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The MPM-2 Antibody Inhibits Mitogen-activated Protein Kinase Activity by Binding to an Epitope Containing Phosphothreonine-183

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Mitogen-activated protein (MAP) kinases are a family of serine/threonine kinases implicated in the control of cell proliferation and differentiation. We have found that activated $p42^{mapk}$ is a target for the phosphoepitope antibody MPM-2, a monoclonal antibody that recognizes a cell cycle–regulated phosphoepitope. We have determined that the MPM-2 antibody recognizes the regulatory region of $p42^{mapk}$. Binding of the MPM-2 antibody to active $p42^{mapk}$ in vitro results in a decrease in $p42^{mapk}$ enzymatic activity. The MPM-2 phosphoepitope can be generated in vitro on bacterially expressed $p42^{mapk}$ by phosphorylation with either isoform of MAP kinase kinase (MKK), MKK1, or MKK2. Analysis of $p42^{mapk}$ proteins mutated in their regulatory sites shows that phosphorylated Thr-183 is essential for the binding of the MPM-2 antibody. MPM-2 binding to Thr-183 is affected by the amino acid present in the other regulatory site, Tyr-185. Substitution of Tyr-185 with phenylalanine results in strong binding of the MPM-2 antibody, whereas substitution with glutamic acid substantially diminishes MPM-2 antibody binding. The MPM-2 phosphoepitope antibody recognizes an amino acid domain incorporating the regulatory phosphothreonine on activated $p42^{mapk}$ in eggs during meiosis and in mammalian cultured cells during the G_0 to G_1 transition.

INTRODUCTION

The mitogen-activated protein (MAP) kinases play critical roles in cell cycle control. MAP kinases or ERKs, also known as extracellular signal-regulated kinases, are a family of serine/threonine specific protein kinases that mediate intracellular signal transmission in response to external stimuli (Errede and Levin, 1992; Ruderman, 1993). $p42^{mapk}$ requires phosphorylation on both Thr-183 and Tyr-185 for full activation (Anderson *et al.*, 1990; Payne *et al.*, 1991). In vivo, $p42^{mapk}$ is phosphorylated and activated by MAP kinase kinase (MKK), also known as MEK, a dual specificity protein threonine/tyrosine kinase (Ahn *et al.*, 1992; Crews and Erikson, 1993). $p42^{mapk}$ homologues have been shown to play a cell cycle–regulating role in the mating/meiosis response in both budding and fission yeast, in the G_0/G_1 transition of mammalian somatic cells, and in the passage of oocytes through meiosis (Pelech and Sanghera, 1992).

The importance of $p42^{mapk}$ function in meiosis is well established in *Xenopus laevis* oocytes (Posada *et al.*, 1991; Posada and Cooper, 1992). A key event in meiotic maturation is the activation of M-phase promoting factor (MPF), a complex of a serine/threonine kinase, $p34^{cdc2}$, and a regulatory subunit, cyclin (Gautier *et al.*, 1988). $p42^{mapk}$, which shares a considerable sequence similarity with $p34^{cdc2}$ kinase (Pelech and Sanghera, 1992; Mazzoni *et al.*, 1993), is also phosphorylated and activated upon the first meiotic division (Haccard *et al.*, 1990; Ferrell *et al.*, 1991; Posada and Cooper, 1992). Activation of the $p34^{cdc2}$ kinase appears to precede the activation of $p42^{mapk}$ (Gotoh *et al.*, 1991a,b) and is thought to lead indirectly to the activation of $p42^{mapk}$, because microinjection of MPF into immature oocytes or addition of MPF to cell-free extracts of interphase

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embryos results in the activation of $p42^{mapk}$ (Gotoh *et al.*, 1991a). However, in contrast to $p34^{cdc2}$ kinase activity, which diminishes between the two meiotic divisions, $p42^{mapk}$ activity remains high (Ferrell *et al.*, 1991). It is thought that these high levels of $p42^{mapk}$ activity may explain why the oocyte remains in M-phase between meiosis I and II, despite the loss of $p34^{cdc2}$ kinase activity (Verlhac *et al.*, 1994). In addition to being essential for proper oocyte maturation, $p42^{mapk}$ activity is critical for arresting matured oocytes in meiosis II (Haccard *et al.*, 1993), presumably due to its indirect activation by the c-mos protooncogene (Posada *et al.*, 1993; Shibuya and Ruderman, 1993). Addition of purified $p42^{mapk}$ to extracts of interphase frog eggs results in the reorganization of microtubules into a metaphase-like pattern (Gotoh *et al.*, 1991b), suggesting that $p42^{mapk}$ may influence the meiotic cell cycle by altering microtubule dynamics.

Whether $p42^{mapk}$ plays a significant role in mitosis remains controversial. $p42^{mapk}$ is apparently reactivated during the early cell divisions after fertilization of sea urchin and sea star eggs (Pelech *et al.*, 1988) but not in the early divisions of *Xenopus* eggs (Ferrell *et al.*, 1991; Posada *et al.*, 1991). Tamemoto et al. (1992) have found evidence for biphasic activation of $p42^{mapk}$ in mammalian cells, with levels of $p42^{mapk}$ activity peaking at S phase and the G_2/M transition.

The MPM-2 monoclonal antibody was originally prepared against extracts of mitotic HeLa cells (Davis and Rao, 1987). MPM-2 recognizes a phosphoepitope expressed on multiple proteins predominantly during M-phase (Davis *et al.*, 1983). In both meiotic and mitotic cells, the MPM-2 antigen is concentrated at key structural elements, including the centrosomes, spindle fibers, kinetochores, and chromosome arms (Taagepera *et al.*, 1993; Vandre *et al.*, 1984). The MPM-2 phosphoepitope appears to be expressed on proteins associated with regulation of the cell cycle. Microinjection of the MPM-2 antibody into *Xenopus* oocytes before progesterone stimulation inhibits the appearance of MPF activity (Kuang *et al.*, 1989). Similarly, addition of MPM-2 to *Xenopus* M-phase egg extracts results in the immunodepletion of MPF activity (Kuang *et al.*, 1989). As the $p34^{cdc2}$ kinase and cyclin B proteins do not express the MPM-2 phosphoepitope (Kuang *et al.*, 1994), these data suggest that the MPM-2 antibody may be interacting with proteins regulating MPF activity.

Several recent advances have been made in the characterization of the molecular nature of the MPM-2 phosphoepitopes. Certain reports suggest that epitopes recognized by the MPM-2 antibody can be created in vitro by proline-directed kinases (Kuang and Ashorn, 1993; Westendorf *et al.*, 1994). Kuang and Ashorn (1993) purified two separate kinase activities from unfertilized *Xenopus* eggs that were capable of generating an MPM-2 phosphoepitope on immature *Xenopus* oocyte proteins: a $p42^{mapk}$-like protein kinase and an unidentified high-molecular-weight protein kinase. Westendorf *et al.* (1994) demonstrated that $p34^{cdc2}$ immunoprecipitated from extracts of M-phase cells was capable of generating an MPM-2 phosphoepitope on artificial substrate peptides. In each case, the purified kinase catalyzed the creation of some, but not all, of the phosphoepitopes formed in vitro by treatment with whole M-phase cell extracts. The data summarized above suggest that, in vivo, it is likely that multiple kinases are involved in the creation of the MPM-2 phosphoepitopes.

Here we show that the phosphoepitope antibody, MPM-2, recognizes the activated form of $p42^{mapk}$. The MPM-2 phosphoepitope is present on $p42^{mapk}$ that has been activated either in vivo or in vitro. Addition of the MPM-2 phosphoepitope antibody to active $p42^{mapk}$ results in a reduction of $p42^{mapk}$ catalytic activity. Using bacterially expressed $p42^{mapk}$ mutants altered in their regulatory sites (Her *et al.*, 1993), we have determined that the MPM-2 phosphoepitope antibody recognizes the activating phosphorylation on $p42^{mapk}$ Thr-183.

MATERIALS AND METHODS

Preparation of Xenopus Egg Extracts

Adult *Xenopus* laevis were purchased from Xenopus 1 (Ann Arbor, MI). Females were induced to ovulate by the injection of human chorionic gonadotropin (ICN Biochemicals, Costa Mesa, CA). Matured eggs were dejellied in 2% cysteine (pH 7.9) as described by Newport and Kirschner (1982). All of the following steps were carried out at 4°C. For immunoblotting of egg extracts, 10 eggs were homogenized for each sample by shearing through a 26-gauge needle in 250 μl of buffer containing 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.5), 100 mM NaCl containing protease inhibitors (aprotinin, leupeptin, antipain, pepstatin, chymostatin; each at 5 μg/ml), and phosphatase inhibitors (100 nM microcystin-LR and 1 mM sodium orthovanadate).

Yolk proteins were extracted from lysates following the procedures of Gurdon and Wickens (1983). Briefly, egg extracts were treated with an equal volume of 1,1,2-trichlorotrifluoroethane (Freon), (T-5271, Sigma Chemical, St. Louis, MO) vortexed, and chilled. Extracts were centrifuged in a microcentrifuge for 10 min at 16 000 × *g*. The upper aqueous phase was recovered and transferred to a clean tube. The extracts were either used immediately by adding 1:5 volume of 5X sodium dodecyl sulfate (SDS) sample buffer and boiling or were frozen in liquid nitrogen and stored at −70°C.

For immunoprecipitation experiments, extracts were prepared in the following manner from eggs before and 50 min after fertilization. Eggs (10 per sample) were disrupted by pipetting in 100 μl of buffer (40 mM NaCl, 10 mM Tris, 10 mM EDTA, pH 7.5) (Ferrell *et al.*, 1991) containing the protease and phosphatase inhibitors described above. Extracts were then centrifuged in a microcentrifuge for 90 s at 16 000 × *g*. The aqueous phase was collected and used immediately for immunoprecipitation experiments.

Cell Culture, Serum Starvation, and Preparation of Cell Extracts

NIH mouse 3T3 fibroblasts were grown to confluence in 100-mm dishes in Dulbecco's modified Eagle's medium with 10% (vol/vol) fetal calf serum. Confluent cells (~10^7 cells per dish) were rinsed and incubated for 18 h in serum-free medium. Quiescent cell were stimulated to reenter the cell cycle with the addition of 10% (vol/vol,

final concentration) fetal calf serum. At various times after stimulation, plates were placed on ice and quickly rinsed with cold phosphate-buffered saline (4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4, pH 7.4, 137 mM NaCl, 2.7 mM KCl). All the subsequent steps were carried out at 5°C. The cells were scraped into ice-cold HO buffer (50 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.5 at 4°C, 100 mM NaCl, 2 mm EDTA, 1% Nonidet P-40) containing the protease inhibitors pepstatin (1 μM), leupeptin (1 μg/ml), aprotinin (2 μg/ml), and phenylmethylsulfonyl fluoride (0.2 mM) and the phosphatase inhibitors sodium orthovanadate (0.2 mM) and 4-nitrophenyl phosphate (PNPP) (40 mM). Crude cell homogenates were clarified by centrifugation in a microcentrifuge for 5 min at 16 000 × *g*, and the supernatants were used directly in the p42^{mapk} immunoprecipitation experiments described below.

Electrophoresis and Immunoblot Analysis

Protein samples were analyzed by electrophoresis on SDS/5–20% gradient polyacrylamide gels. *Xenopus* extract proteins were electrophoresed on single-well gels and transferred onto nitrocellulose. The nitrocellulose was cut into strips, and the strips were blocked for 2 h in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween-20) containing 3% bovine serum albumin (BSA). Nitrocellulose strips were then incubated for 2 h with one of the following antibodies: MPM-2, the phosphoepitope monoclonal antibody, kindly provided by Potu Rao (ascites fluid diluted 1:5000); the anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Lake Placid, NY) at 1.0 μg/ml; or monoclonal anti-p42^{mapk} antibody B3B9, provided by Dr. Michael J. Weber at 0.4 μg/ml. For detection, nitrocellulose strips were rinsed in TBST, incubated with anti-mouse or anti-rabbit horseradish peroxidase antibodies (Jackson Immunoresearch, West Grove, PA) at a concentration of 0.16 μg/ml for 1 h and processed with the Renaissance Western Blot Chemiluminescence System (DuPont NEN, Boston, MA). Immunoprecipitated p42^{mapk} and recombinant p42^{mapk} proteins were similarly processed for immunoblotting with the MPM-2, anti-phosphotyrosine or anti-p42^{mapk} antibodies.

Immunoprecipitation

Before immunoprecipitation, *Xenopus* egg extract proteins were denatured by adding SDS to a final concentration of 1% from a 10% stock solution and boiling for 5 min. The rest of the immunoprecipitation procedure was carried out at 4°C. Extracts were cleared by centrifugation in a microcentrifuge at 16 000 × *g* for 5 min. The SDS was diluted to a final concentration of 0.1% by the addition of 500 μl of 2X immunoprecipitation buffer (final 1× concentration: 150 mM NaCl, 10 mM Tris, pH 7.4, 1% Triton-X 100, 0.5% NP-40 containing the protease and phosphatase inhibitors described above) and 400 μl distilled H_2O. Protease and phosphatase inhibitors, as described in the preparation of *Xenopus* extracts, were used in all buffers. The p42^{mapk} was precipitated by incubating the extracts with 7.5 μl (dry volume) of anti-p42^{mapk} antibody conjugated to agarose beads (cat no. sc-154 AC, Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h. The immune complexes were collected by centrifugation for 30 s at 16 000 × *g* in a microcentrifuge, washed four times in RIPA buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS). After the final centrifugation, the beads were resuspended in 50 μl SDS sample buffer and 5 μl β-mercaptoethanol and then boiled for 2 min. The sample was centrifuged, and the supernatant containing the released proteins was analyzed by electrophoresis and immunoblotting.

Immunoprecipitation of p42^{mapk} from NIH 3T3 cell extracts was performed as follows. For each sample a 10-μl aliquot of rabbit TR-10 anti-p42^{mapk} serum (provided by Dr. Michael Weber) was adsorbed to 50 μl (dry volume) of Protein A trisacryl beads (Pierce, Rockford, IL) in 200 μl HO buffer for 1 h. The antibody-Protein A-trisacryl complex was washed three times with HO buffer by centrifugation and resuspension and then added to 200 μl of cell extract and incubated for 2 h. The immune complexes were collected and washed four times in HO buffer. An aliquot of the sample was assayed for p42^{mapk} enzymatic activity. The rest was resuspended in SDS sample buffer with β-mercaptoethanol and boiled in preparation for electrophoresis and immunoblotting.

p42^{mapk} Activity Assay

Enzymatic activity of the immunoprecipitated p42^{mapk} was measured using MBP as substrate, as described previously (Erickson *et al.*, 1990). Briefly, immunoprecipitated p42^{mapk} was incubated at 30°C for 30 min in 40 μl (total volume) of 25 mM Tris/HCl (pH 7.5 at 20°C), 15 mM $MgCl_2$, 40 mM PNPP, 0.1% (vol/vol) β-mercaptoethanol, 0.1 mM [γ-^{32}P]ATP (5000 cpm/pmol), with MBP at 0.5 mg/ml. The reaction was terminated by spotting 38 μl of each reaction mixture onto (1 cm^2) P81 cellulose paper (Whatman, Maidstone, UK), and immersing the filter in 180 mM H_3PO_4. Filters were washed three times with H_3PO_4 and retained radioactivity was quantified. Incorporated radioactivity, corrected for background, was expressed in pmol/min.

Activation of Recombinant p42^{mapk} and p42^{mapk} Mutants with MKK

Homogeneous nonphosphorylated recombinant p42^{mapk} was purified as described (Her *et al.*, 1993). The p42^{mapk} mutants used in this study were T183A, Y185F, T183A/Y185F, T183E, and Y185E, where the first letter indicates the amino acid at the number indicated in the wild-type protein and the second letter indicates the amino acid present at that position in the mutant protein. (His)6-tagged MKK1 and MKK2 proteins were expressed in insect cells by baculovirus vectors (Dent *et al.*, 1994). Partially active preparations of both MKK proteins were prepared by coinfection of insect cells with additional recombinant viruses expressing the Raf-1, c-Ha-ras, and c-Src (Y527F) proteins, followed by purification to near homogeneity on successive Nickel-chelate and Mono Q ion-exchange chromatography (Fabian *et al.*, 1993; Dent *et al.*, 1994). Purified recombinant p42^{mapk} protein was incubated with partially active MKK protein in a phosphorylation buffer containing 25 mM Tris/HCl (pH 7.5 at 20°C), 15 mM MgCl, and either 0.1 mM labeled [γ-^{32}P]ATP (5000 cpm/pmol) or unlabeled ATP, in a total volume of 40 μl, for 15 min at 30°C. Reactions were terminated by the addition of 10 μl SDS polyacrylamide gel electrophoresis (5×) sample buffer, and samples were electrophoresed on SDS-containing polyacrylamide gels. ^{32}P-labeled p42^{mapk} bands were excised from the gel and their phosphate incorporation determined. Samples prepared with nonradioactive ATP were immunoblotted with the MPM-2, anti-phosphotyrosine, and anti-p42^{mapk} antibodies.

Assay for MPM-2 Inhibition of p42^{mapk} Activity

The MPM-2 monoclonal antibody was purified from ascites fluid by binding to Protein G Sepharose beads in binding buffer containing 50 mM Tris/HCl, pH 7.5 at 4°C, 0.1 mM EDTA, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, and 0.1% Triton-X 100 containing 5 mg/ml BSA. This technique was designed to minimize loss of the MPM-2 phosphoepitope antibody during antibody purification procedures. A control ascites (anti-SV-40 T antigen, Zymed Laboratories, South San Francisco, CA) was used in these experiments to assess nonspecific effects of mouse antibody on p42^{mapk} activity. The antibody/Protein G Sepharose bead complexes were rinsed in binding buffer and incubated with 0.5 μg recombinant activated p42^{mapk} for 2 h at 4°C. Activity of the p42^{mapk} present in these solutions was determined by assaying for MBP kinase activity as described above.

RESULTS

p42^{mapk} in Meiotic Xenopus Eggs Contains the MPM-2 Phosphoepitope

Using proteins extracted from *Xenopus* eggs, we compared the electrophoretic mobilities of MPM-2-anti-

body–positive bands with those of known cell cycle–regulatory proteins. The components of MPF, $p34^{cdc2}$, or cyclin B did not appear to co-migrate with MPM-2 positive bands. We did find that a minor MPM-2 antibody-reactive band co-migrated with a band recognized by monoclonal antibody to $p42^{mapk}$ (Figure 1A). Active $p42^{mapk}$ has previously been identified as a relatively nonabundant protein in extracts of mature *Xenopus* eggs (Gotoh *et al.*, 1991b; Barrett *et al.*, 1992).

To test whether active $p42^{mapk}$ contained the MPM-2 phosphoepitope, $p42^{mapk}$ was immunoprecipitated from extracts prepared from unfertilized meiotic egg extracts and from eggs 50 min after fertilization. The $p42^{mapk}$ immunoprecipitated from unfertilized meiotic egg extract, but not the interphase extract, was recognized by the MPM-2 antibody (Figure 1B). As previously reported (Ferrell *et al.*, 1991), the $p42^{mapk}$ immunoprecipitated from meiotic, but not interphase extracts, was also recognized by phosphotyrosine antibody.

$p42^{mapk}$ Found in Interphase Mammalian Cells Undergoing the G_0/G_1 Transition Contains the MPM-2 Phosphoepitope

In mammalian somatic cells, $p42^{mapk}$ is activated in cells undergoing the G_0/G_1 transition. To examine whether MPM-2 reactivity is generated in concert with $p42^{mapk}$ activation, confluent NIH 3T3 cells, rendered quiescent by overnight incubation in serum-free medium, were stimulated with serum and then lysed at various times. The $p42^{mapk}$ was immunoprecipitated from the cell extracts and analyzed for kinase activity and MPM-2 reactivity (Figure 2). The activity of $p42^{mapk}$ was maximal at 4 min after the addition of serum (Figure 2A). The binding of the MPM-2 antibody to $p42^{mapk}$ coincided with the appearance of kinase activity, with maximal levels of MPM-2 reactivity at 4–6 min after stimulation (Figure 2B). Unstimulated cells did not contain MPM-2 reactive $p42^{mapk}$. The binding of phosphotyrosine antibody to $p42^{mapk}$ followed a similar time course to that of the MPM-2 antibody.

Both MKK1 and MKK2 Generate the MPM-2 Phosphoepitope on $p42^{mapk}$ In Vitro

To determine if the MPM-2 antibody recognized one of the activating phosphorylations on $p42^{mapk}$ catalyzed by MKK, bacterially expressed and purified wild-type $p42^{mapk}$ protein was incubated with ATP in the presence of MKK1 or MKK2 expressed by baculovirus. Treatment of the unphosphorylated $p42^{mapk}$ with either MKK resulted in the activation of $p42^{mapk}$, as assayed by phosphorylation of MBP. Unphosphorylated and MKK-phosphorylated $p42^{mapk}$ were assayed for MPM-2 and phosphotyrosine antibody reactivity on Western blots (Figure 3A). Only the MKK-treated $p42^{mapk}$ was bound by the MPM-2 antibody. As expected, the activated form of $p42^{mapk}$ was also recognized by phosphotyrosine antibody. The $p42^{mapk}$ incubated without MKK showed a basal level of phosphotyrosine antibody-binding due to autophosphorylation activity on Tyr-185. Immunoblotting with the anti-$p42^{mapk}$ antibody showed that equal amounts of $p42^{mapk}$ were present.

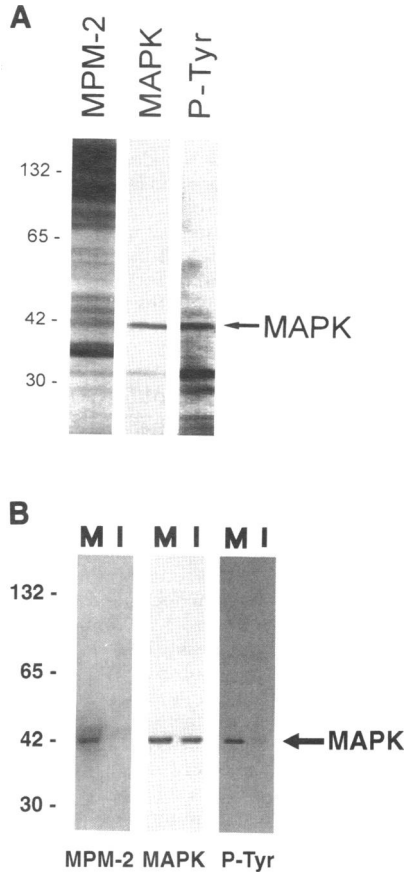

Figure 1. Immunoblotting evidence for the presence of the MPM-2 phosphoepitope on $p42^{mapk}$ from *Xenopus* eggs. (A) Whole cell extracts from mature *Xenopus* eggs were electrophoresed on a SDS/5–20% gradient polyacrylamide gel and transferred onto nitrocellulose. Strips of nitrocellulose were immunoblotted with the MPM-2 phosphoepitope (MPM-2), anti-phosphotyrosine (P-Tyr), or monoclonal anti-$p42^{mapk}$ (MAPK) antibodies. Position of $p42^{mapk}$ is indicated with an arrow. (B) *Xenopus* meiotic egg and interphase one-cell embryo extracts were immunoprecipitated with Sepharose-conjugated anti-$p42^{mapk}$ antibody. The resulting immunoprecipitated proteins were electrophoresed and immunoblotted with MPM-2, monoclonal anti-$p42^{mapk}$ and anti-phosphotyrosine antibodies. The $p42^{mapk}$ protein immunoprecipitated from meiotic extracts (M) is recognized by the MPM-2 and anti-phosphotyrosine antibodies. In contrast, the $p42^{mapk}$ found in interphase extracts (I) does not express the MPM-2 or phosphotyrosine epitopes. The position of $p42^{mapk}$ is indicated by the arrow.

To test whether the MPM-2 phosphoepitope was generated by MKK and or by $p42^{mapk}$ autocatalytic activity, the K52R mutant of $p42^{mapk}$ was used in similar experiments. The substitution of arginine for the active-site lysine in this construct results in a $p42^{mapk}$ protein that has no kinase activity. The K52R $p42^{mapk}$ mutant

was treated with either MKK1 or MKK2 in the presence of ATP and analyzed for MPM-2 reactivity. Both MKK1 and MKK2 were capable of generating the MPM-2 phosphoepitope in the mutant K52R p42^{mapk} (Figure 3B), confirming that p42^{mapk} catalytic activity is not required for the formation of the MPM-2 phosphoepitope.

Identification and Characterization of the MPM-2 Phosphoepitope

Previous work by Zhao *et al.* (1989) suggested that the MPM-2 antibody recognizes a phosphothreonine epitope. Because phosphorylation of p42^{mapk} by MKK creates the MPM-2 epitope and MKK specifically phosphorylates p42^{mapk} on Thr-183 and Tyr-185 (Ahn *et al.*, 1992), it seemed likely that MPM-2 recognized an epitope containing Thr-183. To confirm that the MPM-2 antibody recognized the Thr-183 phosphorylation, we analyzed a series of bacterially expressed p42^{mapk} mutants altered at amino acids 183 and 185, the sites of regulatory phosphorylation (Her *et al.*, 1993). The p42^{mapk} mutants were treated with MKK in the presence of ATP and assayed for MPM-2 and phosphotyrosine reactivity on immunoblots (Figure 4A). Before phosphorylation of the p42^{mapk} mutants with MKK, the mutant proteins were MPM-2 negative. After phosphorylation, the wild-type p42^{mapk} and the mutant in which Tyr-185 was substituted with phenylalanine (Y185F) were strongly reactive with the MPM-2 antibody. However, substitution of Thr-183 with alanine (T183A) resulted in a complete loss of MPM-2 reactivity of the p42^{mapk} protein. These data show that phosphorylated p42^{mapk} Thr-183 is essential for MPM-2 recognition. As expected, the untreated wild-type and T183A p42^{mapk} proteins bound phosphotyrosine antibody, because these proteins were phosphorylated on Tyr-185 either through the activity of MKK or via autophosphorylation.

Substitution of Thr-183 with the negatively charged glutamic acid resulted in loss of MPM-2 recognition similar to the loss seen in the T183A mutant (Figure 4B). This result demonstrates that a negatively charged amino acid in position 183 is not sufficient for binding of the MPM-2 antibody. To test if a nonaromatic amino acid could substitute in the Tyr-185 position, we examined a p42^{mapk} mutant in which this site had been changed to glutamic acid (Y185E), preserving the negative charge of the original phosphotyrosine. After phosphorylation of Thr-183 by MKK, the mutant protein bound the MPM-2 antibody much more weakly than did the wild-type. Both the Y185F and Y185E p42^{mapk} mutants were found to incorporate equal amounts of ^{32}P[ATP], suggesting that MKK phosphorylated each mutant equally. Thus, the binding of MPM-2 to phosphorylated Thr-183 is affected by the amino acid present in p42^{mapk} position 185. The presence of a tyrosine or phenylalanine downstream of the phosphorylated threonine-183 appears to promote MPM-2 binding, whereas substitution of this residue with a glutamic acid hinders MPM-2 antibody binding.

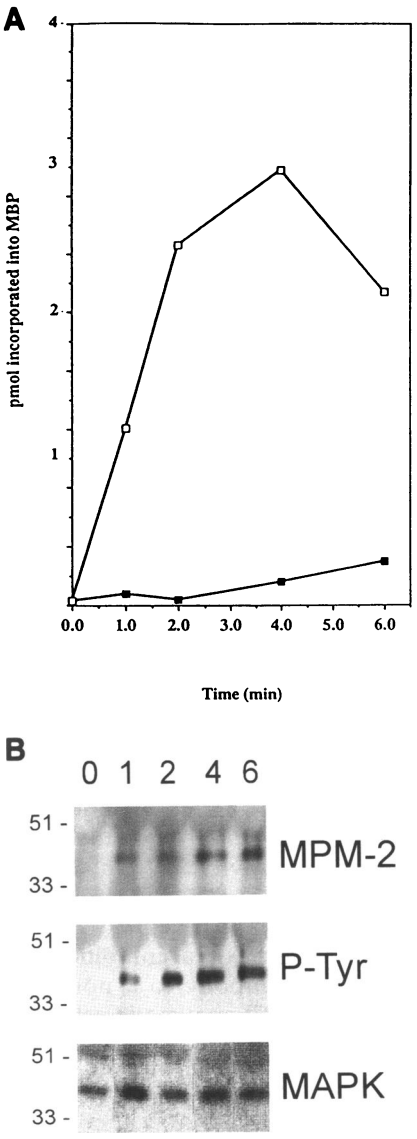

Figure 2. Expression of the MPM-2 phosphoepitope coincides with the appearance of p42^{mapk} enzymatic activity in serum-stimulated cells. (A) NIH 3T3 cells were starved for 18 h in serum-free medium and then stimulated to reenter the cell cycle with the addition of serum. Samples were taken at various times after stimulation and immunoprecipitated with the p42^{mapk} antibody. Immunoprecipitated p42^{mapk} was analyzed for enzymatic activity, as seen by phosphorylation of MBP. (□) Stimulated cells, (■) control nonstimulated cells. (B) The p42^{mapk} protein immunoprecipitated from serum-stimulated NIH 3T3 cells was processed for immunoblotting with the MPM-2, anti-phosphotyrosine, and anti-p42^{mapk} antibodies. Numbers at top indicate minutes after serum stimulation.

Binding of MPM-2 to Active p42^{mapk} In Vitro Inhibits Kinase Activity

We were interested in determining if binding of the MPM-2 antibody to the regulatory site on p42^{mapk} would

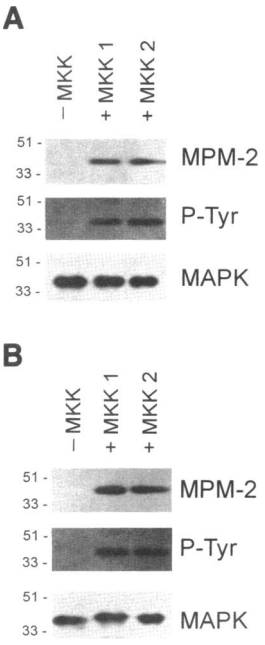

Figure 3. MKK1 and MKK2 generate the MPM-2 phosphoepitope on $p42^{mapk}$ in vitro. (A) Purified wild-type $p42^{mapk}$ was incubated with ATP in the absence (−MKK) or presence (+MKK) of MKK1 and MKK2. The $p42^{mapk}$ proteins were electrophoresed and analyzed for reactivity with MPM-2 and anti-phosphotyrosine antibodies. (B) Purified K52R $p42^{mapk}$, a kinase-defective $p42^{mapk}$ mutant, was similarly incubated with ATP in the absence or presence of MKK1 and MKK2 and analyzed for binding of the MPM-2 antibody. Both wild-type and the kinase-defective $p42^{mapk}$ proteins are recognized by the MPM-2 antibody after phosphorylation with MKK1 or MKK2.

affect the enzymatic activity of $p42^{mapk}$. Purified $p42^{mapk}$ was activated with MKK in the presence of ATP and then incubated with increasing amounts of the MPM-2 or control monoclonal antibody. Binding of the MPM-2 antibody to the active $p42^{mapk}$ resulted in a decrease in $p42^{mapk}$ enzyme activity with 50% inhibition of $p42^{mapk}$ activity occurring at ~15-fold molar excess MPM-2 antibody (Figure 5). Higher concentrations of MPM-2 resulted in a maximal inhibition of 60%. The control monoclonal antibody to a nonrelated protein had no effect on $p42^{mapk}$ enzymatic activity.

DISCUSSION

The reversible phosphorylation of proteins regulates transitions between phases of the cell cycle and the cytoarchitectural changes necessary for cell division. Antibodies specific for phosphoepitopes can be powerful tools in distinguishing various types of in vivo phosphorylations. In some cases, phosphoepitope antibody recognition seems to require only a particular phosphoamino acid. The widely used anti-phosphotyrosine antibodies are examples of this type. However, in some cases, phosphoepitope antibodies include in their antigenic determinants the amino acids adjacent to the target phosphoamino acid, increasing their specificity and allowing for the identification of groups of proteins that share a consensus phosphorylation domain.

The MPM-2 monoclonal antibody reacts with certain phosphothreonine epitopes, its specificity apparently dependent on the amino acids adjacent to the target phosphothreonine. The MPM-2 antibody defines a group of phosphoepitopes that are predominantly expressed during M-phase on key structural elements such as centrosomes, spindle fibers, and chromosomes (Vandre *et al.*, 1984; Taagepera *et al.*, 1993). Some of the proteins expressing the MPM-2 phosphoepitope have been identified (Tombes *et al.*, 1991; Kuang and Ashorn, 1993; Taagepera *et al.*, 1993; Kuang *et al.*, 1994). However, the exact epitopes of proteins from living cells that are recognized by the MPM-2 antibody have not been well characterized at the molecular level.

$p42^{mapk}$ Activated In Vivo Expresses the MPM-2 Phosphoepitope

In this study, we have shown that active $p42^{mapk}$ contains a cell cycle–regulated phosphoepitope recognized by the MPM-2 antibody. The MPM-2 phosphoepitope is present on activated $p42^{mapk}$ that has been in meiotic *Xenopus* eggs or in mammalian somatic cells undergoing the G_0/G_1 transition. In the stimulated somatic cells, the expression of the MPM-2 phosphoepitope coincided with the appearance of $p42^{mapk}$ enzymatic activity. In these cells, maximal expression of the MPM-2 phosphoepitope lagged that of the phosphotyrosine epitope, indicating that phosphorylation of Thr-183 temporally follows that of Tyr-185, as suggested by Haystead *et al.* (1992).

MKK1 and MKK2 Are Members of the MPM-2 Kinase Family

The MPM-2 phosphoepitope can be created on purified $p42^{mapk}$ in vitro by either isoform of MKK: MKK1 or MKK2. In addition, a kinase-defective mutant of $p42^{mapk}$ (K52R) served as an effective in vitro substrate for the generation of the MPM-2 phosphoepitope by MKK1 and MKK2, demonstrating that autophosphorylation by $p42^{mapk}$ was not required. Thus, MKK1 and MKK2 are necessary and sufficient for formation of the MPM-2 phosphoepitope on $p42^{mapk}$. As the MKKs have been shown to phosphorylate $p42^{mapk}$ in vivo (Ahn *et al.*, 1992; Wu *et al.*, 1992), we can identify MKK1 and MKK2 as two enzymes capable of creating the MPM-2 epitope in living cells.

Within cells multiple kinases may be capable of generating an MPM-2 phosphoepitope. The MKKs are highly specific, with no other known substrates besides $p42^{mapk}$ (Seger *et al.*, 1992). In vivo topoisomerase II from mitotic cells is recognized by the MPM-2 antibody (Taagepera *et al.*, 1993). However, we have found that purified topoisomerase II cannot be made reactive with the MPM-2 antibody by treatment with MKK. Thus, in living cells, other kinases are responsible for creating the MPM-2 phosphoepitope on topoisomerase II.

In vitro phosphorylation experiments have suggested that proline-directed kinases are capable of generating an MPM-2 phosphoepitope. Westendorf *et al.* (1994) have shown that $p34^{cdc2}$ immunoprecipitated from mitotic extracts can phosphorylate short peptides and ren-

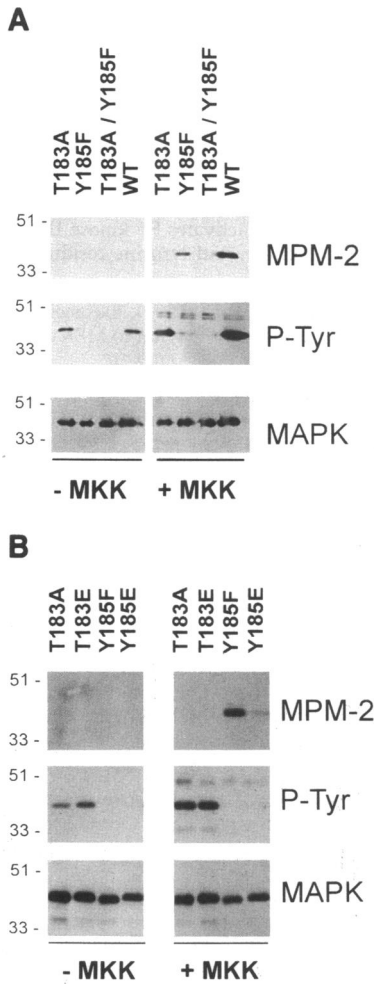

Figure 4. Identification and characterization of the MPM-2 phosphoepitope in p42^{mapk}. (A) Bacterially expressed wild-type p42^{mapk} and mutant p42^{mapk} proteins were incubated with ATP either in the absence (−MKK) or presence (+MKK) of MKK2. The p42^{mapk} protein samples were electrophoresed and immunoblotted with the MPM-2, anti-phosphotyrosine, and p42^{mapk} antibodies. Phosphorylated Thr-183 is essential for recognition by the MPM-2 antibody. (B) Binding of the MPM-2 antibody is affected by substitutions in p42^{mapk} Thr-183 and Tyr-185. Purified recombinant proteins were treated as above, and analyzed for binding of the MPM-2 antibody. Substitution of Tyr-185 with phenylalanine results in strong binding, whereas substitution with glutamic acid diminishes MPM-2 antibody binding.

der them reactive with the MPM-2 antibody. However, crude mitotic extracts, when normalized for equal amounts of p34^{cdc2}/H1 kinase activity, were much more efficient in generating MPM-2 phosphoepitopes, suggesting that other kinases in the mitotic extract were contributing to the creating the MPM-2 phosphoepitopes. Kuang and Ashorn (1993) suggested that p42^{mapk} can generate the MPM-2 phosphoepitope on other proteins. These authors identified a p42^{mapk}-like kinase, which they named ME kinase-L, from *Xenopus* egg extracts. They showed that this kinase was capable of creating the MPM-2 phosphoepitope on microtubule-associated protein 2 (MAP2). These authors did not find that their p42^{mapk}-like enzyme, ME kinase-L, was itself recognized by the MPM-2 antibody. We conclude that either the ME kinase-L identified by these workers is an isoform of p42^{mapk} that does not contain the MPM-2 recognition site, or that the binding of MPM-2 antibody to the enzyme was not detected under the conditions used by these authors. Whether proline-directed kinases participate in creating MPM-2 phosphoepitopes in vivo remains to be determined.

Molecular Characterization of the MPM-2 Phosphoepitope

Using p42^{mapk} mutants that had been altered in their regulatory sites, we demonstrated that phosphorylation of Thr-183 was critical for MPM-2 recognition of active p42^{mapk}. Substitution of Thr-183 with alanine or glutamic acid eliminated all binding of the MPM-2 antibody. Although the phosphotyrosine at position 185 could be substituted with another aromatic amino acid, phenylalaine, substitution of Tyr-185 with glutamic acid greatly reduced the binding of the MPM-2 antibody. These results suggest that the presence of a tyrosine or phenylalanine at the +2 position from the target phosphothreonine is an important determinant of a strong MPM-2 epitope. The importance of other amino acids near Thr-183 to the MPM-2 epitope in p42^{mapk} is uncertain.

We showed previously that DNA topoisomerase IIα and DNA topoisomerase IIβ present in mitotic chro-

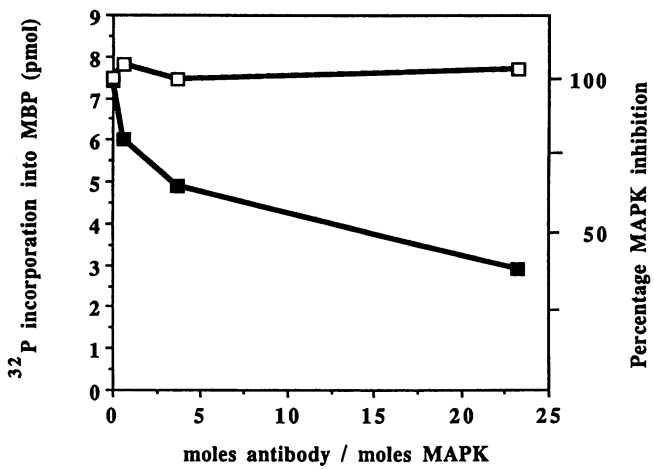

Figure 5. Inhibition of p42^{mapk} enzymatic activity by binding of the MPM-2 phosphoepitope antibody. Purified p42^{mapk} was phosphorylated by MKK in vitro and incubated with either the MPM-2 phosphoepitope antibody (■) or control antibody (□), both of which had been purified from ascites fluid by binding to agarose beads. The enzymatic activity of the p42^{mapk} was assayed, using MBP as a test substrate. Incubation of active p42^{mapk} with the MPM-2 antibody results in substantial inhibition of p42^{mapk} enzymatic activity.

mosomes contain the MPM-2 phosphoepitope (Taagepera *et al.*, 1993). Other proteins known to react with the MPM-2 antibody include the microtubule associated proteins, MAP1B (Tombes *et al.*, 1991), MAP2 (Kuang and Ashorn, 1993), MAP4 (Tombes *et al.*, 1991; Vandre *et al.*, 1991), and *Xenopus* cdc-25 (Kuang *et al.*, 1994). Westendorf *et al.* (1994) reported two additional proteins, termed MPP1 and MPP2, that react with MPM-2 when phosphorylated with mitotic extracts in vitro. By phosphorylating an array of random short peptides with mitotic extracts, these authors described consensus sequences for their in vitro phosphorylations. As reported, the sequence most often recognized by the MPM-2 antibody was LTPLK. The $p42^{mapk}$ protein, which we have shown in this paper to contain an MPM-2 phosphoepitope in vivo, does not contain a domain resembling this sequence. Similarly, the 170- and 180-kDa forms of DNA topoisomerase II, which are major MPM-2 reactive proteins in mitotic cells in vivo (Taagepera *et al.*, 1993), likewise do not contain such a sequence. Thus, further molecular analysis of proteins found to be targets for the MPM-2 antibody in vivo will be required to fully characterize the MPM-2 phosphoepitope.

In conclusion, previously, the MPM-2 antibody had been found to bind phosphorylated forms of several key enzymes and microtubule-associated proteins. However, whether the phosphorylation recognized by the MPM-2 antibody played a significant role in regulating the activity or function of these proteins remained uncertain. In the present work, we have demonstrated that the MPM-2 antibody recognizes a domain previously known to be an important regulator of the function of $p42^{mapk}$ in meiotic maturation and in the G_0 to G_1 transition. Given the diversity of proteins that bind the MPM-2 antibody, the question remains whether the sharing of MPM-2 antibody recognition by various proteins has functional significance or is mere coincidence. We suspect that the MPM-2 phosphoepitope does indeed define a particular class of phosphorylation important in the cell cycle regulation of several enzymes and structural proteins. Only further identification of proteins that bind the MPM-2 antibody, sequencing of the phosphoepitopes, and characterization of function by mutagenesis experiments will resolve this issue.

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