that certain elements of the phosphoepitope site must be conserved. Initial attempts to characterize the MPM-2 epitope utilized an amino acid expression library phosphorylated in vitro, and identified several peptides characterized by a proline residue immediately C-terminal to threonine (Westendorf et al., 1994). Additional characterization of the MPM-2 epitope using synthetic phosphopeptides derived from the sequence of topoisomerase II led to the development of a model that did not require the presence of a proline residue adjacent to the phosphothreonine residue (Ding et al., 1997). In addition, this model predicted that a major influence on the binding of MPM-2 to the epitope was due to the presence of an aromatic amino acid flanking the phosphorylation site to the N-terminal side of the phosphothreonine at the minus 2 position. Further refinement of the MPM-2 epitope site was achieved using a degenerate synthetic peptide library oriented around a phosphoserine-proline sequence (Yaffe et al., 1997). These results indicated that binding of the MPM-2 antibody was strongly influenced by the presence of aromatic amino acid residues at the minus 3 and minus 2 positions. In addition, it was shown in these studies that the peptides recognized by the MPM-2 antibody also bound to the Pin1 peptidyl-proline isomerase. Thus, the MPM-2 phosphorylation site on many proteins may serve as a recognition site for the binding of Pin1. Binding of Pin1 may subsequently induce conformational changes in the phosphoprotein regulating its function, stability, or interactions with other proteins (Shen et al., 1998; Crenshaw et al., 1998; Lavoie et al., 1999). Whether Pin1 interacts with centrosomal components or regulates centrosomal functions remains to be determined.

Some studies have indicated that all MPM-2 phosphoepitope sites may not require the presence of a proline site adjacent to the phosphorylated residue (Taagepera et al., 1994; Ding et al., 1997). We have identified amino acid sequences that fit our model for the MPM-2 epitope in which the phosphorylated residue is not immediately followed by a proline (Ding et al., 1997) in two MPM-2-reactive proteins, topoisomerase II and MAP1B. Using synthetic phosphopeptides corresponding to each of these sites, we generated rabbit polyclonal antibodies that specifically recognize the phosphorylated form of these synthetic peptides. We report here on the immunofluorescence localization of the affinity purified antibodies in cultured mammalian

cells. Both of the phosphopeptide specific antibodies localize to centrosomes in a cell cycle-dependent manner. Thus, we prepared antibodies using defined phosphopeptide sequences that reiterate the centrosomal localization of the MPM-2 phosphoepitope-specific antibody. These results indicate that the topoisomerase II and MAP1B sequences selected have immunological characteristics similar to the MPM-2 epitope, and further demonstrate the cell cycle-dependent phosphorylation of the centrosome during mitosis.

MATERIALS AND METHODS

Materials

HiTrap Protein G columns and CNBr-activated Sepharose 4B columns were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The MPM-2 monoclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY), and the 3F3 antibody was a generous gift from Dr. Gary Gorbsky (Department of Anatomy and Cell Biology, University of Virginia, Charlottesville, VA). Tubulin monoclonal antibody was obtained from Amersham Pharmacia Biotech. Topoisomerase synthetic peptides were prepared by AnaSpec (San Jose, CA), and the MAP1B peptides were prepared by Genosys Biotechnologies Inc. (The Woodlands, TX).

Antibody Production

Topoisomerase synthetic peptides were conjugated to keyhole limpet hemocyanin (KLH) as described (Ding et al., 1997). The MAP1B peptides were also conjugated to KLH, but were cross-linked to the KLH using glutaraldehyde. Briefly, 0.3% glutaraldehyde was slowly added to the peptide/KLH mixture to a final concentration of 0.1% while stirring at room temperature. After a 2-hour incubation, the unreacted glutaraldehyde was blocked by the addition of glycine and the reaction continued for 30 minutes. The MAP1B peptide/KLH conjugate was then dialyzed against distilled water prior to lyophilization. Rabbit polyclonal antibodies were produced following primary subcutaneous immunization using an emulsion of peptide/KLH conjugates (1 mg) in Freund's adjuvant, and secondary immunizations with 0.5 mg of the peptide conjugate using incomplete Freund's adjuvant at 20-day intervals. Total IgG was purified from the immune serum using HiTrap Protein G columns according to the manufacturers instructions. Phosphopeptide-specific antibodies were obtained following affinity purification of antibodies from the isolated IgG fraction using synthetic phosphopeptides coupled to CNBr-activated Sepharose 4B.

Cell Culture and Spindle Isolation

JAR human choriocarcinoma, Chinese hamster ovary (CHO), and LLC-PK pig kidney cell lines were maintained in Dulbecco's Modified Eagle's Medium, Ham's F-10, and Medium 199, respectively. The media were supplemented with 10% fetal bovine serum (FBS) and gentamycin (50 µg/ml) with the exception of Medium 199, which contained 3% FBS and gentamycin. Cells were maintained as monolayer cultures at 37°C in a humidified atmosphere containing 5% CO₂. Taxol-

TABLE 1. Phosphopeptide sequences

Protein	Epitope sequence	Antibody generated
p42mapk*	HTGFLT _p EYVATRW	
MAP1B	LSEFT _p EYLSES	M1BPP
Topoisomerase II	RKEWLT _p NFMDRRC	TiiPP

*Taagepera et al., 1994.

stabilized spindles were isolated from mitotic CHO cells as described (Vandré et al., 1991) with minor modifications. Following release from nocodazole for 12 minutes and taxol stabilization, cells were lysed in isolation buffer (2 mM PIPES, pH 6.9, 0.5% Triton X-100, 5 mM 5,5'-dithiobis(2-nitrobenzoic acid), 100 nM microcystin LR, and 1 mM sodium orthovanadate). Spindles were collected, resuspended, and cytocentrifuged onto glass coverslips.

Immunofluorescence Staining

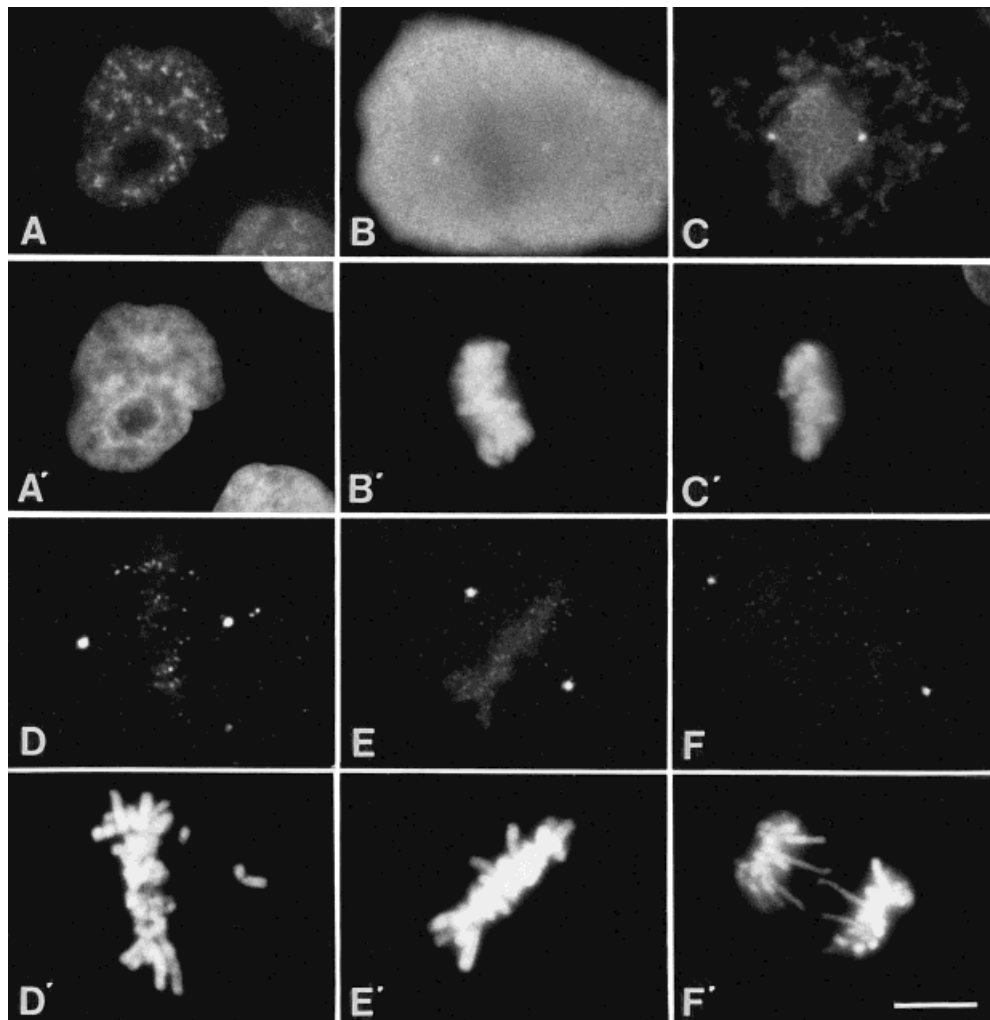
Cells were subcultured onto glass coverslips at least 24 hours prior to fixation in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM MgCl₂, pH 6.9) containing 0.7% glutaraldehyde for 15 minutes at room temperature. Cells were then lysed in PHEM containing 0.2% Triton X-100 for 10 minutes. Unreacted glutaraldehyde was reduced in two changes of Tris-buffered saline, pH 7.4, containing NaBH₄ (1 mg/ml) for 15 minutes each. After blocking in PBS containing 4% normal donkey serum for 30 minutes at 37°C, samples were incubated with primary antibodies for 1 hour at 37°C. After rinsing in PBS, secondary Cy3-conjugated donkey antirabbit or antimouse antibodies were applied for 30 minutes at 37°C. Samples were rinsed in PBS and mounted in mowiol containing 1 mg/ml of p-phenylenediamine and examined by epifluorescence using a Zeiss Axioskop microscope.

In some cases, cells were lysed in PHEM buffer containing 0.2% Triton X-100 for 90 seconds prior to fixation in glutaraldehyde, as described above. Immunostaining with the 3F3 antibody was carried out as described (Gorbsky and Ricketts, 1993). Isolated taxol-stabilized CHO spindles were fixed in 0.7% glutaraldehyde following cytocentrifugation and processed for immunofluorescence staining as described above. To examine antibody specificity with selected synthetic peptides, primary antibodies were preincubated with various concentrations of synthetic peptides for 1 hour at room temperature prior to incubation with the samples.

RESULTS

The identification and characterization of an MPM-2 reactive phosphopeptide derived from the sequence of topoisomerase II (Table 1) has been previously reported (Ding et al., 1997). MAP1B has also been demonstrated to be an MPM-2-reactive protein (Vandré et al., 1991; Tombes et al., 1991). Proteolytic fragments of purified MAP1B were generated following digestion with endoproteinase Asp-N. Immunoblot analysis of the digestion products identified a peptide fragment of approximately 40 kD that retained MPM-2 reactivity and also reacted with a polyclonal antibody prepared

Fig. 1. Immunofluorescence localization of MPM-2 and 3F3 phosphoepitope-specific monoclonal antibodies. Cells were double-labeled with the MPM-2 antibody (A-C) or the 3F3 antibody (D-F), and DAPI to stain the DNA (A'-F'). JAR and LLC-PK cells were used to localize the MPM-2 and 3F3 antibodies respectively. The interphase and mitotic cells in A and B were fixed prior to detergent extraction, while the mitotic cell in C was fixed after detergent extraction. MPM-2 localizes patches within the nucleus of the interphase cells. The cytoplasm of unextracted mitotic cells is labeled by MPM-2, but the centrosomal staining is apparent above this background (B). When antibody localization was carried out on cells detergent-extracted prior to fixation, the cytoplasmic background staining was significantly reduced, and intense label of the two spindle poles is observed (C). 3F3 staining was carried out on cells lysed prior to fixation (see Materials and Methods). In cells with misaligned chromosomes (left side of D'), 3F3 antibody staining of the kinetochore pair is readily detected at the corresponding position in D. While 3F3 kinetochore staining is much reduced or absent in metaphase and anaphase cells, intense staining of the two spindle poles is readily apparent (E,F). Bar = 10 μ M.



against a recombinant MAP1B fragment that included amino acid residues 63–455 (Feng and Vandr , unpublished results). These results suggested that an MPM-2 epitope site was located in the N-terminal region of MAP1B. We examined the protein sequence of MAP1B for sites that fit the proposed model for the MPM-2 epitope (Ding et al., 1997; Yaffe et al., 1997), and two candidate residues were identified in the N-terminal portion of the protein, threonine 230 and threonine 384. We focused on the potential site at Thr230 (Table 1) based on its close resemblance to the MPM-2 site identified in mitogen-activated protein kinase (Tagepera et al., 1994). Rabbit polyclonal antibodies to the synthetic phosphopeptides were prepared as described in Materials and Methods.

The cell cycle-dependent localization of the MPM-2 and 3F3 phosphoepitope-specific monoclonal antibodies (Fig. 1) were compared to the immunofluorescence staining patterns obtained with affinity-purified rabbit polyclonal antibodies generated against the topoisomerase II (TII) and MAP1B (M1B) synthetic phosphopeptides (Figs. 2 and 3, respectively). The results presented in Figure 1 are similar to the more extensive

previous reports describing the localization of the MPM-2 and 3F3 antibodies (Vandr  et al., 1984; Vandr  and Borisy, 1989; Gorbsky and Ricketts, 1993; Bousbaa et al., 1997).

The MPM-2 antibody stains nuclear patches in interphase cells, but the centrosome is not labeled (Fig. 1A). In contrast, MPM-2 staining is present throughout the cytoplasm of mitotic cells, but discrete localization to the spindle poles is evident (Fig. 1B). Much of the cytoplasmic MPM-2 staining is detergent extractable and can be removed prior to fixation, whereas specific localization to the spindle poles remains elevated in the lysed cells (Fig. 1C). Distinct kinetochore staining by the MPM-2 antibody was not observed in the human JAR cells, but is commonly observed in other cell types (see Fig. 4). Cell cycle-dependent localization of the 3F3 antibody to spindle poles and kinetochores was observed in LLC-PK cells that were lysed prior to fixation (Fig. 1D–F). The kinetochore staining by the 3F3 antibody is greatest on those chromosomes not aligned at the metaphase plate (Fig. 1D), whereas kinetochore localization is diminished or absent in metaphase and anaphase cells (Fig. 1E,F). The spindle poles are in-

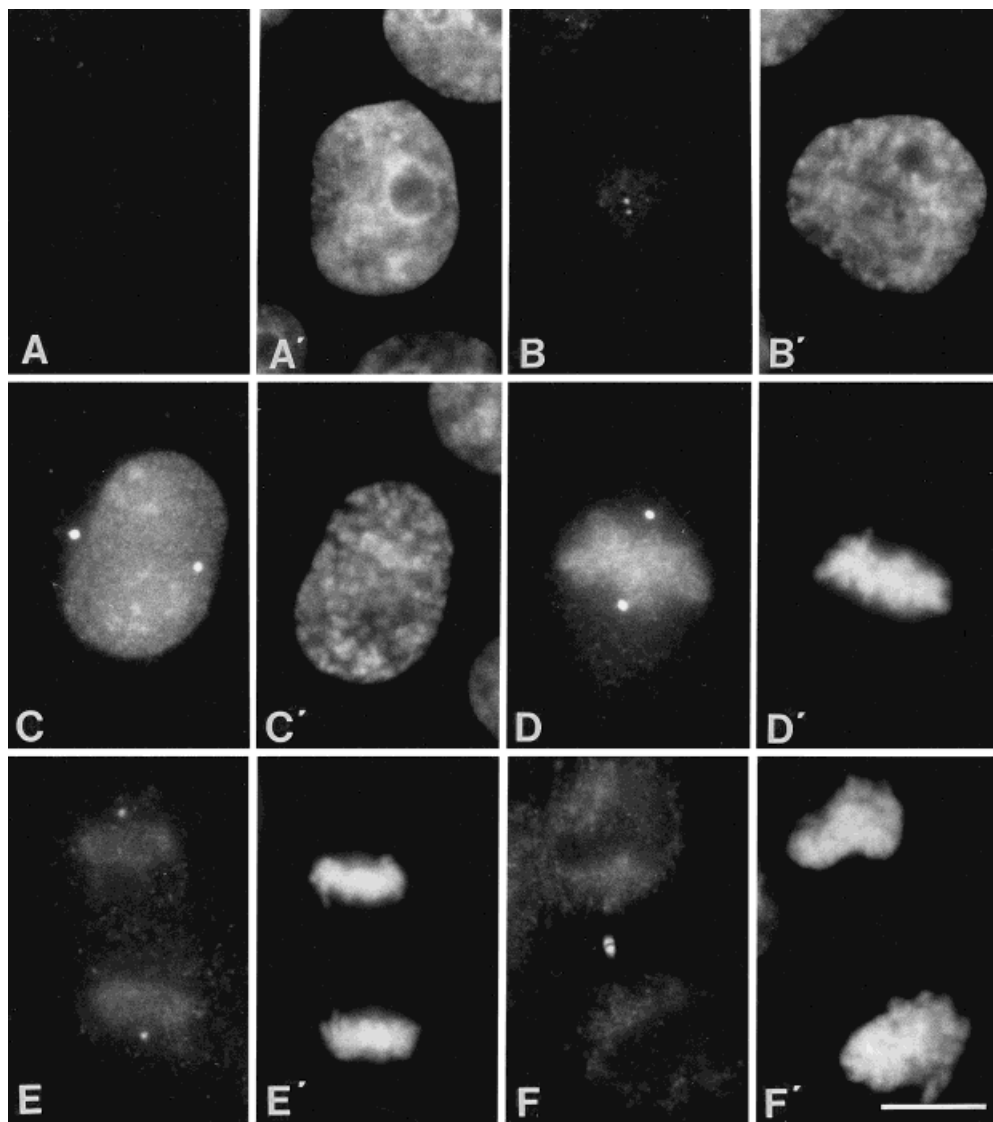


Fig. 2. Immunofluorescence localization of affinity-purified topoisomerase II phosphopeptide (TIIPP) rabbit polyclonal antibody. JAR cells were double-labeled with the TIIPP rabbit polyclonal antibody (A-F), and DAPI (A'-F'). Cells were fixed prior to detergent extraction. In contrast to similarly fixed cells labeled with the MPM-2 antibody (see Fig. 1B), the cytoplasm of mitotic cells is not stained by the TIIPP antibody. The TIIPP antibody does not stain centrosomes in most interphase cells (A), but two closely spaced dots of centrosomal staining are apparent in early prophase cells (central portion of B). These results indicate that the TIIPP antibody labels centrosomes prior to centrosomal separation and migration during prophase. The TIIPP antibody localizes to centrosomes throughout anaphase (C,D,E), and also labels the midbody in telophase cells (F). Bar = 10 μ M.

tensely labeled by the 3F3 antibody in mitotic cells, and this staining remains elevated throughout anaphase (Fig. 1F).

The topoisomerase II (TIIPP) rabbit polyclonal antibody showed cell cycle-dependent staining of the spindle poles in JAR cells similar to that obtained with the MPM-2 antibody (Fig. 2). The TIIPP antibody staining differed from MPM-2, however, since centrosome pairs were also specifically labeled in late G₂/early prophase cells prior to separation of the centrosomes (Fig. 2B). The TIIPP antibody also differed from the MPM-2 antibody in that the cytoplasm of mitotic cells was not heavily stained even in cells that were only detergent extracted after fixation (compare Fig. 1B with Fig. 2D). Kinetochore staining was not observed in any cell line examined with the TIIPP antibody, although metaphase chromosomes were labeled. Centrosomal staining persisted through anaphase (Fig. 2E), but was nearly absent in telophase cells (Fig. 2F). Some anti-

body localization to the midbody was also observed in the telophase cells.

The MAP1B (M1BPP) rabbit polyclonal antibody also localized to the centrosome in a cell cycle-dependent fashion (Fig. 3). Nucleolar staining was observed in interphase cells with the M1BPP antibody (Fig. 3A). This nuclear staining persisted into prophase, although the condensing chromosomes were not labeled (Fig. 3B). Centrosomal localization was present in cells from prophase through telophase, with the maximal intensity of staining being observed at metaphase (Fig. 3C). As with the TIIPP antibody, the M1BPP antibody did not stain the cytoplasm or the kinetochores in mitotic cells. In addition, the M1BPP antibody did not label mitotic chromosomes to the extent of either the MPM-2 or TIIPP antibodies. Similar patterns of centrosomal staining have been obtained in JAR, HeLa, LLC-PK, and CHO cell lines using both the M1BPP and TIIPP antibodies.

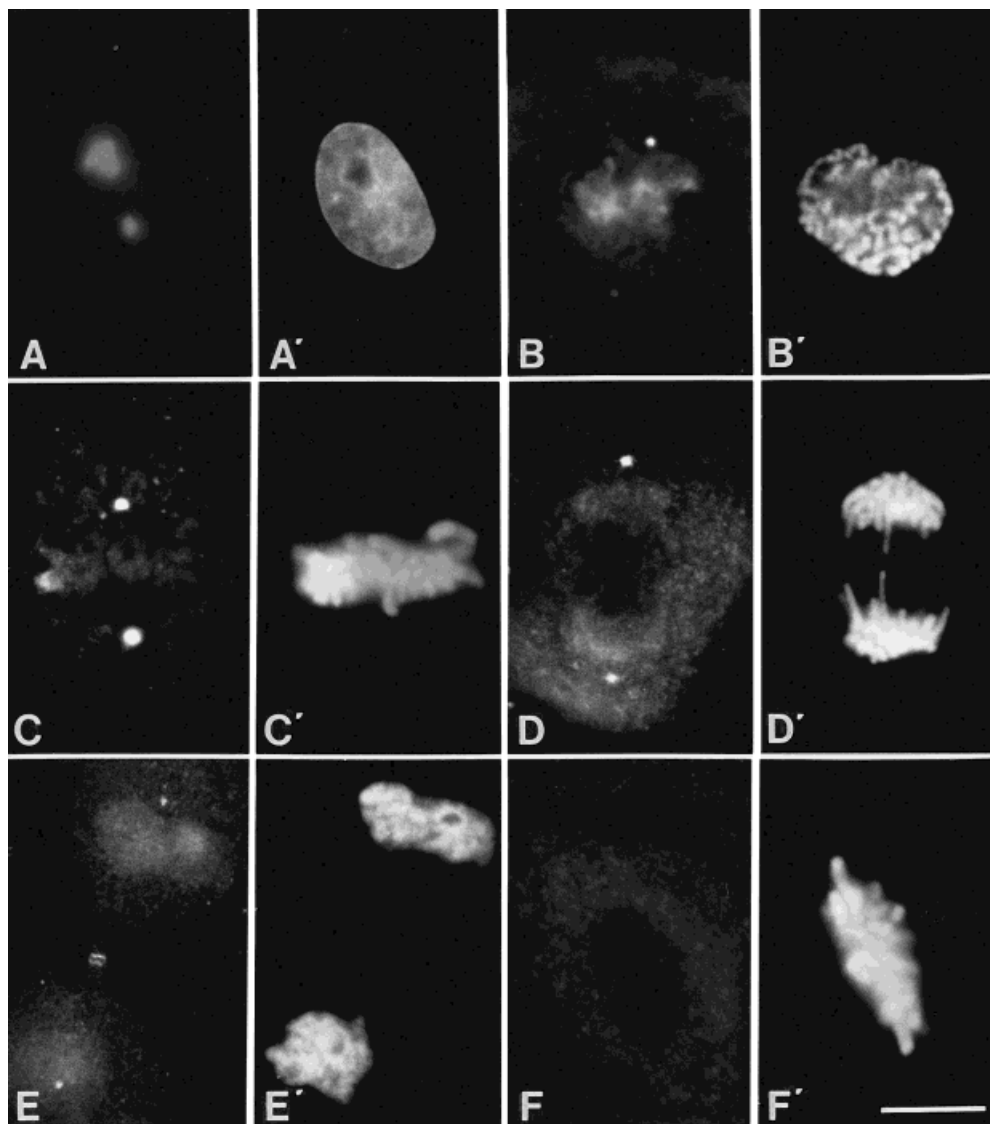


Fig. 3. Immunofluorescence localization of affinity-purified MAP1B phosphopeptide (M1BPP) rabbit polyclonal antibody. LLC-PK cells were double-labeled with the M1BPP rabbit polyclonal antibody (Panels A-E), and DAPI (A'-E'). Cells were fixed prior to detergent lysis. The M1BPP antibody shows some intranuclear staining in interphase cells (A), and localizes to the centrosomes throughout mitosis (B,C,D,E). The most intense staining is observed at metaphase (C). Preimmune serum (F) and the corresponding DAPI staining (F'), show that centrosomal staining in mitotic cells is specific to the immune serum. Bar = 10 μ M.

The localization of each phosphopeptide-specific antibody was also examined using taxol-stabilized spindles isolated from CHO cells (Fig. 4). Tubulin staining of the isolated spindle preparation showed that while bipolar structures dominated the preparation, midbodies were also present (Fig. 4A). This indicated that spindles ranging from metaphase through telophase were present in the sample. Typical of MPM-2 staining, a metaphase spindle showed localization to the kinetochores, spindle fibers, and spindle poles (Fig. 4B). In comparison, the primary staining obtained with the affinity-purified M1BPP antibody was restricted to the spindle poles (Fig. 4C). Both the MPM-2 and M1BPP antibodies labeled spindle poles in anaphase and telophase mitotic structures as well (data not presented). The affinity-purified T11PP antibody also labeled the spindle poles of metaphase spindles, and weak staining of the kinetochores was apparent (Fig. 4D). T11PP staining in addition to the spindle poles was also observed in

spindles derived from cells in later stages of mitosis. In spindles that appeared to be derived from cells in early anaphase, not only were the spindle poles labeled, but T11PP antibody staining was also localized to the region of overlap between the microtubule fibers radiating from each aster (Fig. 4E). The labeling of the microtubule overlap zone was more apparent in later anaphase structures derived from cells undergoing the initial stages of cleavage (Fig. 4F). Ultimately, this T11PP antibody staining of the midzone was concentrated at the central portion of the midbody (Fig. 4G).

DISCUSSION

Cell cycle-dependent phosphorylation of the centrosome has been demonstrated by indirect immunofluorescence labeling using the phosphoepitope specific monoclonal antibodies MPM-2 and 3F3 (Vandré et al., 1984; Vandré and Borisy, 1989; Gorbsky and Ricketts, 1993; Bousbaa et al., 1997). Both of these monoclonal

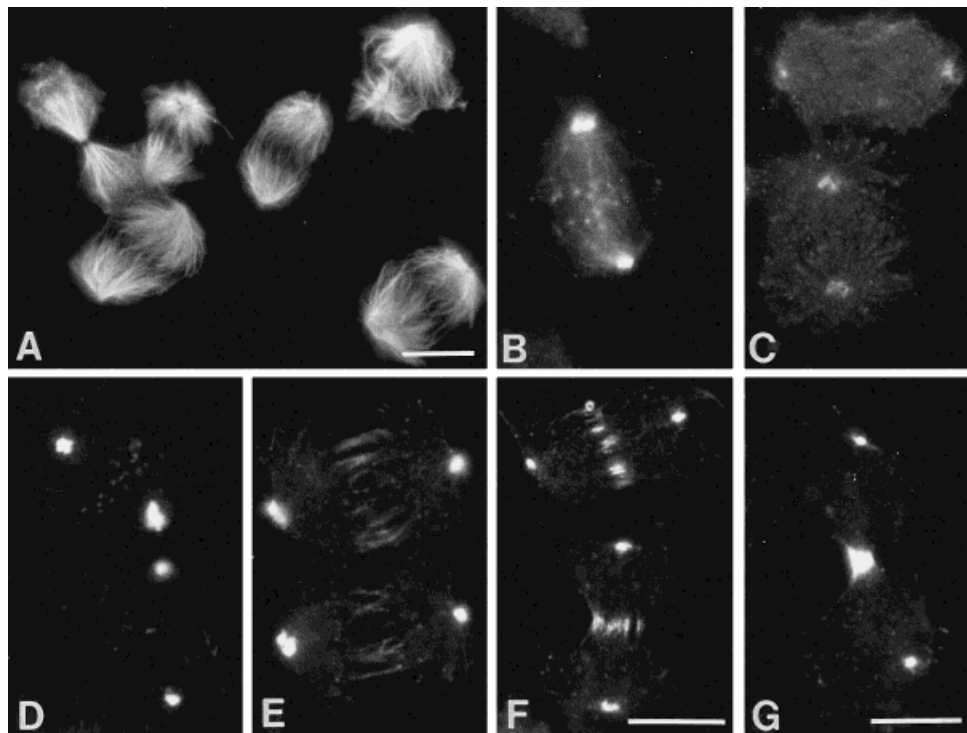


Fig. 4. Immunofluorescence staining of isolated taxol-stabilized CHO spindles. Tubulin staining demonstrates that both mitotic spindles and midbody structures are present in the sample (A). MPM-2 antibody localizes to the spindle pole, spindle fibers, and kinetochores (B). The M1BPP antibody localizes to the spindle poles (C). The TIIPP antibody is localized to the spindle poles in metaphase spindles (D), but in addition to the spindle poles it also stains the region of microtubule overlap in the midzone of anaphase and telophase spindles (E-G). Bar = 10 μ M A and F; bar = 7.5 μ M B, C, D, E, and G.

antibodies were prepared against a mixture of proteins present in mitotic cell extracts (Davis et al., 1983; Cyert et al., 1988); therefore, the specific antigen recognized by these antibodies cannot be determined. Progress has been made in determining the epitope recognized by the MPM-2 antibody through analysis of specific MPM-2-reactive proteins (Taagepera et al., 1993, 1994), expression cloning techniques (Westendorf et al., 1994; Matsumoto-Taniura et al., 1996), and analysis of synthetic phosphopeptides (Ding et al., 1997; Yaffe et al., 1997). These efforts have led to the development of a model for the MPM-2 epitope site that is strongly influenced by the presence of aromatic amino acids near the phosphorylated residue (Ding et al., 1997; Yaffe et al., 1997).

Unlike the MPM-2 and 3F3 antibodies, polyclonal antibodies were generated using a defined antigen. With information based on the MPM-2 epitope models, potential MPM-2-reactive phosphopeptides present in the sequences of topoisomerase II and MAP1B were identified. Synthetic phosphopeptides that corresponded to these sites were conjugated to keyhole limpet hemocyanin and used to immunize rabbits. The resulting antibodies were shown to localize to the centrosomes of mammalian cells in a cell cycle-dependent manner. The staining obtained with these new phosphopeptide specific antibodies was similar to that obtained with the MPM-2 antibody. Thus, antibodies that share many of the staining properties of the MPM-2 antibody have been produced using a defined phosphopeptide antigen that was predicted to have characteristic features similar to those found in MPM-2 epitopes. These localization results further demon-

strate the specific association of phosphorylated proteins with the centrosome during mitosis, and suggest that specific kinases are associated with and/or targeted to the centrosome during mitotic progression.

Emerging studies have indicated a specific role for protein kinases in centrosome replication, separation, and maturation. The role of kinases in the regulation of centrosomal function was recently reviewed by Mayor et al. (1999). The POLO kinase in particular has been shown to have a role both in centrosomal function and is linked to the MPM-2 epitope. Wild-type POLO kinase activity was required for generation of normal levels of MPM-2 epitopes in *Drosophila* cells in vivo (Logarhino and Sunkel, 1998). In addition, POLO or POLO-like kinase (Plk) present in mammalian cells were both demonstrated to restore MPM-2 reactive phosphoepitopes in extracted cell model systems (Renzi et al., 1997; Logarhino and Sunkel, 1998). A *Xenopus* homolog of Plk, Plx1, was also shown to phosphorylate and generate MPM-2 reactive epitopes on the cell cycle regulatory protein phosphatase Cdc25 (Kumagai and Dunphy, 1996). Lastly, microinjection of antibodies to Plk inhibits both γ -tubulin recruitment to and MPM-2 phosphorylation of the centrosome (Lane and Nigg, 1996).

The activity of a second kinase, Nek2 (for NIMA related kinase 2), has recently been shown to be required for centrosome separation during prophase (Fry et al., 1998). Nek2 phosphorylates a protein, designated C-Nap1 (centrosomal Nek2-associated protein 1), which may function to link centrosomes together throughout interphase. Phosphorylation of C-Nap1 by Nek2 at an early stage of prophase appears to regulate

the stability of this interaction, disrupting the bridge linking the centrosomes and allowing for centrosome separation. Interestingly, the T₁PP antibody reported here localizes to the centrosome pair in late G₂/early prophase cells prior to separation of the centrosomes. Thus, the T₁PP may be recognizing phosphorylated centrosomal components linked to this early event in mitotic progression. Further studies will be required to determine if the T₁PP antibody recognizes phosphorylated C-Nap1.

While cell cycle-dependent phosphorylation of the centrosome was initially detected more than 15 years ago (Vandré et al., 1984), our understanding of the molecular events regulating centrosomal function have only recently begun to be unraveled. It is clear that phosphorylation of centrosomal components and/or the localization of kinases and their target substrates to the centrosome play an important role in the proper assembly and function of the mitotic spindle. Either the abnormal phosphorylation of centrosomal components or activity of centrosomal kinases may contribute to the development of aneuploid cells characteristic of most tumor types. Therefore, as additional centrosomal kinases and substrates are identified a more complete understanding for the role of this complex organelle in normal, as well as abnormal, cellular growth will emerge. It is likely that phospho-epitope specific reagents such as the MPM-2, 3F3, and new T₁PP and M1BPP antibodies will continue to prove valuable as tools for probing the functional properties of the centrosome.

REFERENCES

- Bousbaa H, Correia L, Gorbsky GJ, Sunkel CE. 1997. Mitotic phosphoepitopes are expressed in Kc cells, neuroblasts, and isolated chromosomes of *Drosophila melanogaster*. *J Cell Sci* 110:1979–1988.
- Brinkley BR, Goepfert TM. 1998. Supernumerary centrosomes and cancer: Boveri's hypothesis resurrected. *Cell Motil Cytoskel* 41:281–288.
- Centonze VE, Borisy GG. 1990. Nucleation of microtubules from mitotic centrosomes is modulated by phosphorylated epitope. *J Cell Sci* 19:405–411.
- Crenshaw DG, Yang J, Means AR, Kornbluth S. 1998. The mitotic peptidyl-prolyl isomerase, Pin1, interacts with Cdc25 and Plx1. *EMBO J* 17:1315–1327.
- Cyert MS, Scherson T, Kirschner MW. 1988. Monoclonal antibodies specific for thiophosphorylated proteins recognize *Xenopus* MPF. *Dev Biol* 129:209–216.
- Davis FM, Tsao TH, Fowler SK, Rao PN. 1983. Monoclonal antibodies to mitotic cells. *Proc Natl Acad Sci USA* 80:2926–2930.
- Dietenberg JB, Zimmerman W, Sparks CA, Young A, Vidair C, Zheng Y, Carrington W, Fay FS, Doxsey SJ. 1998. Pericentriolar and gamma-tubulin form a protein complex and are organized into a novel lattice at the centrosome. *J Cell Biol* 141:163–174.
- Ding M, Feng Y, Vandré DD. 1997. Partial characterization of the MPM-2 phosphoepitope. *Exp Cell Res* 231:3–13.
- Félix M-A, Antony C, Wright M, Maro B. 1994. Centrosome assembly in vitro: role of γ -tubulin recruitment in *Xenopus* sperm aster formation. *J Cell Biol* 124:19–31.
- Fry AM, Mayor T, Meraldi P, Stierhof Y-D, Tanaka K, Nigg EA. 1998. C-Nap1, a novel centrosomal coiled-coil protein and candidate substrate of the cell cycle-regulated protein kinase Nek2. *J Cell Biol* 141:1563–1574.
- Fukasawa K, Choi T, Kuriyama R, Rulong S, Vande Woude GF. 1996. Abnormal centrosome amplification in the absence of p53. *Science* 271:1744–1747.
- Gorbsky GJ, Ricketts WA. 1993. Differential expression of a phospho-epitope at the kinetochores of moving chromosomes. *J Cell Biol* 122:1311–1321.
- Gould RR, Borisy GG. 1978. The pericentriolar material in Chinese hamster ovary cells nucleates microtubule formation. *J Cell Biol* 73:265–275.
- Kuang J, Ashorn CL, Gonzalez-Kuyvenhoven M, Penkala JE. 1994. cdc25 is one of the MPM-2 antigens involved in the activation of maturation-promoting factor. *Mol Biol Cell* 5:135–145.
- Kumagai A, Dunphy WG. 1996. Purification and molecular cloning of Plx1, a cdc25-stimulatory kinase from *Xenopus* egg extracts. *Science* 273:1377–1380.
- Kuriyama R, Borisy GG. 1981. Microtubule-nucleating activity of centrosomes in Chinese hamster ovary cells is independent of the centriole cycle but coupled to the mitotic cycle. *J Cell Biol* 91:822–826.
- Lane HA, Nigg EA. 1996. Antibody microinjection reveals an essential role for human polo-like kinase (Plk1) in the functional maturation of mitotic centrosomes. *J Cell Biol* 135:1701–1713.
- Lingle WL, Lutz WH, Ingle JN, Mailhe NJ, Salisbury JL. 1998. Centrosome hypertrophy in human breast tumors: implications for genomic stability and cell polarity. *Proc Natl Acad Sci USA* 95:2950–2955.
- Logarinho E, Sunkel CE. 1998. The *Drosophila* POLO kinase localizes to multiple compartments of the mitotic apparatus and is required for the phosphorylation of MPM2 reactive epitopes. *J Cell Biol* 111:2897–2909.
- Martin MA, Osmani SA, Oakley BR. 1997. The role of γ -tubulin in mitotic spindle formation and cell cycle progression in *Aspergillus nidulans*. *J Cell Sci* 110:623–633.
- Masuda H, Sevik M, Cande WZ. 1992. In vitro microtubule-nucleating activity of spindle pole bodies in fission yeast *Schizosaccharomyces pombe* spindle pole body. *J Cell Biol* 117:1055–1066.
- Matsumoto-Taniura N, Pirolet F, Monroe R, Gerace L, Westendorf JM. 1996. Identification of novel M phase phosphoproteins by expression cloning. *Mol Biol Cell* 7:1455–1469.
- Mayor T, Meraldi P, Stierhof Y-D, Nigg EA, Fry AM. 1999. Protein kinases in control of the centrosome cycle. *FEBS Lett* 452:92–95.
- Moritz M, Braunfeld MB, Sedat JW, Alberts B, Agard DA. 1995. Microtubule nucleation by γ -tubulin containing rings in the centrosome. *Nature* 378:638–640.
- Mueller PR, Coleman TR, Dunphy WG. 1995. Cell-cycle regulation of a *Xenopus* wee1-like kinase. *Mol Biol Cell* 6:119–134.
- Oakley CE, Oakley BR. 1989. Identification of gamma-tubulin, a new member of the tubulin superfamily encoded by mipA gene of *Aspergillus nidulans*. *Nature* 338:543–547.
- Ohta K, Shiina N, Okumura E, Hisanaga S-I, Kishimoto T, Endo S, Gotoh Y, Nishida E, Sakai H. 1993. Microtubule nucleating activity of centrosomes in cell-free extracts from *Xenopus* eggs: involvement of phosphorylation and accumulation of pericentriolar material. *J Cell Sci* 104:125–137.
- Pihan GA, Purohit A, Wallace J, Knecht H, Woda B, Quesenberry P, Doxsey SJ. 1998. Centrosome defects and genetic instability in malignant tumors. *Cancer Res* 58:3974–3985.
- Renzi L, Gerseh MS, Campbell MS, Wu L, Osmani SA, Gorbsky GJ. 1997. MPM-2 antibody-reactive phosphorylations can be created in detergent-extracted cells by kinetochore-bound and soluble kinases. *J Cell Sci* 110:2013–2025.
- Rieder CL, Borisy GG. 1982. The centrosome cycle in PtK2 cells: asymmetric distribution and structural changes in the pericentriolar material. *Biol Cell* 44:117–132.
- Salisbury JL. 1995. Centrin, centrosomes, and mitotic spindle poles. *Curr Opin Cell Biol* 7:39–45.
- Schackenberg BJ, Khodjakov A, Rieder CL, Palazzo RE. 1998. Disassembly and reassembly of functional centrosomes in vitro. *Proc Natl Acad Sci USA* 95:9295–9300.
- Shen M, Stukenberg PT, Kirschner MW, Lu KP. 1998. The essential mitotic peptidyl-prolyl isomerase Pin1 binds and regulates mitosis-specific phosphoproteins. *Genes Dev* 12:706–720.
- Stearns T, Evans L, Kirschner MW. 1991. γ -Tubulin is a highly conserved component of the centrosome. *Cell* 65:825–836.
- Stukenberg PT, Lustig KD, McGarry TJ, King RW, Kuang J, Kirschner MW. 1997. Systematic identification of mitotic phosphoproteins. *Curr Biol* 7:338–348.
- Taagepera S, Rao PN, Drake FH, Gorbsky GJ. 1993. DNA topoisomerase IIa is the major chromosome protein recognized by the mitotic phosphoprotein antibody MPM-2. *Proc Natl Acad Sci USA* 90:8407–8411.
- Taagepera S, Dent P, Her J-H, Sturgill TW, Gorbsky GJ. 1994. The MPM-2 antibody inhibits mitogen-activated protein kinase activity by binding to an epitope containing phosphothreonine-183. *Mol Biol Cell* 5:1243–1251.
- Tombs RM, Peloquin J, Borisy GG. 1991. Specific association of an M-phase kinase with isolated mitotic spindles and identification of two of its substrates as MAP4 and MAP1B. *Cell Reg* 2:861–874.

- Vandré DD, Burry RW. 1992. Immunoelectron microscopic localization of phosphoproteins associated with the mitotic spindle. *J Histochem Cytochem* 40:1837-1847.
- Vandré DD, Davis FM, Rao PN, Borisy GG. 1984. Phosphoproteins are components of the mitotic microtubule organizing centers. *Proc Natl Acad Sci USA* 81:4439-4443.
- Vandré DD, Davis FM, Rao PN, Borisy GG. 1986. Distribution of cytoskeletal proteins sharing a conserved phosphorylated epitope. *Eur J Cell Biol* 41:72-81.
- Vandré DD, Centonze VE, Peloquin J, Tombes RM, Borisy GG. 1991. Proteins of the mammalian mitotic spindle: phosphorylation/dephosphorylation of MAP-4 during mitosis. *J Cell Sci* 98:577-588.
- Verde F, Labbe JC, Doree M, Karsenti E. 1990. Regulation of microtubule dynamics by Cdc2 protein kinase in cell-free extracts of *Xenopus* eggs. *Nature* 343:233-238.
- Yaffe MB, Schutkowski M, Shen M, Zhou XZ, Stukenberg PT, Rahfeld J-U, Xu J, Kuang J, Kirschner MW, Fischer G, Cantley LC, Lu KP. 1997. Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism. *Science* 278:1957-1960.
- Ye XS, Xu G, Pu RT, Fincher RR, McGuire SL, Osmani AH, Osmani SA. 1995. The NIMA protein kinase is hyperphosphorylated and activated downstream of p34cdc2/cyclin B: coordination of two mitosis-promoting kinases. *EMBO J* 14:986-994.
- Wang X-J, Greenhalgh DA, Jiang A, He D, Zhong L, Medina D, Brinkley BR, Roop DR. 1998. Expression of a p53 mutant in the epidermis of transgenic mice accelerates chemical carcinogenesis. *Oncogene* 17:35-45.
- Westendorf JM, Rao PN, Gerace L. 1994. Cloning of cDNAs for M-phase phosphoproteins recognized by the MPM2 monoclonal antibody and determination of the phosphorylated epitope. *Proc Natl Acad Sci USA* 91:714-718.
- Zheng Y, Wong ML, Alberts B, Mitchison T. 1995. Nucleation of microtubule assembly by a γ -tubulin-containing ring complex. *Nature* 378:578-583.