Partial Characterization of the MPM-2 Phosphoepitope

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The MPM-2 monoclonal antibody recognizes a distinctive group of proteins that are associated with structural components of the mitotic apparatus. These proteins become phosphorylated and MPM-2 reactive during M-phase and appear to be required for both the onset and completion of M-phase. Based upon the analysis of reported MPM-2 reactive sequences, we have developed a model for the essential elements that comprise the MPM-2 epitope. This model was tested by employing a series of synthetic phosphopeptides. We show here that a 14 amino acid synthetic phosphopeptide, derived from a potential MPM-2 site on human DNA topoisomerase II, is recognized by the MPM-2 antibody. This phosphopeptide was sufficient to compete for MPM-2 antibody recognition of (1) an isolated native mitotic MPM-2 antigen on dot blots, (2) proteins on immunoblots of mitotic cell lysates, and (3) specific immunostaining of mitotic cells. These results indicated that the topoisomerase peptide contained all of the essential elements of the MPM-2 epitope. By substituting selected amino acids with alanine, we were able to examine the contribution of different amino acids to the binding between the MPM-2 antibody and the epitope. Changing the amino acid that was adjacent to the phosphorylated threonine residue on the C-terminal side (the +1 position) had no effect on MPM-2 antibody binding. However, substitution of aromatic amino acids at either the −2 or +2 positions reduced antibody recognition. The aromatic amino acid at the −2 position appeared to be the most critical residue of those tested that influenced antibody binding. These results provide information required for the molecular definition of the MPM-2 epitope and should aid in the identification of potential MPM-2 reactive sites on other mitotic phosphoproteins.

INTRODUCTION

MPM-2 is a monoclonal antibody that was raised against mitotic HeLa cells and is characterized by its preferential staining of mitotic cells following indirect immunofluorescence localization [1]. A dramatic increase in the levels of MPM-2 reactive proteins is observed during the G2/M transition in all eukaryotic species examined. Some of the MPM-2 antigens are present on important components of the mitotic apparatus, including chromosomes, kinetochores, centrosomes, and midbodies [2, 3]. Among the identified cell cycle-dependent MPM-2 reactive proteins are microtubule-associated proteins 1 and 4 (MAP1, MAP4) [4, 5], DNA topoisomerase II [6], p42 mapk [7], cdc25 [8], myt1 [9], and wee1 [10]. The importance of these MPM-2 antigens was confirmed following microinjection of the MPM-2 antibody into fertilized Xenopus eggs and HeLa cells, which blocked both the onset and the completion of M-phase [11, 12]. It was proposed that a single MPM-2 kinase was responsible for phosphorylating these cell cycle-regulated MPM-2 reactive proteins. It was also hypothesized that this kinase, together with these MPM-2 reactive proteins, constitute an integral part of the M-phase regulatory mechanism. Despite significant effort, the MPM-2 epitope and the putative MPM-2 kinase have not been clearly identified.

A recent report by Westendorf et al. [13] indicated that the MPM-2 epitope contains a proline residue located on the C-terminal side of a phosphorylated threonine or serine residue. These epitope sites were identified following the in vitro phosphorylation of protein fragments expressed using a λgt11 system or following phage display using a library of pentadecapeptides. Following phosphorylation, the MPM-2 reactive proteins or peptides generated were then isolated and sequenced. The identification of these MPM-2 reactive sites required the presence of an active proline-directed kinase in the mitotic cell extract used to phosphorylate the expressed proteins or peptides. Therefore, it is possible that only a subset of MPM-2 reactive epitopes, those dependent upon the activity of particular kinase(s) in the mitotic cell extract under the selected experimental conditions used, were identified by this approach.

Another MPM-2 reactive site has been identified by Taagepera et al. [7] on p42mapk, a protein first identified by its comigration with an MPM-2 reactive band in...
extracts of Xenopus eggs. This MPM-2 reactive site was shown to contain a phosphorylated threonine residue in the sequence T EY, the regulatory sequence on p42mapk phosphorylated by MAP kinase kinase [7]. These results suggest that the proline residue C-terminal to the phosphorylated residues is not required for MPM-2 recognition. The tyrosine residue in this sequence of p42mapk is also phosphorylated in vivo and limited mutagenesis of this site showed that the Y → F mutation maintained MPM-2 reactivity, while the Y → E mutation showed weakened reactivity. Thus, it was demonstrated that neither the phosphorylation of the tyrosine residue nor the negative charge at that position were required for MPM-2 reactivity. In addition, the downstream F/Y appeared to promote MPM-2 binding while E hindered binding [7], suggesting the importance of the aromatic residue. Since this epitope was identified on a protein whose phosphorylation is not typically associated with mitosis, it could be argued that this proline-independent site is not reflective of the majority of the MPM-2 reactive epitopes found during mitosis that might contain a proline-directed phosphorylation site.

Based solely upon these two previous reports [7, 13], it is difficult to identify common elements that would be required to define an epitope recognized by the same monoclonal antibody. However, upon careful analysis of the reported epitope sites certain common features are detected. In order to further define the MPM-2 epitope, we have designed a series of synthetic peptides based upon a potential MPM-2 reactive site associated with human topoisomerase II [14].

We demonstrate here that the phosphorylated form of a 13 amino acid synthetic peptide corresponding to the potential MPM-2 site in topoisomerase II is indeed MPM-2 reactive, while the corresponding dephosphopeptide is not recognized by the antibody. We have also shown that this synthetic phosphopeptide is sufficient to compete for antibody binding to a native mitotic MPM-2 antigen, MAP4, which blocks MPM-2 recognition of proteins in mitotic cell lysates examined by immunoblot, and prevents MPM-2 antibody localization to cellular structures by indirect immunofluorescence. We also designed and synthesized three more phosphopeptides each containing a single amino acid change from the native sequence. These altered peptides allowed us to examine the contribution of aromatic residues both N- and C-terminal to the phosphothreonine residue on antibody recognition. We show that the alteration of the aromatic residue to the N-terminal side nearly eliminated MPM-2 reactivity. Changing the C-terminal aromatic residue to alanine also reduced antibody binding, but the reduction in binding was not to the same extent as the alteration of the N-terminal aromatic residue. These results strongly support a role for these flanking aromatic residues in defining the MPM-2 epitope. Further, a proline residue adjacent to the phosphorylated serine or threonine on the C-terminal side is not required for antibody recognition of the epitope.

Our findings provide the basis for a more complete analysis of the MPM-2 epitope and may be useful in identifying potential MPM-2 target sequences. In addition, our results provide a model for the MPM-2 epitope that includes both proline-directed as well as proline-independent phosphorylation sites.

**MATERIALS AND METHODS**

**Materials.** Peptides P1 and P2 were synthesized by AnaSpec Inc. (San Jose, CA). Peptides P3, P4, and P5 were synthesized by Princeton BioMolecules Corp. (Columbus, OH). The HPLC purity of these peptides was greater than 95%. MPM-2 mouse monoclonal antibody was a generous gift from Dr. Potu Rao (Department of Chemotherapy Research, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, TX) or purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Peroxidase-conjugated goat anti-mouse antibody was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Keyhole limpet hemocyanin was obtained from CalBiochem (La Jolla, CA), and m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) from Pierce (Rockford, IL). Prepared PD-10 Sephadex columns were obtained from Pharmacia (Uppsala, Sweden). Fetal bovine serum was obtained from Intergen Co. (Purchase, NY), and all other tissue culture components from Gibco BRL Products (Gaithersburg, MD). Cy3-conjugated donkey anti-mouse secondary antibody was obtained from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania). Mitotic HeLa MAP4 was kindly provided by Yunihi Choi (this lab) and was purified essentially as described by Vallee and Collins [15].

**Cell culture.** The HeLa human cervical carcinoma, Chinese hamster ovary (CHO), and LLC-PK pig kidney cell lines were maintained in DMEM, Ham’s F-10, and Medium 199, respectively. The media were supplemented with 10% fetal bovine serum (FBS) and gentamicin (50 μg/ml) with the exception of the Medium 199, which contained 3% FBS and gentamicin. Cells were maintained as monolayer cultures at 37°C in a humidified atmosphere containing 5% CO2.

Conjugating peptides to KLH. Synthetic peptides were conjugated to keyhole limpet hemocyanin (KLH) using a modification of the procedure described by Coligan et al. [16]. Briefly, KLH (5 mg) was dissolved in 0.5 ml Buffer A (0.01 M phosphate buffer, 0.9 M NaCl, 0.01 M EDTA, pH 7.0) and dialyzed against Buffer A overnight at 4°C. MBS was dissolved in dimethylformamide (15 mg/ml) immediately before use, and 70 μl was added to the dialyzed KLH. The mixture was gently stirred for 30 min at room temperature, at which time it was applied to a PD-10 column and eluted with Buffer B (0.05 M phosphate buffer, 0.9 M NaCl, 0.01 M EDTA, pH 6.0). The first peak eluted (KLH-MBS) was pooled and mixed with 5 mg of synthetic peptide that was dissolved in 1 ml of Buffer B. The pH was adjusted to 7.3 and the sample was gently stirred for 3 h at room temperature. The reaction mixture was dialyzed twice against distilled water at 4°C, lyophilized, and stored at −20°C.

**Immunoblot blot and competition experiments.** A Bio-Dot apparatus (Bio-Rad Laboratory, Hercules, CA) was used for applying samples to nitrocellulose paper. Samples were first diluted to a total volume of 50 μl in TBS (154 mM NaCl, 10 mM Tris-Base, pH 7.4) and added to each well. The samples were applied to the nitrocellulose under vacuum, and the wells were then washed several times with a large volume of TBS. The nitrocellulose was removed from the Bio-Dot apparatus, rinsed in TBS, and then blocked in 10% heat-inactivated horse serum in TBS for 1 h. Dot blots were incubated with various dilutions of the MPM-2 antibody for 1 h, washed in...
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RESULTS

The MPM-2 Antibody Recognizes a Phosphopeptide Conserved in Human DNA Topoisomerase IIα and IIβ

We examined the sequences of the previously reported MPM-2 reactive peptides and protein epitopes [7, 13], in an effort to identify similarities in their structures that may be related to antibody recognition (Table 1). The presence or absence of structurally related groups of amino acids were determined for each residue of the MPM-2 reactive peptides on both the N-terminal and C-terminal sides of the phosphorylated amino acid.

A group of amino acids was considered irrelevant to the MPM-2 epitope if none of the amino acids within the group appeared consistently. As a result of this analysis, we found that 90% of the sequences examined contained an aromatic amino acid (most commonly F or W and to a lesser extent Y) on the N-terminal side of the phosphorylated T/S residue. Most frequently, this aromatic residue was located at the –2 position. Many of the MPM-2 reactive sequences also contained either an aromatic amino acid or one or more positively charged amino acids (most commonly R, K) on the C-terminal side of the phosphorylated T/S residue. Threonine was clearly the most frequent amino acid located at the phosphorylation site. In addition, while the majority of the MPM-2 reactive peptides contained a proline residue adjacent to the phosphorylated residue on the C-terminal side, the presence of a proline was not required at this position since the MPM-2 epitope site reported for p42mapk lacked this proline residue [7].

Based upon our analysis, we developed a hypothetical model that would define critical components of the MPM-2 epitope. Our model would predict that maximal MPM-2 antibody binding would be achieved if (1) the phosphorylated residue was a threonine, (2) the phosphorylated amino acid was located near an aromatic amino acid to its N-terminal side, and (3) either an aromatic or positively charged amino acid was located to its C-terminal side.

In order to test the validity of this model, we designed a series of synthetic peptides that could be used to examine the potential contribution of specific amino acids to the MPM-2 epitope. We first needed to establish that a small synthetic peptide obtained from a defined MPM-2 reactive protein was capable of binding

<p>| TABLE 1 |</p>
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<th>MPM-2 Reactive sequences*</th>
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*Sequence 1 was reported to be MPM-2 reactive by Taaagepera et al. [7] and corresponds to amino acids 164–176 of human p42mapk. The remaining sequences were reported by Westendorf et al. and represent the most commonly identified MPM-2 reactive peptides in this report [13]. The sequences were aligned along the position of a potential phosphorylated residue, and multiple alignments are possible with some peptides as indicated. The presence of an aromatic amino acid on the N-terminal side of the potential phosphorylation site is highlighted in bold type. Peptides 2 and 3 represent synthetic peptides generated by Westendorf et al. [13]. Interestingly, peptide 2 was reported to have strong affinity for MPM-2, while peptide 3 was reported to have weak affinity for MPM-2. Peptide 3 lacks an N-terminal aromatic residue; however, the N-terminal aromatic residue is present on the related peptide 4, which was reported to bind the MPM-2 antibody.
to the MPM-2 antibody. Further, this phosphopeptide needed to have an affinity for the MPM-2 antibody that was comparable to a native in vivo MPM-2 antigen. It has been reported that topoisomerase IIα and IIβ were MPM-2 reactive in a cell cycle-dependent fashion [6]. Thus, we prepared a synthetic peptide containing 13 amino acids based upon a putative MPM-2 epitope site in topoisomerase II [14]. An amide-cystine residue was added to the C-terminal end of the peptide to allow for conjugation to carrier protein (Table 2, P1). The phosphorylated form of this peptide was also synthesized (Table 2, P2). This phosphopeptide met the three characteristics we proposed for an MPM-2 epitope, it contained a phosphothreonine residue, an aromatic residue at the −2 position relative to the phosphothreonine, and an aromatic residue at the +2 position. These peptides were individually conjugated to KLH as described under Materials and Methods and tested for their ability to react with the MPM-2 antibody. While an unconjugated KLH control along with the P1-KLH conjugate showed no affinity for the MPM-2 antibody (see below, Fig. 4A, KLH and P1), the P2-KLH conjugate exhibited strong affinity for the MPM-2 antibody similar to that for a human MPM-2 reactive protein, mitotic HeLa cell MAP4 (Fig. 1A). We observed a titration of the MPM-2 antibody against a fixed amount of the P2-KLH conjugate (Fig. 1C), similar to that observed using mitotic MAP4 (Fig. 1B).

There was a slight possibility that certain amino acid/motifs on KLH may contribute to the MPM-2 reactivity of the peptide after conjugation. In order to obtain an unambiguous result, we tested the affinity of the unconjugated peptides for the MPM-2 antibody. Varying amounts of each peptide were preincubated with a constant amount of the MPM-2 antibody. This peptide–antibody mixture was then incubated with nitrocellulose dot blots containing mitotic HeLa cell MAP4, a native MPM-2 reactive protein. If the unconjugated peptide could effectively bind to the MPM-2 antibody, it would compete for reaction with the immobilized MAP4. We demonstrated that the P2 phosphopeptide could effectively block the binding of the MPM-2 antibody to mitotic MAP4 (Fig. 2A, P2). This inhibition was clearly dependent upon the amount of peptide preincubated with the MPM-2 antibody (Fig. 2B). On the other hand, the nonphosphorylated P1 peptide failed to exhibit any inhibition of the binding between the MPM-2 antibody and MAP4 (Fig. 2A, P1). Similar results were observed when the P2 phosphopeptide–KLH conjugate was used in place of MAP4 (Figs. 3A and 3B). These results not only showed that the unconjugated 14 amino acid phosphopeptide could bind to the MPM-2 antibody in solution, but that it could also compete

### TABLE 2

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<th>Peptide constructs</th>
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<td>[NH₂]-RKEWLT NFMEDRRC-[CONH₂]</td>
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<td>[NH₂]-RKEWLTpAFMEDRRC-[CONH₂]</td>
<td>[NH₂]-RKEALTpNFMEDRRC-[CONH₂]</td>
<td>[NH₂]-RKEWLTpAMEDRRC-[CONH₂]</td>
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*a The first 13 amino acids in P1 correspond to aa660–aa672 in human DNA topoisomerase IIa and aa682–aa694 in human DNA topoisomerase IIb.

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**FIG. 1.** Recognition of the P2-KLH conjugate by the MPM-2 antibody. (A) Dot-blot of mitotic MAP4 and the P2-KLH conjugate at different MPM-2 antibody concentrations (1: 1:1,000,000; 2: 1:100,000; 3: 1:20,000; 4: 10,000; 5: 1:1,000). Mitotic MAP4 (1.5 μg) and P2-KLH conjugate (1.25 μg) were applied to each dot. (B) Corresponding plot of the amount of bound MPM-2 antibody to mitotic MAP4 (Integrated Optical Density) against the antibody concentrations used, based upon the data from A. (C) Corresponding plot of the amount of bound MPM-2 antibody to the P2-KLH conjugate (Integrated Optical Density) against the antibody concentrations used, based upon the data from A.
Extracts were prepared from synchronized populations of mitotic CHO or HeLa cells, and examined by immunoblot of nitrocellulose transfers following electrophoretic separation on polyacrylamide gels (Fig. 4). As can be seen in the immunoblots of both cell types, preincubation of the MPM-2 antibody with increasing concentrations of P1 (up to 100 µg/ml) had no effect on the recognition of mitotic phosphoproteins in these samples. However, preincubation of the MPM-2 antibody with the phosphorylated topoisomerase II peptide (P2) inhibited the binding of MPM-2 to all of the mitotic phosphoproteins. Similar results were obtained with populations of S phase cells synchronized using hydroxyurea and cells in logarithmic growth (data not presented).

The immunofluorescence localization of the MPM-2 antibody in LLC-PK cells was also inhibited by preincubation with the P2 synthetic peptide regardless of cell cycle stage (Fig. 5). In control cells, weak MPM-2 antibody staining of patches in the nuclei of interphase cells was observed, while more intense nuclear staining and centrosomal staining was apparent in prophase cells. Extensive cytoplasmic staining with more intense specific localization to the centrosomes and spindle was for the binding of the antibody to a mitotic protein known to be MPM-2 reactive in vivo. This suggested that the P2 phosphopeptide, derived from human topoisomerase II, contains all the elements of the MPM-2 epitope necessary for MPM-2 binding.

Recognition of Mitotic Phosphoepitopes by the MPM-2 Antibody Is Blocked by Preincubation with the Synthetic Topoisomerase II Phosphopeptide But Not by the Corresponding Dephosphopeptide

It was possible that the P2 phosphopeptide represented only a subset of the mitotic phosphoepitopes recognized by the MPM-2 antibody since it lacked the proline residue C-terminal to the phosphorylated amino acid that is common both to most previously identified MPM-2 reactive peptides [7], as well as consensus phosphorylation sites for kinases that are known to be active at mitosis, such as the cyclin-dependent kinases. To ensure that the topoisomerase II phosphopeptide was indeed representative of all MPM-2 reactive epitopes, we determined whether preincubation of the MPM-2 antibody with peptide in either the dephosphorylated (P1) or phosphorylated form (P2) would inhibit antibody reactivity.

FIG. 2. Competition of synthetic peptides for MPM-2 antibody binding to mitotic MAP4. (A) Peptides P1 or P2 were used to compete for binding of MPM-2 antibody against mitotic MAP4 blotted onto nitrocellulose paper. Mitotic MAP4 (1.5 µg) was applied to each dot. Different amounts (µg) of the P1 or P2 synthetic peptides were added to 3 ml of MPM-2 antibody solution diluted to 1:20,000 and preincubated for 1 h prior to application to the dot-blot. (B) The amount of bound MPM-2 antibody was determined using image analysis software and plotted against the amounts of synthetic peptides P1 (□) or P2 (■) preincubated with the MPM-2 antibody.

FIG. 3. Competition of synthetic peptides for MPM-2 antibody binding to the P2-KLH conjugate. (A) Peptides P1 or P2 were used to compete for binding of MPM-2 antibody against P2-KLH conjugate blotted onto nitrocellulose paper. P2-KLH conjugate (1.25 µg) was applied to each dot. Different amounts (µg) of the P1 or P2 synthetic peptides were added to 3 ml of MPM-2 antibody solution diluted to 1:20,000 and preincubated for 1 h prior to application to the dot-blot. (B) The amount of bound MPM-2 antibody was determined using image analysis software and plotted against the amounts of synthetic peptides P1 (□) or P2 (■) preincubated with the MPM-2 antibody.
eliminated and the centrosomal staining intensity was reduced. At concentrations of 100 μg/ml, preincubation with P2 totally abolished MPM-2 staining, while similar levels of P1 had no effect on MPM-2 staining patterns.

These results strongly indicate that the synthetic phosphopeptide P2 that is derived from the sequence of topoisomerase II represents a typical MPM-2 epitope in that this peptide can compete for the binding of the MPM-2 antibody with all mitotic phosphoproteins recognized by the antibody.

Critical Components of the MPM-2 Phosphoepitope Include Aromatic Amino Acids Located Both to the N- and C-Terminal of the Phosphorylated Residue

Having shown that the P2 phosphopeptide derived from topoisomerase II was indeed sufficient to serve as an MPM-2 epitope, we further examined the characteristics of this epitope by changing selected amino acids to alanine (Table 2, peptides P3, P4, and P5). These residues were selected to examine the importance of the aromatic amino acids on either side of the phosphothreonine residue. Each of these phosphopeptides were also conjugated to KLH and tested on dot-blots for their MPM-2 reactivity as described above. We compared the staining of the KLH conjugates containing the modified peptides with the original P2-KLH conjugate (Fig. 7A). Changing from N at the +1 position to A did not generate any observable difference in the affinity of this peptide conjugate for the MPM-2 antibody (Fig. 7, P3). This result was not unexpected, since this modification did not affect either of the flanking aromatic residues. In addition, the minimal effect of this alteration further indicated that proline was not required at the +1 position in the MPM-2 epitope.

In contrast, changing the aromatic amino acid W to A at the –2 position greatly reduced this peptide’s ability to bind to MPM-2 antibody. Binding of MPM-2 antibody to the P4-KLH conjugate was observed only at extremely high substrate amounts, and also higher antibody concentrations (Fig. 7B, P4). The P4-KLH conjugate was not detected using lower antibody concentrations that still reacted with the P2-KLH conjugate (Fig. 7A, P4). Interestingly, changing the aromatic acid at the +2 position from F to A produced little difference in the ability of this peptide conjugate, P5-KLH, to react with the MPM-2 antibody (Fig. 7A, 10 and 2 μg). Decreased staining was only observed when the amount of the P5-KLH conjugate present on the dot blot was greatly reduced (Fig. 7A, 0.4 μg).

These results showed that the aromatic amino acid on the N-terminal side of the phosphothreonine is critical for the binding of MPM-2 antibody. However, we had also proposed an important role in the binding of MPM-2 for the aromatic amino acid at the +2 position,
FIG. 5. Inhibition of MPM-2 immunofluorescence staining by preincubation with the phosphopeptide P2. LLC-PK cells were double-labeled by indirect immunofluorescence with the MPM-2 antibody at a dilution of 1:16,000 (A, C, E, G, I, and K) and DAPI to stain cellular DNA (B, D, F, H, J, and L). Control MPM-2 staining showed weak staining of patches within the nucleus of interphase cells (A), and intense nuclear staining of the nucleus in prophase cells (E). The two centrosomes were also stained by MPM-2 in the prophase cell (E, arrowheads). Both the cytoplasm and the mitotic spindle were intensely stained in control metaphase cells (I). Following preincubation with 10 μg/ml of the phosphorylated peptide P2, MPM-2 staining of the interphase nucleus was abolished (C). Specific immunolocalization of the MPM-2 antibody was also nearly eliminated in both prophase and metaphase cells following preincubation with P2 (G and K, respectively). Some weak staining was still observed over the metaphase spindle by the preincubated MPM-2 antibody. Similar reduction in the level of MPM-2 staining was observed in cells regardless of cell cycle stage following preincubation with the P2 peptide (data not presented). Bar, 10 μm.

but the dot-bLOTS suggested that this amino acid has only a limited effect on the antibody–antigen binding. Since the conjugation efficiency of each synthetic peptide with KLH may not be identical, it was not possible to use the peptide conjugate alone to quantitatively assess the level of MPM-2 reactivity. In order to obtain a more precise indication as to the extent with which these amino acids might contribute to the MPM-
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FIG. 7. MPM-2 antibody binding to modified synthetic peptide-KLH conjugates. Different amounts (10, 2, and 0.4 μg) of KLH (KLH) or peptide-KLH conjugates (P1, P2, P3, P4, and P5) were applied to the nitrocellulose paper and tested for their MPM-2 binding ability at different antibody dilutions (A, 1:10,000; B, 1:1,000). Antibody binding was compared between the control topoisomerase peptide P1 and P2, and these modified phosphopeptides P3, P4, and P5 (see Table 2).

2 epitope, we again examined each peptide using the competition experiments. As expected, preincubation with peptide P3 inhibited the ability of the MPM-2 antibody to bind to the P2-KLH conjugate (Fig. 8). In contrast, peptide P4 did not inhibit binding of the MPM-2 antibody, except at relatively high phosphopeptide concentrations (Fig. 8). Both the P3 and P4 competition experiments were consistent with the previous dot blot results (Fig. 7), indicating that the weak reaction of the MPM-2 antibody with the P4-KLH conjugate was not simply due to differences in the level of peptide conjugation.

In contrast to the dot blot results, which demonstrated a similar ability of the P5-KLH conjugate to bind the MPM-2 antibody (Fig. 7), the P5 phosphopeptide showed an intermediate ability to compete against the P2-KLH conjugate (Fig. 8). Thus the competition experiment gave a more precise quantitative measurement of the differences between each of the synthetic phosphopeptides and their interaction with the MPM-2 antibody than that which could be achieved using the dot blots.

In summary, our results indicate that in addition to the phosphorylated threonine, the aromatic residue at the −2 position was essential for MPM-2 recognition of the P2 phosphopeptide. The aromatic residue at the +2 position was also important, but contributes less to the antibody binding than that at the −2 position. A diagram that depicts our model of the MPM-2 epitope, which is based upon the results obtained in this study, is presented in Fig. 9.

DISCUSSION

In this report, we have shown that a synthetic phosphopeptide composed of 14 amino acids has the capacity to serve as an epitope for the MPM-2 monoclonal antibody. This phosphopeptide can compete against both native and synthetic MPM-2 antigens for the binding of the MPM-2 antibody. This peptide was derived from the sequence of topoisomerase II, which has been reported to be an MPM-2 reactive protein that is phosphorylated in a cell cycle-dependent fashion [6]. We have demonstrated the importance of an aromatic amino acid on the N-terminal side of the phosphorylated threonine for the antibody—antigen interaction. Our data on the +2 position indicates that an aromatic amino acid at the C-terminal side will also enhance the binding between the MPM-2 antibody and antigen, confirming results reported for the MPM-2 epitope of p42mapk [7].

In contrast to the previously reported requirement for a proline at the +1 position of the MPM-2 epitope [13], we have shown that proline is not required at this position for antibody recognition. The identification and characterization of proline-directed MPM-2 reactive epitopes reported by Westendorf et al. [13], required that the peptide substrates were phosphorylated with kinases present in a mitotic cell extract prior to reaction with the MPM-2 antibody. This was necessary, since posttranslational modifications such as phosphorylation are not carried out in the expression systems which were utilized [13]. Thus, these MPM-2 reactive phosphopeptides were preselected by

FIG. 6. Inhibition of the MPM-2 mitotic immunostaining is dependent upon preincubation of the antibody with phosphorylated peptide. Micrographs of double-labeled metaphase LLC-PK1 cells are presented. Preincubation of the MPM-2 antibody (1:16,000 dilution) with increasing concentrations of dephosphorylated peptide P1 had no effect on the staining of mitotic structures, including the mitotic cytoplasm, spindle poles, kinetochores, or kinetochore fibers (A, C, E, and G). The corresponding DAPI staining patterns are also shown (B, D, F, and H). Preincubation of the MPM-2 antibody with increasing concentrations of the phosphorylated peptide P2 inhibited the MPM-2 staining of all mitotic structures (I, K, M, and O). Corresponding DAPI staining demonstrates that all cells were in metaphase (J, L, N, and P). Peptide concentrations used during the preincubation with the MPM-2 antibody were 0.1 μg/ml (A and I), 1.0 μg/ml (C and K), 10.0 μg/ml (E and M), and 100.0 μg/ml (G and O). Concentrations of the P2 peptide as low as 1.0 μg/ml were able to significantly reduce the level of MPM-2 staining of the mitotic cells, whereas 100.0 μg/ml completely abolished MPM-2 staining. Bar, 10 μm.
their ability to be phosphorylated in vitro, and it is possible that other MPM-2 reactive substrates were not detected due to the conditions used to phosphorylate the peptide samples. Our results suggest that the +1 position has little or no influence on the recognition of the phosphoepitope by the MPM-2 antibody. Similarly, a proline was not present in the +1 position of the MPM-2 epitope reported on p42\textsuperscript{mapk} [7]. Therefore, while mitosis-specific substrates for the MPM-2 antibody may contain a proline residue at the +1 position, this is not a required element of the epitope. Whether the proline-directed MPM-2 epitope sites are associated with known mitosis-specific kinases is irrelevant in defining the components required for recognition of the MPM-2 epitope by the MPM-2 antibody. Clearly, some of the MPM-2 epitope sites may contain proline-directed sites, but they need not be restricted to proline-directed sites.

Based upon the analysis of the MPM-2 reactive sequences reported here, and those previously described [7, 13], we propose a model for the interaction between the MPM-2 antibody and the MPM-2 epitope (Fig. 9). Most importantly, the epitope requires a phosphorylated amino acid, preferably phosphothreonine, although phosphoserine appears to be able to substitute. However, the phosphothreonine itself is not sufficient for the binding of antibody. An aromatic amino acid on the N-terminal side of the phosphothreonine appears to have a major positive influence on forming a stable complex between the MPM-2 antibody and the antigen. The stability of the complex will be further enhanced if there is an aromatic amino acid or positively charged amino acid on the C-terminal side of the phosphothreonine. Thus, an MPM-2 phosphoepitope will exhibit maximal antibody affinity when both the N- and C-terminal elements are present. We speculate that native MPM-2 reactive proteins will contain these epitope determinants. All of the factors that define the MPM-2 epitope have not been fully delineated, however, and we would expect that other elements having either positive and negative effects remain to be defined. For example, a proline residue located at the +1 position, while not required for antibody binding, may enhance antibody binding.

Several protein kinases have been implicated in the phosphorylation of MPM-2 epitope, including MEK [7], MAPK [17], and cdc2 [13]. In addition, a novel and as yet unidentified kinase that phosphorylates the MPM-2 epitope site of topoisomerase II corresponding to peptide P2, as defined here, may also exist. Although it is possible that a single MPM-2 kinase phosphorylates most MPM-2 sites in vivo, our data on the structural requirements of the MPM-2 epitope, as well as the multiple MPM-2 kinases that have been characterized to date, indicates that the MPM-2 epitope may coexist or partially overlap with a number of kinase consensus sequences. Each of these different kinases may be responsible for the phosphorylation of one or more MPM-2 reactive proteins. It is likely that only some of the substrate proteins for these kinases contain all of the essential elements of the MPM-2 epitope, and only these selected substrates will become MPM-2 reactive following phosphorylation. For example, this would explain how the cdc2 kinase might restore MPM-2 reactivity on some proteins, whereas most cdc2 phosphorylation sites would not be recognized by the MPM-2 antibody.

Several proteins recognized by MPM-2 have been shown to be present during interphase, but they only become MPM-2 reactive during M-phase [5, 6, 18]. Thus, the phosphorylation of the MPM-2 site may serve to regulate the functions of the various MPM-2 reactive proteins.
proteins and their respective kinases. Here is modified in vivo similar interphase cell lysates. These results suggest (1990) mitotic cell lysates. These proteins are not detected in 20. Anderson, N. G., Maller, J. L., Tonks, N. K., and Sturgill, T. W. M-phase. Preliminary evidence indicates that antibod-

Thus, the MPM-2 reactive topoisomerase II peptide sequence we have identified here could be used as a unique substrate to identify/isolate the novel in vivo topoisomerase II kinase, which may be important in the regulation of topoisomerase activity. Coupled with our knowledge of the MPM-2 epitope, this approach could be applied in general to purify or identify the corresponding in vivo kinases of other MPM-2 reactive proteins. This approach would clearly establish whether there is a single in vivo MPM-2 epitope kinase, or whether there are multiple kinases involved in establishing MPM-2 reactivity of different proteins during M-phase. Preliminary evidence indicates that antibodies generated against the phosphorylated topoisomerase II peptide P2 used in these studies specifically recognize a number of proteins by immunoblot analysis of mitotic cell lysates. These proteins are not detected in similar interphase cell lysates. These results suggest that the topoisomerase II phosphorylation site reported here is modified in vivo in a cell cycle-dependent fashion and may represent a common phosphoepitope present in many mitotic proteins. It appears likely that a single MPM-2 epitope kinase does not exist, but that the MPM-2 epitope will prove important for the identification of a number of unique mitosis-specific phosphoproteins and their respective kinases.

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REFERENCES


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