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Cloning of cDNAs for M-phase phosphoproteins recognized by the MPM2 monoclonal antibody and determination of the phosphorylated epitope

(cell cycle/cdc2/cyclins)

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ABSTRACT **The MPM2 monoclonal antibody binds to a phospho amino acid-containing epitope present on more than 40 proteins of M-phase eukaryotic cells. We have developed a technique for cloning cDNAs encoding MPM2-reactive phosphoproteins from bacteriophage λ expression libraries. Proteins from phage plaques were adsorbed to nitrocellulose filters, phosphorylated by M-phase kinases, and screened for MPM2 binding. Partial-length cDNAs encoding two MPM2-reactive proteins termed MPM2-reactive phosphoproteins 1 and 2 (MPP1 and MPP2) were isolated. The deduced MPP1 and MPP2 amino acid sequences are not closely related to any previously described proteins. To determine which amino acid stretches contained the MPM2 epitope, sequences from a 15 amino acid peptide expression library were selected for binding to MPM2 after phosphorylation by M-phase kinases. A string of five amino acids was similar among all selected peptides, and the sequence reflecting the most frequent amino acid at each position was Leu-Thr-Pro-Leu-Lys (LTPLK). MPP1 and MPP2 proteins, respectively, contained five and nine sites closely related to LTPLK, including two that were common to both proteins, (F/T)TPLQ and SSP(I/S)D. Peptides containing LTPLK and FTPLQ were strongly phosphorylated by M-phase, but not interphase, cytosolic kinases, and the phosphorylated peptides were bound by MPM2. Thus, we have identified M-phase-specific phosphorylation sites bound by MPM2 and two putative M-phase phosphoproteins containing these sites.**

Progression of cells from interphase to mitosis involves major alterations in cellular structures and activities. The G_2/M transition is induced by M-phase-promoting factor (MPF), which is composed of a protein kinase, $p34^{cdc2}$, and its associated regulatory subunit, cyclin (reviewed in ref. 1). Upon entry into M phase, many proteins are phosphorylated directly by MPF or indirectly by kinases that are activated by MPF. Forty or more of the M-phase phosphoproteins are reactive with MPM2, a monoclonal antibody that binds an epitope containing a phospho amino acid (2). The proteins are synthesized during interphase and become MPM2-reactive when cells enter M phase, apparently due to the action of an M-phase-specific kinase (3, 4). It is likely that phosphorylation at MPM2 antigenic sites regulates mitotic processes, because some of the MPM2 antigens are present in components of the mitotic machinery such as the centrosome, kinetochores, mitotic spindle, and chromosome axis (5, 6), and microinjected MPM2 inhibits entry into and exit from mitosis (3, 4). As yet very few of the MPM2-reactive proteins have been identified (7, 8), and definition of more would contribute to understanding of the mitotic process.

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By modifying bacterially expressed proteins with a mammalian cell lysate, we have extended standard immunoscreening and immunoselection techniques to the isolation of cDNAs encoding MPM2-reactive phosphoproteins (MPPs) and peptides encoding the MPM2 epitope. Two putative M-phase phosphoproteins and a set of peptide sequences containing potential MPM2 sites were identified and, together, permitted determination of the M-phase-specific phosphorylation site bound by MPM2. The methods we have developed will identify a substantial number of M-phase kinase substrates, even those of low abundance, whose phosphorylation may be important for the process of cell division.[§]

MATERIALS AND METHODS

Preparation of Cell Extracts. Cell extracts were prepared from HeLa S3 cells treated with nocodazole (0.1 μg/ml) to enrich for M-phase cells (85–95%) (9). To separate kinases from endogenous MPM2 antigens, M-phase extract was fractionated on phosphocellulose. Kinase(s) capable of inducing MPM2 reactivity in MPP1 fusion protein was coeluted with the major histone H1 kinase at about 0.5 M NaCl.

Isolation of MPM2-Reactive Plaques. Protein replica filters prepared for cDNA library immunoscreens (10) were blocked with KPM (50 mM KCl/50 mM Pipes/1.92 mM $MgCl_2$/10 mM EGTA, pH 7.0) plus 2% gelatin and 0.5% Tween-20 at 37°C and then incubated for 45 min at 37°C with extracts of M-phase HeLa cells diluted 1:16 or 1:30 in KPM plus 0.5% Tween-20, 25 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM adenosine 5′-[γ-thio]triphosphate (ATP[γS]), and 1 mM ATP. [In subsequent screens, more MPM2-reactive plaques were obtained by replacing the NaF, $Na_4P_2O_7$, and ATP[γS] with 1 μM okadaic acid or microcystin (R. Monroe, J.M.W., and L.G., unpublished results).] Filters were processed for immunodetection with MPM2 (1 μg/ml) and anti-mouse antibodies (Clontech).

DNA Sequencing. Sequencing was by the Sanger dideoxy method (11). The primer for the peptide library was 5′-TTCTGTATGGGGTTTTGCTAA-3′.

Production of Fusion Proteins. T7 protein 10–MPP1 and –MPP2 fusion proteins were produced by using plasmids pGEMEX1 and pGEMEX1-HIS (GEMEX1 modified to contain the nucleotide sequence ATGGTGATGGTGATGATG between the *Sac* I and *Eco*RI sites), respectively (Promega). Histidine-tagged protein 10 and protein 10–MPP2 were purified under denaturing conditions on Probond resin (Invitrogen). Protein 10–MPP1 from inclusion bodies was purified

Abbreviations: MPF, M-phase-promoting factor; MPP, MPM2-reactive phosphoprotein; ATP[γS], adenosine 5′-[γ-thio]triphosphate.

[§]cDNA sequences for MPP1 and MPP2 have been deposited in GenBank (accession nos. L16782 and L16783, respectively).

on S Sepharose (Pharmacia) under denaturing conditions (8 M urea/50 mM Tris·HCl, pH 8.0/1% 2-mercaptoethanol). The amount of full-length fusion protein recovered after purification and dialysis into 50 mM KCl/50 mM Pipes/10 mM EGTA, pH 7.0, containing leupeptin, pepstatin, and aprotinin (each at 1 μg/ml) was determined by densitometry of SDS/polyacrylamide gels stained with Coomassie blue.

Design of Peptide Substrates. Peptide kinase substrates were designed with arginine residues added to the carboxyl terminus, the end of the MPM2-selected peptides (see *Results*) that contained more basic amino acids, for easy separation from ATP on phosphocellulose paper (12). QFTPLQPRRR-amide corresponded to amino acids 372–378 of MPP1 plus three arginines, ALTPLKGRR-amide to amino acids 4–9 of clone 7 (see *Results*) plus a linker glycine and two arginines, and CAEKTPVKA-amide to cysteine plus amino acids 13–20 of rabbit thymus histone H1 (13).

Kinase Assays. Assay mixtures (20 μl) contained 1–5 μl of cell extract or $p34^{cdc2}$ (precipitated with a polyclonal antibody against the human cdc2 carboxyl-terminal 12 amino acids conjugated to keyhole limpet hemacyanin), various amounts of substrate, 1 mM ATP, 50 mM KCl, 50 mM Pipes, (pH 7.0), 10 mM $MgCl_2$, 5 μM okadaic acid, 1 μM protein kinase A inhibitor, and leupeptin, pepstatin, and aprotinin each at 1 μg/ml, as well as, in some experiments, 10–20 μCi of [γ-^{32}P]-ATP (1 μCi = 37 kBq). To measure MPM2 reactivity, reaction mixtures were stopped by addition of SDS sample buffer, separated by SDS/PAGE, transferred to nitrocellulose, and reacted with MPM2. For dephosphorylation, an equal volume of 80 mM Pipes (pH 6.0) with or without 0.2 units of potato acid phosphatase was added before addition of SDS sample buffer.

Binding of Phosphorylated Peptides to MPM2. Phosphorylated peptides produced in a kinase reaction were partially purified on Sep-Pak cartridges (Waters) and suspended in 10 μl of Dulbecco's phosphate-buffered saline containing leupeptin, pepstatin, and aprotinin at 100 μg/ml and bovine serum albumin at 10 mg/ml. Five microliters of phosphopeptide-containing solution was mixed overnight at 4°C with 50 μg of HA4 (mouse IgG1 of irrelevant specificity) or MPM2 coupled to 10 μl of Sepharose 4B. The beads were washed rapidly (<3 sec) with 3 ml of phosphate-buffered saline, and bound substances were eluted with 10% acetic acid, chromatographed on cellulose-coated thin-layer plates (14), and detected by autoradiography.

Isolation and Characterization of Peptide-Bearing Phage That Bind MPM2. M13 phage bearing 15-amino acid peptides (15) were selected on MPM2 as described (16, 17), except that prior to each round of purification, phage were phosphorylated for 2 hr at 37°C in a reaction mixture (200 μl) which contained 10^{11} phage from an amplified peptide library, 50 μl of partially purified M-phase kinases (A_{280} = 1.0; see above), 2 mM ATP[γS], 5 μM okadaic acid, 10 mM $MgCl_2$, 50 mM Hepes (pH 7.4), 10 mM EGTA, 50 mM KCl, 1 μM protein kinase A inhibitor, 2 mM ATP, and aprotinin, leupeptin, and pepstatin each at 10 μg/ml. Individual MPM2-selected clones were subjected to micropan assay (17) with and without previous kinase treatment. Only those phage clones that bound to MPM2 better after kinase treatment were sequenced.

RESULTS

Isolation of cDNAs Encoding MPPs. Bacteriophage λ cDNA expression libraries were immunoscreened with MPM2 after phosphorylation of bacterially expressed proteins with M-phase cytosol, which contains the relevant kinase(s). A single positive plaque encoding MPP1 was isolated from 600,000 plaques of a λZAPII (Stratagene) cDNA library made from RNA of growing HeLa cells; a second positive cDNA, encoding MPP2, was isolated from 200,000 plaques of a λgt11 cDNA library made from MOLT-4 cell RNA. From sequencing the cDNA inserts, we found that MPP1 cDNA was a partial-length cDNA containing 2565 bp and encoding an open reading frame of 566 amino acids (Fig. 1*A*), whereas MPP2 cDNA was a partial length cDNA containing 1659 bp and encoding an open reading frame of 221 amino acids (Fig. 1*B*). The cDNAs corresponded to different proteins because the partial-length clones encoded two different carboxyl-terminal sequences. Neither encoded protein was identical to or substantially homologous with any known sequence. For MPP1 protein, analysis (18) indicated that the first 302 amino acids had a high probability (≥75%) of forming a coiled-coil α-helix.

Phosphorylation of MPP1 and MPP2 Is Required for Reactivity with MPM2. MPP1 and MPP2 fusion proteins were very sensitive to proteolysis *in vivo* in bacteria and *in vitro* during purification and, despite the use of protease inhibitors and strongly denaturing conditions, were always partially degraded. Typically, 20–50% of the mass of purified MPP1 and MPP2 fused to protein 10 of the T7 phage migrated at the predicted sizes in SDS/polyacrylamide gels (Fig. 2*A*, lanes 2 and 3). When incubated with ATP and M-phase extracts and analyzed on immunoblots with MPM2, MPP1 and MPP2 fusion proteins became strongly immunoreactive (Fig. 2*B*, lanes 8 and 11) above the background of MPM2 antigens endogenous to the M-phase extract (Fig. 2*B*, lane 2), and the mobility of MPP2 fusion protein was substantially reduced (compare the strongest protein bands of Fig. 2*A*, lane 3, with Fig. 2*B*, lane 11). In contrast, fusion proteins incubated only with ATP and buffer (Fig. 2*B*, lanes 6 and 9) did not react with MPM2 at all, and those incubated with ATP and extracts of exponentially growing cultures (containing 5% M-phase cells) (Fig. 2*B*, lanes 7 and 10) reacted comparatively weakly above a low background of MPM2 antigens (Fig. 2*B*, lane 1). This indicated that an enzymatic activity enriched in M-phase extract was required for conversion of the fusion proteins to MPM2 antigens. In addition, the amino acid sequences encoded in the cDNAs were required for gaining immunore-

A

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QETEKLKEELSASSARTQNLKADLQRKEEDYADLKEKLTDAKKQIKQEQKEVSVM   55

RDEDKLLRIKINELEKKKNQCSQELDMKQRTIQQLKEQLNNQKVEEAIQQYERAC  110

KDLNVKEKIIEDMRMTLEEQEQTQVEQDQVLEAKLEEVERLATELEKWKEKCNDL  165

ETKNNQRSNKEHENNTDVLGKLTNLQDELQESEQKYNADSKKWLEEKMMLITQAK  220

EAENIRNKEMKKYAEDRERFFKQQNEMEILTAQLTEKDSDLQKWREERDQLVAAL  275

EIQLKALISSNVQKDNEIEQLKRIISETSKIETQIMDIKPKRISSADPDKLQTEP  330

LSTSFEISRNKIEDGSVVLDSCEVSTENDQSTRFPKPELEIQFTPLQPNKMAVKH  385

PGCTTPVTVKIPKARKRKSNEMEEDLVKCENKKNATPRTNLKFPISDDRNSSVKK  440

EQKVAIRPSSKKTYSLRSQASIIGVNLATKKKEGTLQKFGDFLQHSPSILQSKAK  495

KIIETMSSSKLSNVEASKENVSQPKRAKRKLYTSEISSPIDISGQVILMDQKMKE  550

SDHQIIKRRLRTKTAK                                          566
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B

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RSRRKQHLLPPCVDEPELLFSEGPSTSRWAAELPFPADSSDPASQLSYSQEVGGP   55

FKTPIKETLPISSTPSKSVLPRTPESWRLTPPAKVGGLDFSPVQTSQGASDPLPD  110

PLGLMDLSTTPLQSAPPLESPQRLLSSEPLDLISVPFGNSSPSDIDVPKPGSPEP  165

QVSGLAANRSLTEGLVLDTMNDSLSKILLDISFPGLDEDPLGPDNINWSQFIPEL  220

Q                                                         221
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FIG. 1. Predicted amino acid sequences of cDNA fragments encoding MPP1 (*A*) and MPP2 (*B*). Amino acid residues are numbered on the right. Potential phosphorylation/MPM2 binding sequences are underlined.

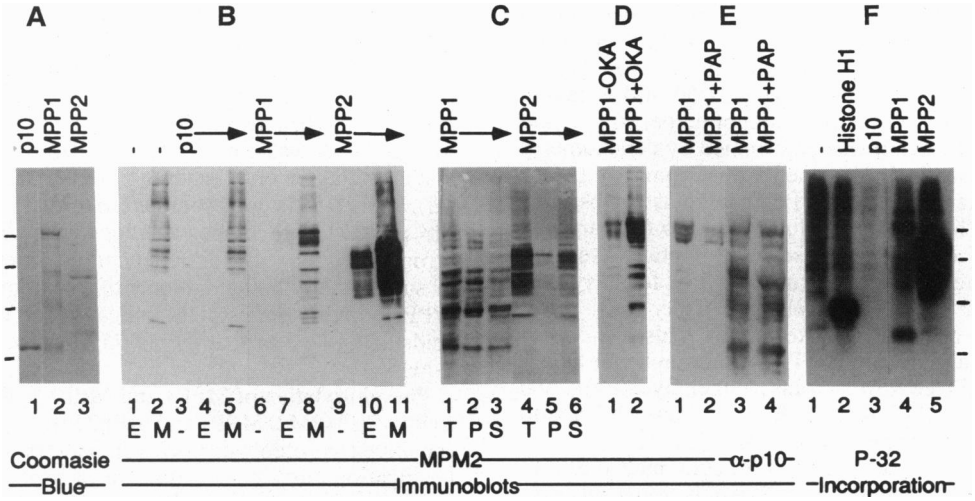

FIG. 2. Cloned protein fragments become MPM2 antigens after phosphorylation by M-phase cell extracts. (*A*) Protein 10 (p10), p10–MPP1 (MPP1), and p10–MPP2 (MPP2) (1 μg samples) were separated by SDS/PAGE and stained with Coomassie blue. (*B*) Kinase assay mixtures containing extract (1 μl) of exponentially growing (E) or M-phase (M) cells or no extract (−) were incubated with 1 μg of substrate as indicated for 1 hr at 37°C and processed for detection of MPM2 reactivity. (*C*) Kinase assay mixtures containing extract of M-phase cells (T), $p34^{cdc2}$ precipitated with antibodies to its carboxyl terminus (P), or extract immunodepleted of $p34^{cdc2}$ (S) were incubated at 37°C for 30 min and processed for detection of MPM2 reactivity. Each sample contained 1 μg of substrate and 1 μl of extract (or its equivalent). (*D*) Kinase assay mixtures containing M-phase extract with or without 5 μM okadaic acid (OKA) were incubated for 2 hr at 37°C and processed for detection of MPM2 reactivity. (*E*) p10–MPP1 was incubated with M-phase extract for 1 hr at 37°C and then potato acid phosphatase (PAP) for an additional 4 hr. Reaction mixtures were processed for immunoblotting with MPM2 (lanes 1 and 2) or anti-p10 (α-p10) (Novagen) (lanes 3 and 4). (*F*) Kinase assay mixtures containing M-phase extract were incubated in the presence of [γ-^{32}P]ATP for 15 min at 37°C with substrate as indicated. Reaction mixtures were subjected to SDS/PAGE and proteins that had incorporated $^{32}PO_4$ were detected by autoradiography. Molecular sizes indicated on the far right and left from top to bottom are 97, 66, 45, and 31 kDa.

activity, since protein 10 alone (Fig. 2*A*, lane 1) did not become reactive even when incubated in the presence of ATP and cell extracts (Fig. 2*B*, lanes 3–5). Further, since the fusion proteins were dissociated from other proteins during SDS/PAGE, the acquisition of MPM2 reactivity must have been due to a covalent modification rather than association of another MPM2-reactive protein.

Three types of experiments indicated that the covalent modification leading to MPM2 reactivity was phosphorylation. First, potato acid phosphatase (19), an enzyme that has broad specificity against phosphoproteins, greatly reduced MPM2 immunoreactivity of protein 10–MPP1 (Fig. 2*E*, lanes 1 and 2) and -MPP2 (data not shown) fusion proteins that had been reacted with ATP and M-phase extracts. In contrast, immunoreactivity toward the protein 10 epitope of the fusion protein was unaffected by phosphatase treatment (Fig. 2*E*, lanes 3 and 4). This indicated that loss of MPM2 reactivity was due to dephosphorylation of fusion protein rather than proteolytic degradation. Second, ^{32}P from [γ-^{32}P]ATP was incorporated into MPP1 and MPP2 fusion proteins and some of their degradation products in the presence of M-phase extracts (Fig. 2*F*, lanes 4 and 5), as seen above a background of weakly labeled endogenous extract proteins (Fig. 2*F*, lane 1). In contrast, protein 10 alone did not become radioactive (Fig. 2*F*, lane 3). This indicated that the fusion proteins were protein kinase substrates. MPP2 appeared to incorporate at least as much phosphate in the presence of M-phase kinases as histone H1 (Fig. 2*F*, lane 2), a frequently used indicator of M-phase kinase activity. Third, when MPP1 and MPP2 fusion proteins were incubated with M-phase extracts for an extended period of time in the presence of microcystin-LR (data not shown) or okadaic acid, inhibitors of the phosphoserine/threonine phosphatases PP1 and PP2A (20, 21), the amount of MPM2 immunoreactivity associated with either MPP1 (Fig. 2*D*) or MPP2 (data not shown) fusion protein was much greater than in the absence of inhibitor (Fig. 2*D*). This indicates that MPM2 reactivity was stabilized by phosphatase inhibitors. Taken together, the data indicate that the cDNAs we have isolated encode proteins with the properties of M-phase phosphoproteins.

When $p34^{cdc2}$ kinase or MPF was immunoprecipitated (85% of total) from M-phase extracts and added to kinase reactions, both MPP1 and MPP2 fusion protein substrates became MPM2-reactive (Fig. 2*C*). MPF accounted for most (75%) of the M-phase kinase activity toward MPP1 (Fig. 2*C*, lanes 1–3) and a substantial amount (20%) of activity toward MPP2 (Fig. 2*C*, lanes 4–6).

Determination of the MPM2-Reactive Phosphorylated Site. We used a pentadecapeptide expression library (15) of 2×10^7 different peptide sequences to determine the MPM2 epitope. The peptide sequences, each fused to M13 coat protein III and displayed on the surface of a phage, were incubated with M-phase kinases and ATP to phosphorylate potential MPM2 sites. Phage that bound to MPM2 were amplified by growth in bacteria and subjected to two more rounds of selection and amplification. From the second round of purification, 14 clones were sequenced and 6 different sequences were obtained, and from the third round 42 clones were sequenced and 13 different sequences were obtained. A string of 5 amino acids similar among all derived amino acid sequences (Table 1) contained a hydrophobic amino acid in position 1 (amino acid, number of different isolates: L, 6; P, 4; Y, 3; I, 2; F, 2; E, 2; V, 2; A, 1; T, 1; N, 1; or K, 1), the putative phosphorylated amino acid in position 2 (T, 19; or S, 6), P in position 3, a hydrophobic amino acid in position 4 (L, 6; I, 5; V, 4; M, 3; P, 3; A, 1; N, 1; W, 1; or Q, 1), and a basic or hydrophobic amino acid in position 5 (K, 13; R, 4; L, 2; P, 2; H, 1; T, 1; A, 1; or F, 1). The sequence consisting of the most frequently used amino acid at each position was LTPLK.

In the amino acid sequences of MPP1 and MPP2 we found five and nine occurrences, respectively, of the central (T/S)P motif (positions 2 and 3) of the MPM2 binding site (Fig. 1). For each occurrence, we compared the amino acid in each surrounding position (1, 4, or 5) with all amino acids found in the corresponding position of the 5-amino acid strings within the MPM2-selected peptides. The amino acid in each position

Table 1. Predicted peptide sequences of clones affinity-purified with MPM2

Clone	No. isolated	Displayed peptide
		Third round with 0.1 nM MPM2
1	2	LP LTPLT KTSRQPFH
2	1	FPEFTGLQPY PTPVK
3	1	LLP LTPLR PLKSSMR
4	1	SMI FSPLR ATKFQPH
5	1	IKQFQKTYD ITPIK K*
6	15	SSDNTQFY ATPIK TPP
		SSDNTQFYATPI KTPPP
7	9	APFA LTPLK MQKGPS
		APFALTPLKMQKG PSPPP
8	4	LWETLS YTPWL TPIK
		LWETLSYTPW LTPIK
9	3	KTYFL PTPIK LSPMK
		KTYFLPTPIK LSPMK
10	2	ETPNH DWTITPMRPSR*
		TPNHDWT ITPMR PSR*
11	1	HLQFL PTPVK LTPQR
		HLQFLPTPVK LTPQR
12	1	NSPMF HVTPLKVNVR
		NSPMFH VTPLK VNVR
13	1	ETPAA PNANIFSPPLKR
		TPAAPNANI FSPPL KR
		Second round with 10 nM MPM2
14	4	NYTHVSMP VTPVK GI
15	3	TSMQPLYYM YSPLK R
16	2	PSVTNNL YTPVK HRK
17	1	IKQFQKTYD ITPIK K*
18	1	AHDLMAFP TTPIK HV
19	3	ETPNH DWTITPMRPSR*
		TPNHDWT ITPMR PSR*
		Most common amino acids
		LTPLK

Underlined amino acids are present in the linker, which fuses the peptide to M13 phage protein III (clones 6, 7, 10, 13, and 19). Peptides containing two similar 5-amino acid strings are aligned twice.
*Identical sequences, isolated from both the second and third round of selection.

within sequence 389–393 of MPP1 and within sequences 84–88 and 129–133 of MPP2 (Fig. 1) was found in the corresponding position of at least one 5-amino acid string within an MPM2-selected peptide (Table 1). Matches were found at zero to two of the three positions (Fig. 1) surrounding the other (T/S)P sites of MPP1 and MPP2. Interestingly, two of the (T/S)P sites of MPP1 (FT^{374}PLQ and SS^{533}PID) were almost identical to two sites of MPP2 (TT^{120}PLQ and SS^{151}PSD).

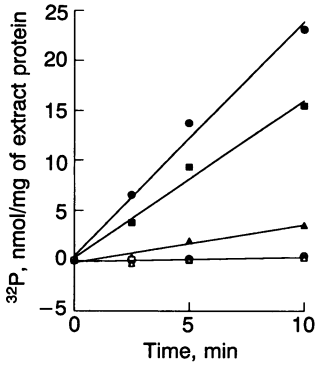

FIG. 3. Peptides containing the putative sequences for binding MPM2 are phosphorylated by extracts of M-phase cells. Two peptides, ALTPLKGRR (LTPLK) and QFTPLQPRRR (FTPLQ), containing potential binding sequences for MPM2 were phosphorylated in the presence of extracts of M-phase (M) or exponentially growing (E) cells and [γ-^{32}P]ATP. Background phosphorylation, obtained in the absence of extracts, was subtracted from each value. ●, 200 μM LTPLK, M; ■, 500 μM FTPLQ, M; ▲, no peptide, M; ○, 200 μM LTPLK, E; □, 500 μM FTPLQ, E; △, no peptide, E.

Synthetic peptides containing the potential MPM2 binding sites LTPLK and FTPLQ were readily phosphorylated by extracts of M-phase cells, but not by extracts of exponentially growing cells (Fig. 3). Although extracts of M-phase cells contain multiple kinases that might phosphorylate the synthetic peptides, K_m and V_{max} values were calculated to permit evaluation of the peptides as substrates for phosphorylation by M-phase kinase(s). Calculated K_m values for the two peptides were similar (average K_m in four experiments: LTPLK, 152 μM; FTPLQ, 181 μM), but the calculated V_{max} for LTPLK was almost double that for FTPLQ (average V_{max} in four experiments: LTPLK, 1700 fmol/min per μg of extract protein; FTPLQ, 1000 fmol/min per μg). When p34^{cdc2} was immunoprecipitated from extracts of M-phase cells and used to phosphorylate the peptides, the K_m values for the two peptides were again similar, although lower than those obtained with crude extracts (average K_m in two experiments: LTPLK, 47 μM; FTPLQ, 73 μM), and the V_{max} values were only 20% of the values obtained with crude extracts in the same experiment.

To test whether the sequences could become MPM2 antigenic sites, LTPLK, FTPLQ, and KTPVK (a sequence corresponding to a mitotic phosphorylation site of histone H1) peptides were phosphorylated to an equal extent by extracts of M-phase cells and incubated with an irrelevant IgG or MPM2 immobilized on Sepharose (Fig. 4). In our initial experiments, which included prolonged washes of immune complexes, only phospho-FTPLQ bound selectively to MPM2. When we shortened our postbinding wash conditions to detect lower affinity binding, both phospho-FTPLQ and phospho-LTPLK, but not phospho-KTPVK, bound selectively to MPM2 (Fig. 4). These results suggest that monovalent phospho-FTPLQ binds to MPM2 with high affinity and that monovalent phospho-LTPLK binds with low affinity. Nevertheless, when presented as a pentavalent ligand concentrated at one end of an M13 virion (22) (Table 1, clone 7), phospho-LTPLK bound stably to MPM2. Thus, LTPLK- and

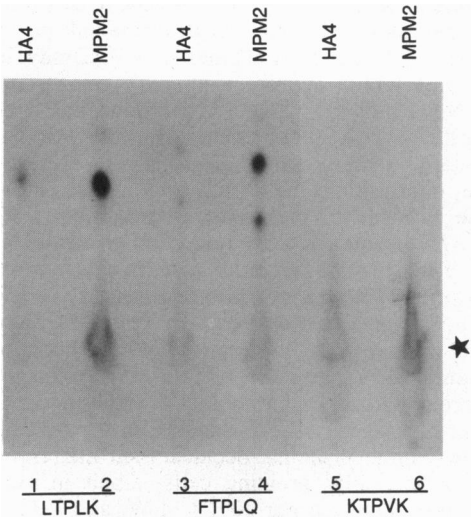

FIG. 4. Phosphorylated peptides containing potential MPM2 sites are bound by MPM2. Peptides containing the sequences LTPLK, FTPLQ, and KTPVK were phosphorylated by extracts of M-phase cells for 10 min at 30°C, immunoabsorbed with a control antibody (HA4) or MPM2, eluted from the antibodies, and detected as described in *Materials and Methods*. Star indicates the position of migration of background ATP.

FTPLQ-containing peptides were phosphorylated selectively by M-phase kinases to give epitopes that bind to MPM2. Further, when sequences 3′ of base 1145 of MPP1 cDNA were deleted, the resulting encoded protein, which lacked all (T/S)P sites except FTPLQ, was still immunoprecipitated by MPM2 after phosphorylation with M-phase kinases (data not shown). This result indicates that the phosphorylated FTPLQ sequence within MPP1 is sufficient for recognition by MPM2.

DISCUSSION

We have devised a technique for cloning cDNAs encoding a specific set of proteins that are phosphorylated by M-phase kinases. Taken together, the following criteria indicate that the isolated cDNAs encode proteins (MPP1 and MPP2) with the properties of genuine M-phase phosphoproteins. (*i*) The encoded proteins react with MPM2 after, but not before, treatment with MPF or M-phase extract, which is much more effective than interphase extract. (*ii*) The MPM2 immunoreactivity is reversed by dephosphorylation with potato acid phosphatase and enhanced by the protein phosphatase inhibitors okadaic acid and microcystin. (*iii*) The addition of [γ-^{32}P]ATP to M-phase kinase assays results in incorporation of ^{32}P into MPP1 and MPP2 substrates. In addition, we have defined stretches of amino acids that will bind MPM2.

The sequence we determined for MPM2 binding overlaps with the consensus for phosphorylation by two known protein kinases, p34^{cdc2}, (S*/T*)PXR/K (23, 24), and mitogen-activated protein (MAP) kinase, PX(S*/T*)P (25), both of which may confer MPM2 reactivity on proteins containing appropriate sequences. The results of our kinase assays with immunoadsorbed p34^{cdc2} indicate that LTPLK and FTPLQ peptides and MPP1 and MPP2 fusion proteins are substrates for p34^{cdc2}, which may be a major or predominant kinase giving rise to MPM2 reactivity *in vivo*. Nevertheless, since all kinase activity toward LTPLQ and FTPLQ peptides and MPP2 is not removed from extracts of M-phase cells with antibodies to p34^{cdc2}, other kinases must also contribute to generation of MPM2 reactivity. In support of this possibility, recent data suggest that MAP kinase and another kinase unrelated to p34^{cdc2} can phosphorylate some proteins to create MPM2 antigens (26).

Although we have determined the recognition sequence for MPM2, we cannot predict whether a particular protein is an MPM2 antigen because (*i*) we do not believe that the MPM2-selected peptides exhaustively describe all possible sequences that will bind to MPM2 and (*ii*) MPM2 may recognize multiple sites within a protein, thereby greatly stabilizing binding. Nevertheless, when we searched the Protein Identification Resource sequence data base (April 1993) for proteins containing the 5 amino acids of the MPM2 binding sequence, microtubule-associated proteins 1B and 4, which comigrate with MPM2 antigens on immunoblots of mitotic spindles (7, 8), (along with many other proteins), were selected. Thus the MPM2 binding motif may be useful for choosing proteins to test for MPM2 reactivity *in vivo*.

It has not been possible to confirm that MPP1 and MPP2 are MPM2 antigens *in vivo* because we have been unable to produce antibodies that will detect the HeLa cell proteins by immunoprecipitation, immunoblot, or immunocytochemistry. Nevertheless, we believe that MPP1 and MPP2 cDNAs do encode cellular proteins, because their mRNAs are present in exponentially growing cells, albeit in very small amounts (less than one per cell; J.M.W. and L.G., unpublished results). The apparently low abundance of these proteins and their mRNAs in cells suggests that our technique is capable of isolating rare mitotic phosphoproteins that would be difficult to discover and characterize by other means. Further, because so few proteins encoded by cDNA libraries are reactive with MPM2 after phosphorylation, we believe that our method is highly selective for proteins that are excellent substrates for M-phase kinases and contain high-affinity binding sites for MPM2.

To our knowledge, this is the first method described for cDNA expression cloning with an antibody whose epitope requires a postsynthetically modified amino acid. Because MPM2 recognizes many proteins that are substrates for M-phase kinases, we have recently been able to isolate 11 additional cDNAs whose encoded proteins, which have not been previously described, bind to MPM2 after phosphorylation with M-phase kinases (J.M.W., R. Monroe, N. Taniura, F. Pirollet, and L.G., unpublished results). Thus, it is likely that our method of cloning M-phase phosphoproteins will generate new probes for understanding how cells enter and proceed through mitosis. This technique could be further extended by use of recombinant kinases or appropriate cellular extracts and [γ-^{32}P]ATP or antibodies against phospho amino acids (27) to identify proteins regulated by phosphorylation in other signal transduction pathways.

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1. Nurse, P. (1990) *Nature (London)* **344,** 503–508.
2. Davis, F., Tsao, T., Fowler, S. & Rao, P. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2926–2930.
3. Davis, F. M. & Rao, P. N. (1987) in *Molecular Regulation of Nuclear Events in Mitosis and Meiosis*, eds. Schlegel, R. A., Halleck, M. S. & Rao, P. N. (Academic, New York), pp. 259–294.
4. Kuang, J., Zhao, J., Wright, D. A., Saunders, G. F. & Rao, P. N. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 4982–4986.
5. Vandre, D., Davis, F., Rao, P. & Borisy, G. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 4439–4443.
6. Hirano, T. & Mitchison, T. J. (1991) *J. Cell Biol.* **115,** 1479–1489.
7. Vandre, D. D., Centonze, V. E., Peloquin, J., Tombes, R. M. & Borisy, G. G. (1991) *J. Cell Sci.* **98,** 577–588.
8. Tombes, R. M., Peloquin, J. G. & Borisy, G. G. (1991) *Cell Regul.* **2,** 861–874.
9. Suprynowicz, F. A. & Gerace, L. (1986) *J. Cell Biol.* **103,** 2073–2081.
10. Huynh, T. V., Young, R. A. & Davis, R. W. (1984) in *DNA Cloning: A Practical Approach*, ed. Glover, D. (IRL, Oxford), pp. 49–78.
11. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
12. Casnellie, J. E. (1991) *Methods Enzymol.* **200,** 115–120.
13. Cole, R. D. (1977) in *The Molecular Biology of the Mammalian Genetic Apparatus*, ed. Ts'o, P. (Elsevier, Amsterdam), pp. 93–104.
14. Ottaviano, Y. & Gerace, L. (1985) *J. Biol. Chem.* **260,** 624–632.
15. Devlin, J. J., Panganiban, L. C. & Devlin, P. E. (1990) *Science* **249,** 404–406.
16. Scott, J. K. & Smith, G. P. (1990) *Science* **249,** 386–390.
17. Parmley, S. F. & Smith, G. P. (1988) *Gene* **73,** 305–318.
18. Lupas, A., Van Dyke, M. & Stock, J. (1991) *Science* **252,** 1162–1164.
19. Cooper, J. A. & King, C. S. (1986) *Mol. Cell. Biol.* **6,** 4467–4477.
20. Cohen, P., Holmes, C. F. B. & Tsukitani, Y. (1990) *Trends Biochem. Sci.* **15,** 98–102.
21. MacKintosh, C., Beattie, K. A., Klumpp, S., Cohen, P. & Codd, G. A. (1990) *FEBS Lett.* **264,** 187.
22. Grant, R. A., Lin, T., Konigsberg, W. & Webster, R. E. (1981) *J. Biol. Chem.* **256,** 539–546.
23. Langan, T. A., Zeilig, C. & Leichtling, B. (1981) *Cold Spring Harbor Conf. Cell Prolif.* **8,** 1039–1052.
24. Chambers, T. C. & Langan, T. A. (1990) *J. Biol. Chem.* **265,** 16940–16947.
25. Clark-Lewis, I., Sanghera, J. S. & Pelech, S. L. (1991) *J. Biol. Chem.* **266,** 15180–15184.
26. Kuang, J. & Ashorn, C. L. (1993) *J. Cell Biol.* **123,** 859–868.
27. Wang, J. Y. (1988) *Anal. Biochem.* **172,** 1–7.