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Identification of Novel M Phase Phosphoproteins by Expression Cloning

Naoko Matsumoto-Taniura,*† Fabienne Pirollet,*‡ Robert Monroe,*§ Larry Gerace,* and Joanne M. Westendorf*‡‖

*Departments of Cell and Molecular Biology, The Scripps Research Institute, La Jolla, California 92037; and ‡Laboratoire du Cytosquelette Commissariat à l'Energie Atomique, Institut National de la Santé et de la Recherche Médicale Unité 366, Département de Biologie Moléculaire et Structurale, CEA/Grenoble, 38054 Grenoble Cedex 9, France

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Using an expression cloning technique, we isolated cDNAs for eight M phase phosphoproteins (MPPs 4–11). We then used affinity-purified antibodies to fusion proteins to characterize the intracellular localization and some biochemical properties of these proteins and two others that we identified previously (MPPs 1–2). Each antibody immunoprecipitated one or two protein species of a characteristic size ranging from 17,000 to 220,000 Da. Each MPP, when immunoprecipitated from lysates of M phase cells, was reactive with MPM2, a monoclonal antibody that recognizes a group of related M phase phosphorylation sites, including F-phosphoT-P-L-Q. This reactivity indicated that all the MPPs encoded genuine M phase phosphoproteins. When antibodies to the MPPs were used for immunofluorescence microscopy, each anti-MPP gave a characteristic pattern of localization. In interphase, several of the MPPs were nuclear proteins, whereas others were cytoplasmic or distributed throughout the cell. Three MPPs were strikingly localized to interphase structures: MPP7 to centers of DNA replication, MPP9 to the Golgi complex, and MPP10 to nucleoli. In mitosis, most of the MPPs were distributed throughout the cells. Further studies of the 10 MPPs, most of which are previously undescribed, are expected to provide new understandings of the process of cell division.

INTRODUCTION

A family of cyclin-dependent kinases (cdks) drives progression through the cell cycle by phosphorylating numerous cellular proteins (Draetta, 1994; Heichman and Roberts, 1994; Hunt and Sherr, 1994; Hunter and Pines, 1994; King *et al.*, 1994; Nurse, 1994; O'Connell and Nurse, 1994; Sherr, 1994). These kinases consist of two subunits: a catalytic subunit that is related to the yeast cdc2/CDC28 gene and a cyclin regulatory subunit. The catalytic subunit is present throughout the cell cycle, and the cyclin subunit, which is required for activity, appears and disappears at a time appropriate to its function. The activity of cyclin-dependent kinases is further regulated by phosphorylation and dephosphorylation of the catalytic subunit (Coleman and Dunphy, 1994) and by binding to several small peptide kinase inhibitors (Elledge and Harper, 1994). Although much information has accumulated on regulation of cdk activity, less is now known about the kinase substrates that perform cell-cycle progression.

The first known cyclin-dependent kinase, $p34^{cdc2}$, associates primarily with cyclin B to generate M phase-promoting factor (MPF; Dunphy *et al.*, 1988; Gautier *et al.*, 1988, 1990; Draetta *et al.*, 1989; Labbé *et al.*, 1989, Meijer *et al.*, 1989), which causes G2 phase cells to enter M phase. Upon progression into M phase, many proteins, which include mitotic regulators and interphase structural proteins, become hyper-

Present addresses: †Department of Biochemistry, Biomedical Research Center, Osaka, Japan; §Present address: Children's Hospital, Harvard Medical School, Boston, MA 02115.
‖Corresponding author: Laboratoire du Cytosquelette CEA, INSERM U366, DBMS, CEA/Grenoble, 17 rue des Martyrs, 38054 Grenoble Cedex 9, France.

phosphorylated by MPF or other activated M phase kinases (Maller *et al.*, 1977; Karsenti *et al.*, 1987; Lohka *et al.*, 1987). These M phase phosphoproteins, many of which have not yet been identified, permit disassembly of interphase structures and generation of M phase enzymatic activities and structures.

A subset of the M phase phosphoproteins (at least 40 polypeptides) is reactive with MPM2 (Davis *et al.*, 1983), a monoclonal antibody that recognizes a set of related phosphorylation sites, including F-phosphoT-P-L-Q (Westendorf *et al.*, 1994). The MPM2-reactive phosphoproteins, which are abundant in M phase cells and strongly reduced in interphase cells, can be generated by phosphorylation of pre-existing interphase proteins (Kuang *et al.*, 1989). As a group, MPM2-reactive phosphoproteins are present throughout the M phase cell but are most concentrated in several structures involved in M phase functions, including centrosomes, kinetochores, spindle fibers, the chromosomal axis, and the midbody (Vandre *et al.*, 1984; Hirano and Mitchison, 1991). Because addition of MPM2 to centrosomes prevents microtubule nucleation in vitro (Centonze and Borisy, 1990), the concentration of MPM2-reactive proteins in spindle poles is suggested to be of functional importance. Furthermore, when cells are microinjected with MPM2, entry into (Kuang *et al.*, 1989) and exit from M phase (Davis and Rao, 1987) are inhibited. Recently, a molecular basis for these results has been suggested; MPM2-reactive forms of cdc25, a tyrosine phosphatase, and wee1, a kinase, have been shown to be involved in activation of MPF (Kuang *et al.*, 1994; Mueller *et al.*, 1995), and cyclin destruction activity has been shown to be MPM2-reactive (King *et al.*, 1995), probably because of an MPM2-reactive ubiquitin-protein ligase subunit CDC27, which contains several potential MPM2 binding sites. Several other proteins, including topoisomerase II (Taagepera *et al.*, 1993), MAP4 (Vandre *et al.*, 1991), and nimA (Ye *et al.*, 1995), known to have M phase functions have also been shown to be MPM2 antigens. It seems likely that phosphorylation of these and other proteins at MPM2 sites imparts key M phase functions.

We have originated a method for cloning cDNAs encoding proteins that are efficient substrates for M phase kinases that act at MPM2 sites, and in this manner we have identified two novel proteins (Westendorf *et al.*, 1994). In this paper, we have isolated cDNA clones for eight more proteins, most of which have not yet been described. Further, for each of the 10 proteins, we have produced fusion proteins that were then used to generate specific antisera. These antisera have allowed us to characterize biochemical properties and the intracellular localization of these 10 proteins in interphase and M phase. Importantly, we are now able to show that all of the proteins are authentic MPM2 antigens in vivo during M phase. Among them are molecules with a possible role in regulating structure and functions of nucleoli, Golgi, and DNA replication centers.

MATERIALS AND METHODS

Isolation of M Phase Phosphoproteins (MPP) cDNAs

cDNAs were isolated essentially as described previously (Westendorf *et al.*, 1994). Protein replica filters of λ cDNA libraries were incubated with extracts of M phase HeLa cells to phosphorylate substrate proteins, washed, and incubated with the phospho-epitope-binding antibody MPM2. Immunoreactivity was detected with horseradish peroxidase anti-mouse (Clontech, Palo Alto, CA) and 4-chloro-1-napthol. The following improvements were made to the procedure. Before reaction with M phase cell extracts for isolation of MPPs 4–11, replica filters were denatured with 6 M guanidine in buffer A (25 mM HEPES, 25 mM NaCl, and 5 mM $MgCl_2$) and renatured by incubation with four successive twofold dilutions of the guanidine solution with buffer A containing 0.5 mM dithiothreitol (DTT). This improved the kinase substrate accessibility of MPP protein expressed in λ plaques. For the kinase incubations using extracts of M phase cells, 1 μM microcystin LR was substituted for NaF and $Na_4P_2O_7$, which were found to inhibit kinase activity in the extract. Finally, 1.5 M NaCl was added to the MPM2 incubation to decrease background antibody binding. To allow selection of cDNAs different from those already isolated, we compared the positive plaques of the protein replica filters reacted with M phase cell extracts and MPM2 with the positives obtained with a second replica filter hybridized with probes corresponding to our previously isolated cDNAs by standard procedures (Sambrook *et al.*, 1989).

Preparation of Bacterially Expressed MPPs

All MPPs were fused to T7 protein 10, and all of those except MPP1 also contained a hexahistidine (his) tag. MPP1 and MPP2 fusion proteins were prepared as described previously (Westendorf *et al.*, 1994). Vectors encoding his-tagged protein 10–MPP fusion proteins for MPP5, 6, and 9 were prepared exactly as described for MPP2. Vectors encoding the other MPP fusion proteins were prepared as follows. MPP7 cDNA, which contained internal *Eco*RI sites, was amplified from λ DNA, subcloned into the pCRII vector (Invitrogen, San Diego, CA), excised with *Hin*dIII and *Eco*RV, and ligated to *Hin*dIII-cut GEMEX1HIS. The ends of the resulting linear product of this ligation were blunted with mung bean nuclease and recircularized. MPP8 cDNA, which lacked a 5′ *Eco*RI site, was amplified from λ DNA, subcloned into the pCRII vector, methylated at *Eco*RI sites, excised together with 61 base pairs (bp) of vector DNA with *Bst*XI, blunted with T4 DNA polymerase, ligated to 12 mer *Eco*RI linkers, and cloned into the *Eco*RI site of GEMEX1HIS. MPP10 cDNA was subcloned into the *Eco*RI site of pBluescript, excised with *Bam*HI (which resulted in a 144 bp 5′ end deletion), blunted with T4 DNA polymerase, ligated to 10 mer *Eco*RI linkers, and cloned into the *Eco*RI site of GEMEX1HIS. MPP11 cDNA, which contained internal *Eco*RI sites, was amplified from λ DNA and subcloned into the pCRII vector. The largest *Eco*RI (starting at bp 607) fragment was excised, blunted with T4 DNA polymerase, ligated to 8-mer *Bam*HI linkers, and cloned into GEMEX1HIS-*Bam*HI (GEMEX1HIS modified by digesting with *Eco*RI, blunting with T4 DNA polymerase, and ligating in an 8-mer *Bam*HI linker). The MPP4 cDNA insert 4-3 was subcloned into GEMEX2, but because of a 5′ stop codon, it was not fused to protein 10. This construct was used for production of a nonfusion protein, which was used for immunization. Subsequently, a PCR primer containing an *Eco*RI site and the region surrounding the apparent start codon and the T3 promoter primer were used to produce a PCR product lacking the 5′ stop. This PCR

product was cut with *Eco*RI and subcloned into pGEMEX1HIS. This construct was used for preparation of an affinity column for purification of antisera. Plasmids were transfected into JM109(DE3) or BL21plysS (Glass *et al.*, 1993) and grown in minimal media plus casamino acids and 200 μg/ml ampicillin at 30 or 37°C. Just before induction, cells were centrifuged and resuspended in media containing fresh ampicillin and 1–10 mM isopropyl-β-D-thiogalactopyranoside. Cells were centrifuged and resuspended in 6 M guanidine for purification on Probond resin, according to the manufacturer's instructions (Invitrogen, San Diego, CA). MPP4 that was not fused to protein 10 HIS was isolated from bacterial inclusion bodies, purified by SDS-PAGE, and electroluted from gel slices.

Production of Antisera

Guinea pigs were injected intramuscularly and subcutaneously with bacterially expressed MPP proteins in Freund's complete adjuvant and boosted five times with MPP proteins in Freund's incomplete adjuvant. Terminal bleeds were by cardiac puncture 7–10 d after the final injection. For each protein, antisera were generated in two guinea pigs, and for all of the MPPs except MPP9, antisera from both animals gave similar immunofluorescent patterns. For MPP9, antibodies from one of the animals were reactive almost exclusively with protein 10. Antibodies were purified on affinity columns containing fusion proteins (1 mg/ml) coupled to cyanogen bromide-activated Sepharose CL4 beads (Pharmacia, Piscataway, NJ), as described by the manufacturer. One-half milliliter aliquots of antisera were bound to 1–2 ml of beads and eluted with 3 M potassium thiocyanate, 0.5 M NH_4OH, 5 mg/ml bovine serum albumin, or 4.9 M $MgCl_2$. For some experiments, antisera were passed over beads containing a heterologous protein 10 fusion protein to remove cross-reacting antibodies before purification on homologous protein 10 fusion protein beads.

Immunoprecipitation

HeLa S3 cells were grown as spinner cultures in Joklik's media containing 10% equine serum. M phase cells (85–98%, as determined by staining with lacto-orcein) were produced by treating exponentially growing cultures with 0.1 μg/ml nocodazole for 18–24 h. Most of the immunoprecipitations were performed by lysing cells at a concentration of 10^7 per milliliter in high-salt RIPA, which contained 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 mM EDTA, 10 mM ethylene glycol bis(β-aminoethyl)ether-*N,N,N',N'*-tetra-acetic acid (EGTA), 10 mM benzamidine, 10 mM tosylarginyl methyl ester (TAME), 50 mM NaF, 10 mM tetrasodium pyrophosphate, 1 mM sodium orthovanadate, 80 mM β-glycerophosphate, and 500 nM to 5 μM microcystin LR. Others were performed by first lysing cells in hot 50 mM Tris-HCl, pH 7.4, 0.4% SDS, 10 mM EDTA, 10 mM EGTA, 10 mM benzamidine, 50 mM NaF, 10 mM tetrasodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM TAME, and 80 mM β-glycerophosphate and then adding appropriate stock solutions to yield final concentrations of 2% Triton X-100, 2 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 2–5 μM microcystin LR. Lysates were centrifuged at 100,000 × *g* and 20–40 μg of antibody cross-linked to cyanogen bromide-activated Sepharose CL4B, or protein A Sepharose 6 MB was added to 10^7 or 10^8 cell equivalents of lysate. After a 2 h to overnight incubation at 4°C, beads were washed three times with immunoprecipitation buffer, and bound protein was eluted with SDS sample buffer. In some experiments, after washing with immunoprecipitation buffer, the beads were further washed with 40 mM 1,4-piperazinediethanesulfonic acid (PIPES) buffer, pH 6.0, and incubated with 0.2 U of potato acid phosphatase in the same buffer for 15–60 min before addition of SDS sample buffer.

Immunoblotting

Proteins were separated by SDS-PAGE, blotted to supported nitrocellulose in 50 mM Tris, 380 mM glycine, 0.3% SDS, and 20% methanol or Immobilon-P in 25 mM Tris, 180 mM glycine, reacted with primary antibodies overnight at 4°C, reacted with secondary antibodies conjugated to horseradish peroxidase for 1–2 h, and detected by enhanced chemiluminescence reagents (Amersham, Arlington Heights, IL).

Immunofluorescence

HeLa, CV1, or mycNAGT I HeLa (Nilsson *et al.*, 1993) cells were grown on coverslips in DMEM plus 10% fetal bovine serum and nonessential amino acids (plus 450 μg/ml Geneticin for the mycNAGT I HeLa cells) and fixed in methanol at −20°C for 10 min or in 4% formaldehyde, PBS, and 1 mM $MgCl_2$ at room temperature for 6–30 min, followed by permeabilization with 0.2% Triton X-100, PBS. Generally, formaldehyde and methanol fixations gave similar results, and, therefore, results of only one fixation, the one that gave the strongest antibody reaction or best preserved the structures stained by the antibody, are shown. Fixed cells were blocked with 0.2% gelatin in PBS and incubated with primary antibodies (2–10 μg/ml) for 1–2 h at room temperature or overnight at 4°C. After washing, cells were incubated with cyanine3-labeled donkey anti-guinea pig (Jackson ImmunoResearch, West Grove, PA) for 30 min to 2 h at room temperature, stained with 1 μg/ml Hoechst 33258, and mounted in PBS, 50% glycerol, or MOWIOL. For double immunofluorescence with anti-myc (mouse) or MPM2 (mouse), cells were incubated with the two primary antibodies, followed by both secondary antibodies (cya3-labeled donkey anti-guinea pig and fluorescein-labeled donkey anti-mouse). For double immunofluorescence with anti-MPP7 and anti-BrdU (bromodeoxyuridine), rapidly growing, unsynchronized cells were incubated for 10 min with 10 μM BrdU, fixed with formaldehyde, and stained as described above with anti-MPP7 and cyanine3-labeled anti-guinea pig. Then cells were again fixed with formaldehyde for 6 min, treated with 4 N HCl for 10 min, washed with PBS, and stained with fluorescein-labeled anti-BrdU (Becton Dickinson, San Jose, CA).

Fractionation

HeLa S3 cells (exponentially growing and nocodazole-arrested M phase) grown as for immunoprecipitations were harvested by centrifugation, washed with 10 mM HEPES, pH 7.4, 10 mM KCl, 150 mM NaCl, 1.5 mM $MgCl_2$, and lysed by nitrogen cavitation in two pellet volumes of 10 mM HEPES, pH 7.4, 10 mM KCl, 250 mM sucrose, 2 mM $MgCl_2$, 5 μM microcystin LR, 2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 mM TAME, 10 mM benzamidine, 5 μM E-64, 1 mM EDTA, and 10 μg/ml aprotinin. Cell lysates were loaded onto 30% sucrose and centrifuged at 800 × *g* to obtain the nuclear pellet. The nuclear-free lysate above the 30% sucrose layer was collected, and part of it was centrifuged at 39,000 rpm in a Beckman Ti70 rotor for 1 h to obtain cytosol and crude high-speed pellets. The remaining portion was brought to 1.4 M sucrose, layered underneath 0.25 M sucrose, and centrifuged at 25,000 rpm in a Beckman SW28 rotor for 1.5 h. The membrane fraction at the interface between the two layers was collected, diluted to 0.25 sucrose, and centrifuged at 39,000 rpm in a Beckman Ti70 rotor for 1 h. Portions of the membrane pellets were further fractionated by resuspension at 2 mg/ml in 0.5 M NaCl or 0.1 M NaOH and 1 mM DTT, incubation on ice for 15 min, and centrifugation at 70,000 rpm in a Beckman TLA rotor for 30 min. Samples of unfractionated cell extract, nuclear pellet, cytosol, crude high-speed pellet, gradient pellet, and membranes containing 25 μg protein and the supernatants and pellets from salt and alkaline fractionation of 25 μg of membranes were subjected to SDS-PAGE and immunoblot procedures.

RESULTS

Expression Screening Isolates cDNAs for MPM2-reactive Phosphoproteins

We improved our previous protocol (Westendorf *et al.*, 1994) for isolating cDNAs encoding MPM2-reactive phosphoproteins (MPPs) by using microcystin LR as phosphatase inhibitor during phosphorylation of phage plaque proteins with M phase cell extracts. In addition, we improved the accessibility of protein kinase substrates bound to nitrocellulose by pretreatment of filters with denaturing solutions of guanidine hydrochloride. This permitted us to isolate a total of 30 cDNA clones (Tables 1 and 2). Cross-hybridization experiments and the sequences of the 5′ and 3′ termini of each clone indicated that we have isolated cDNAs for 16 different mRNAs. Ten of these encoded previously undescribed proteins, MPP 1–2, and MPP 4–11 (Table 1), which we further characterized in this study. The other six sequences encoded known proteins (Table 2: casein kinase II β-subunit, ribosomal protein L18a, and the 33-kDa laminin binding protein) or did not clearly encode any human protein (Table 2: 3′ noncoding sequences of cofilin; X-1, which encodes a partial open reading frame (ORF) of 226 amino acids with 39% identity to a segment of *Escherichia coli* DNA helicase II and may be of bacterial origin; and X-2, an uncharacterized sequence containing many *Eco*RI sites). Further sequencing of the clones for MPPs 4–11 indicated that the predicted protein sequences of all MPPs except MPP8 have one or more 5-amino-acid strings that correspond to sequences previously shown to be reactive with MPM2 (Table 3; Westendorf *et al.*, 1994). This finding strengthens our method for cloning MPM2-reactive phosphoproteins and the sequence that we have determined for binding of MPM2.

To study further the 10 previously undescribed proteins, we made T7 protein 10 fusion proteins, most of which contained a six histidine tag, to allow easy purification on a metal chelate column. As shown in Figure 1 and previously described for MPP1 and MPP2 fusion proteins (Westendorf *et al.*, 1994), all of the fusion proteins were at least partially degraded owing to their extreme sensitivity to proteolysis. Nev-

Table 1. Characteristics of cDNA clones for MPPs 1–2 and 4–11

MPP	Library[a]	Clone	Length cDNA (bp)	A tail?	5′stop before ORF?	Amount of protein encoded by ORF (kDa)	Size HeLa protein (by IP)	Intracellular localization, interphase	Intracellular localization, M phase
1	HeLa λZap	6-1	2565	no	no	66	220	cytoplasmic foci and cytoplasm	cytoplasmic foci and cytoplasm
2	MOLT4λgt11	5-3	1659	yes	no	24	120	nucleus	mitotic centrosomes and spindle, cytoplasmic foci, and cytoplasm
4	HeLa λZap	4-3	2042	no	yes	66	90, 110	nucleus and nucleolus	cytoplasm
	HeLa λZap	5-1	1386	no	yes	48			
	MOLT4λgt11	14	1373	no	yes	43			
5	MOLT4λgt11	3	687	no	no	12	130	cytoplasmic foci, cytoplasm, and nucleus	cytoplasmic foci and cytoplasm
6	MOLT4λgt11	6	1017	yes	no	20	17	nucleus and nucleolus	cytoplasm
		13	1099	yes	no	20			
7	MOLT4λgt11	7	2200 (744)[b]	yes[b]	no	9	75	centers of DNA replication, nucleus	cytoplasm
8	MOLT4λgt11	8	918	no	no	35	130	nucleus	cytoplasm
9	MOLT4λgt11	9	925	yes	no	24	150, 160	Golgi	cytoplasm
10	MOLT4λgt11	2	2130	yes	no	78	120	nucleolus	perichromosomal plaques
11	MOLT4λgt11	14	1859	yes	no	67	130	cytoplasmic foci, cytoplasm, and nucleus	cytoplasmic foci and cytoplasm

[a] The origins of cDNA libraries: HeLa λZap, Drs. Helena Richardson and Steven Reed; MOLT4λgt11, Dr. Kevin Sullivan.

[b] The first "A tail" occurs after nucleotide 744 of the clone.

Table 2. Characteristics of other cDNAs whose encoded proteins become MPM2 antigens

Protein	Library[a]	Clone	Length cDNA (bp)	Portion of Protein Encoded
Casein Kinase II β (regulatory) subunit[c] (242aa)	MOLT4λgt11	4	1200	aa −42 to 242[d]
ribosomal protein L18a[e] (175aa)	MOLT4λgt11	5	300	aa 121 to 175
laminin binding protein[f] (295aa)	MOLT4λgt11	1-1	345	aa 222 to 295
	MOLT4λgt11	1-2	200	ND[b]
	MOLT4λgt11	2-1	312	aa 223 to 295
	MOLT4λgt11	3-1	679	aa 105 to 295
	MOLT4λgt11	4-1	342	aa 222 to 295
	MOLT4λgt11	5-2	448	aa 177 to 295
	MOLT4λgt11	9-1	349	aa 221 to 295
3′ noncoding sequence of cofilin[g] (166 aa)	MOLT4λgt11	1	550	63nt beyond aa166 codon to A tail
	MOLT4λgt11	10	650	aa 160 to A tail
	MOLT4λgt11	11	500	123 nt beyond aa166 codon to A tail
	MOLT4λgt11	12	450	126 nt beyond aa166 codon to A tail
X-1[h]	HeLa λgt11	4	2284	226 C terminal aa
		3	1835	222 C terminal aa
		16	2260	218 C terminal aa
X-2	HeLa λZap	4-2	3000	ND[b]

[a] The origins of cDNA libraries: HeLa λZap, Drs. Helena Richardson and Steven Reed; MOLT4λgt11, Dr. Kevin Sullivan; HeLa λgt11, Clontech.

[b] The cDNA sequence was not determined. Laminin binding protein clone 1-2 was identified by cross-hybridization with the other clones. Clone 4-2 did not hybridize with any other isolated clones.

[c] A. Voss, U. Wirkner, R. Jacobi, N. Hewitt, C. Schwager, J. Zimmermann, W. Ansorge, and W. Pyerin (1991). Structure of the gene encoding human casein kinase II subunit beta. J. Biol. Chem. *266*, 13706–13711.

[d] The 126 nt of 5′ "noncoding" sequence contain no inframe stop codons.

[e] Y. Aoyama, Y.-L. Chan, O. Meyuhas, and I.G. Wool. (1989). The primary structure of rat ribosomal protein L18a. FEBS Lett. 247, 242–246.

[f] H. Yow, J.M. Wong, H.S. Chen, C. Lee, G.D. Steele, Jr., and L.B. Chen. (1988). Increased mRNA expression of a laminin-binding protein in human colon carcinoma: complete sequence of a full-length cDNA encoding the protein. Proc. Natl. Acad. Sci. USA *85*, 6394–6398.

[g] K. Ogawa, M. Tashima, Y. Yumoto, T. Okuda, H. Sawada, M. Okuma, and Y. Maruyama. (1990). Coding sequence of human placental cofilin cDNA. Nucleic Acids Res. *18*, 7169.

[h] The X1 clones contain two apparent ORFs in one cDNA. The first ORF contains 2 regions with 94% (18 of 19 amino acids) and 100% (9 of 9 amino acids) identity to replicative protein motifs V and VI, respectively. T.C. Hodgman. (1988). A new superfamily of replicative proteins. Nature *333*, 22–23; the second has 39% identity with *E. coli*. DNA ligase. The presence of two ORFs in a cDNA of eukaryotic origin is an uncommon occurrence and suggests that these clones may be bacterial contaminants of the HeLa cDNA library. The accession number for X-1 is X98266.

ertheless, if the largest fusion protein band is compared with the size of the T7 protein 10 fusion partner (40 kDa), the sequences contained in the cDNA clones are found to code for ~10-110 kDa of MPP protein (Figure 1). For most of the clones, the apparent amount of protein mass present in the fusion protein is almost the same as that derived from the predicted amino acid sequence (Figure 1 and Table 1). For MPP10, by contrast, there seems to be substantially more protein mass in the fusion protein (130 kDa) than would be predicted from the sequence (78–5 kDa not included in fusion protein + 40 kDa protein 10 = 113 kDa). This could be due to anomalous migration of a highly charged protein. Purified fusion proteins for MPPs 1–2 and 4–11 were injected into guinea pigs to generate antisera, all of which were purified on MPP fusion protein affinity columns for use in the following experiments.

Each MPP cDNA Encodes a Human Protein(s) of a Characteristic Size(s)

To determine the molecular size of the proteins in cells, we immunoprecipitated lysates of 10^7 to 10^8 exponentially growing HeLa cells with each anti-MPP and reacted immunoblots of the immunoprecipitates with homologous antibodies (Figure 2). The blots indicate that the MPPs are HeLa cell proteins of various sizes from 17,000 to 220,000 Da. Their molecular sizes are MPP1, 220,000; MPP2, 120,000; MPP4, 90,000 and 110,000; MPP5, 130,000; MPP6, 17,000 (and, possibly, a minor high molecular weight species); MPP7, 75,000;

Table 3. Potential MPM2 binding sites in MPPs 1 to 2 and 4 to 11.

cDNA Clone	Potential MPM2 sites–TP	Potential MPM2 sites–SP
MPP1	FT_{374}PLQ*	HS_{486}PSI
	TT_{390}PVT*	SS_{533}PID
	AT_{421}PRT	
MPP2	KT_{58}PIK	FS_{96}PVQ*
	ST_{69}PSK	ES_{130}PQP*
	RT_{78}PES	SS_{151}PSD
	LT_{85}PPA*	GS_{162}PEP
	TT_{120}PLQ*	
MPP4	IT_{368}PMK*	TS_{173}PVV
	ST_{504}PSA	KS_{382}PSK
	DT_{596}PLA	
MPP5	ET_{51}PRQ	PS_{65}PLQ*
		AS_{78}PVV
		VS_{92}PWP*
MPP6	IT_{158}PIK*	
MPP7	TT_{23}PRR	GS_{65}PPE
	LT_{30}PLK*	
	DT_{36}PPS	
	ST_{48}PST	
	ET_{58}PGD	
MPP8	KT_{107}PRK	DS_{45}PFP
	QT_{158}PKG	
	LT_{227}PEE	
MPP9	LT_{11}PVT*	ST_{2}PTK
	VT_{130}PQG	YS_{18}PKR
	QT_{185}PQI	RS_{22}PKE
		LS_{28}PGF
		SS_{41}PIR
		PS_{171}PGG
MPP10		GS_{52}PLQ
		KS_{154}PVF*
MPP11	FT_{197}PVF*	
	AT_{500}PSE	
	FT_{513}PWT*	
	NT_{534}PER	

Amino acid numbering begins with the first 5′ inframe amino acid coded for all clones except MPP4, which contains a stop codon before the open reading frame and is numbered from the start methionine. All TP and SP sites within each protein are listed.

* Starred sequences are identical to or closely resemble sequences previously shown to be reactive with MPM2 in that each of the five positions in the string contains an amino acid previously identified in the corresponding position of MPM2-reactive sequences (Westendorf *et al.*, 1994). MPM2 binding at any site also depends on phosphorylation on the T or S and the number of reactive sites within the protein. The accession numbers for the cDNAs are as follows: MPP1, L16782; MPP2, L16783; MPP4, X98264 (clones 14 and 5-1) and X98265 (clone 4-3); MPP5, X98261; MPP6, X98263; MPP7, X98262; MPP8, X98259; MPP9, X98258; MPP10, X98494; and MPP11, X98260.

MPP8, 70,000 and 130,000; MPP9, 150,000 and 160,000; MPP10, 120,000; MPP11, 130,000. The multiple molecular species recognized by antibodies to MPPs 4, 8, and 9 could be due to the existence of multiple genes, alternatively spliced mRNAs, proteolysis (despite the use of many protease inhibitors), or various phosphorylation states. For MPP8, the 70-kDa species is never seen in immunoblots of total cell lysates and is likely

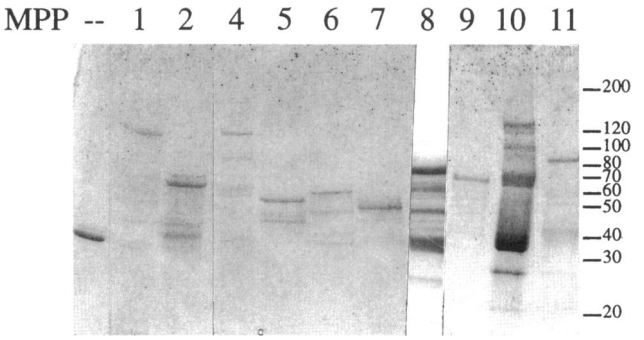

Figure 1. Fusion proteins for cloned MPPs. Fusion proteins produced and purified as discussed in MATERIALS AND METHODS were subjected to electrophoresis on an SDS gel and stained with Coomassie blue. The far left lane contains protein 10 not fused with an MPP protein. The other lanes contain the protein 10 fusion protein for the indicated MPP. Migration of molecular weight markers in kDa is indicated on the right.

to be a proteolytic product of full-length MPP8. For MPP4 and MPP9, phosphorylation is apparently not responsible for multiple molecular species, because dephosphorylation of protein from exponentially growing cells with a phosphatase of broad specificity (potato acid phosphatase) does not change the sizes of the two proteins (MPP4, our unpublished observations; MPP9, Figure 3). The reported molecular weights of all HeLa cell MPPs, which, like their bacterial fusion proteins, are very sensitive to proteolytic degradation, will be reassessed when full-length sequences for each protein are known.

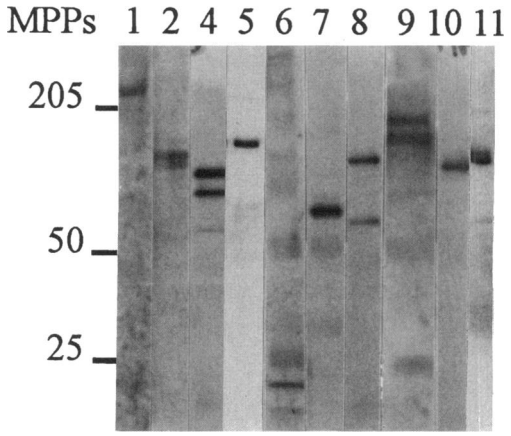

Figure 2. Immunoprecipitation of MPPs from extracts of exponentially growing HeLa cells. MPPs were immunoprecipitated with affinity-purified antibodies from lysates of exponentially growing HeLa cells in high-salt RIPA buffer. Lane, antibody, and cell equivalents: 1, αMPP1, 10^8; 2, αMPP2, 10^7; 3, αMPP4, 10^8; 4, αMPP5, 10^8; 5, αMPP6, 10^8; 6, αMPP7, 10^8; 7, αMPP8, 10^7; 8, αMPP9, 10^8; 9, αMPP10, 10^7; 10, αMPP11, 10^8. In the MPP8 precipitate, the 70-kDa band is apparently an artifact of immunoprecipitation, because it is never seen in immunoblots of whole-cell extracts. Migration of molecular weight markers in kDa is indicated on the left.

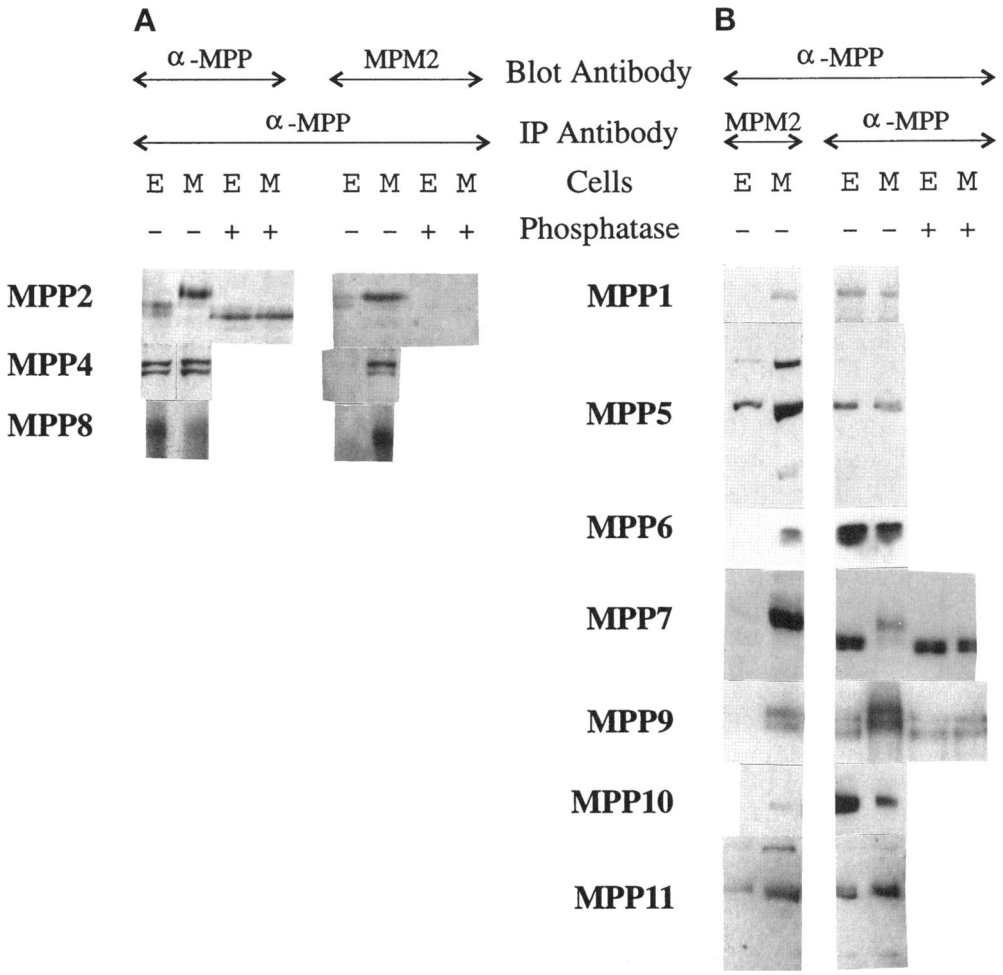

Figure 3. MPM2 reactivity of MPPs immunoprecipitated from extracts of HeLa cells. (A) MPPs were immunoprecipitated (IP) from lysates of exponentially growing (E) or M phase (M) HeLa cells with MPM2 or antibodies directed against an MPP (α-MPP). Immunoprecipitated proteins were separated by SDS-PAGE, and immunoblots were reacted with the α-MPP indicated at the left. (B) MPPs immunoprecipitated with α-MPP as described above were separated by SDS-PAGE, and immunoblots were incubated with MPM2 or the α-MPP indicated at the left. (A and B) When the protein from M phase cells was larger than the protein from exponentially growing cells (MPP2, MPP7, and MPP9), immunoprecipitation pellets were treated with a broad specificity phosphatase, as described in MATERIALS AND METHODS, before addition of SDS sample buffer.

Each MPP Is Reactive with MPM2 in M Phase

To learn whether the proteins encoded by the cDNAs were MPM2-reactive phosphoproteins in vivo, we performed one of the two following types of experiments. In the first type of experiment, MPP proteins were immunoprecipitated from exponentially growing and M phase cells with MPP antibodies. Then, one-half of the resulting immunoprecipitates were immunoblotted with MPP antibodies and the other one-half with MPM2 (Figure 3A). The MPM2 reactivity of MPPs 2, 4, and 8 could be visualized clearly with this procedure. For each of these MPPs, the respective anti-MPP reacted approximately equally well with the MPP precipitated from exponentially growing and M phase cells, whereas MPM2 reacted much more strongly with the MPP precipitated from M phase cells. The other MPPs could not be clearly visualized with MPM2 by this protocol, possibly because they are dephosphorylated in cell lysates even in the presence of phosphatase inhibitors, or of too low abundance to be detected with MPM2 after precipitation with anti-MPP. Therefore, a second procedure was used to analyze their reactivity with MPM2 during M phase. Proteins were immunoprecipitated from lysates of exponentially growing or M phase cells with MPM2 or MPP antibodies and detected on immunoblots with antibodies against MPPs (Figure 3B). For each of these MPPs the anti-MPP precipitates from exponentially growing and M phase cells were approximately equally reactive with the respective anti-MPP on immunoblots. In contrast, the MPM2 precipitates from M phase cells were more strongly reactive with anti-MPP than those from exponentially growing cells. Thus, the results of these experiments indicate that all the MPPs are immunoreactive with MPM2 in M phase cells.

For most of the MPPs, the gel mobilities of the polypeptides from exponentially growing and M phase cells (including the MPM2-reactive species) are indistinguishable (Figure 3). Nevertheless, in M phase cells compared with exponentially growing cells, the gel mobilities of MPP2, MPP7, and MPP9 are substantially decreased, and these slowly migrating forms of the proteins are MPM2-reactive (Figure 3). For MPP2 and MPP9, the shifts are equivalent to ~20 kDa; for

MPP7, the observed shift is variable, but in some of our unpublished observations the shift is as much as 90 kDa. These mobility changes can be reversed by treatment with a broad specificity phosphatase (potato acid phosphatase), which also destroys reactivity with MPM2 (Figure 3). All detectable molecules of MPP2, MPP7, and MPP9 are shifted in mobility, indicating that phosphorylation in M phase is quantitative. Interestingly, MPP2 also seems to be phosphorylated in interphase, because a small shift in size of the interphase protein is seen after dephosphorylation. In addition, immunoprecipitation with MPM2 permits detection of additional protein species of 50,000, 65,000, and 200,000 daltons for MPP5 and of 80,000 and 200,000 daltons for MPP11. The origin of these other anti-MPP–reactive protein species for MPPs 5 and 11 is uncertain, but some could be due to proteolysis and dephosphorylation artifacts.

Although all the MPPs are reactive with MPM2 in M phase, they differ in the strength of reactivity with MPM2 and the conditions required for maintaining MPM2 reactivity. For instance, most of the MPPs react with MPM2 only in M phase, but MPP5 and MPP11 also react more weakly with MPM2 in interphase (Figure 3). Also, MPP6, which contains only one potential MPM2 site, was found to be an MPM2 antigen only when precipitated from a cell lysate containing 5 μM microcystin LR, whereas MPP2, which contains nine potential sites, was an MPM2 antigen when precipitated from lysates containing only 500 nM microcystin LR (Figure 3; our unpublished observations). Further, MPP10, which contains only S-P, as opposed to T-P, sites was less strongly reactive with MPM2 than most of the other MPPs, possibly because MPM2 reacts more strongly with T-P sites than S-P sites (Zhao *et al.*, 1989; Westendorf *et al.*, 1994).

Each MPP Is Localized to Characteristic Cell Structures

When MPP proteins were studied by immunofluorescence microscopy, a diverse range of localization patterns was obtained. For each protein, similar results were obtained with HeLa (human) and CV1 (African green monkey) cells. The antibody staining patterns were also confirmed by preincubation with protein 10 alone, which did not alter the staining, or protein 10-MPP fusion protein, which diminished or abolished staining.

Antibodies to MPPs 1, 2, 5, and 11 all recognize some punctate cytoplasmic foci as well as diffuse staining during at least part of the cell cycle. Anti-MPP1 labeled punctate cytoplasmic foci in both interphase and mitotic cells (Figure 4). In mitotic cells, the staining was concentrated near the mitotic spindle (Figure 4) and, in some interphase cells, the centrosomes (our unpublished observations). In both interphase and mitotic cells, MPP5 and MPP11 were distributed throughout the cell, including the nuclei, in fine punctate foci (Figure 4). In some mitotic cells, MPP5 and MPP11 seemed to be associated with the mitotic spindle.

MPP2 was localized throughout the nucleus except for the nucleoli in interphase (Figure 4). The nuclear localization of MPP2 was difficult to preserve in fixed cells and was obtained only after lengthy fixation with paraformaldehyde. After fixation with methanol or shorter fixations with paraformaldehyde, anti-MPP2 yielded punctate cytoplasmic labeling. A nuclear localization of MPP2 in interphase was consistent with the results of our subcellular fractionation experiments. In cells entering M phase, some MPP2 became concentrated at centrosomes (Figure 4; white arrows in MPP2 interphase panel), and in later stages of M phase, MPP2 was present at the centrosome, in the spindle, and in punctate foci throughout the cell.

MPP4, MPP6, MPP8, and MPP10 were localized to the nucleus in interphase (Figure 5). MPP4 and MPP6 were distributed throughout the nucleus and showed a modest concentration in nucleoli, whereas MPP8 was excluded from the nucleoli, and MPP10 was almost entirely nucleolar. In mitosis, MPP10 adhered to the chromosomes in a manner similar to the nucleolar protein fibrillarin (Yasuda and Maul, 1990); in contrast, MPP4, MPP6, and MPP8 were present throughout the mitotic cell (Figure 5).

MPP7 was localized to distinct spots within the nuclei of some cells and was diffusely localized throughout the nuclei of other cells (Figures 6 and 7). During M phase it was distributed throughout the cells (our unpublished data). When centers of DNA replication were labeled by a 10-min treatment with bromodeoxyuridine, which incorporates into DNA that is being synthesized, and were detected with antibodies to bromodeoxyuridine, the MPP7 spots corresponded exactly to the positions in which DNA was being replicated (Figure 6). Because MPM2 also stains spots within the nucleus (Vandre *et al.*, 1984), it was of interest to know whether the MPM2 reactivity of interphase nuclei was due to MPP7 present in DNA replication centers. Double immunofluorescence with MPM2 and MPP7, however, indicates that the MPM2 and MPP7 spots are not the same (Figure 7). Furthermore, whereas the MPP7 spots are present only in S phase cells, the MPM2 spots are present in all interphase cells. Apparently, the MPP7 associated with centers of DNA replication is not the predominant source of interphase nuclear MPM2 reactivity. This observation is also supported by the data in Figure 3, indicating that MPP7 is not an MPM2 antigen of exponentially growing (92–97% interphase) cells.

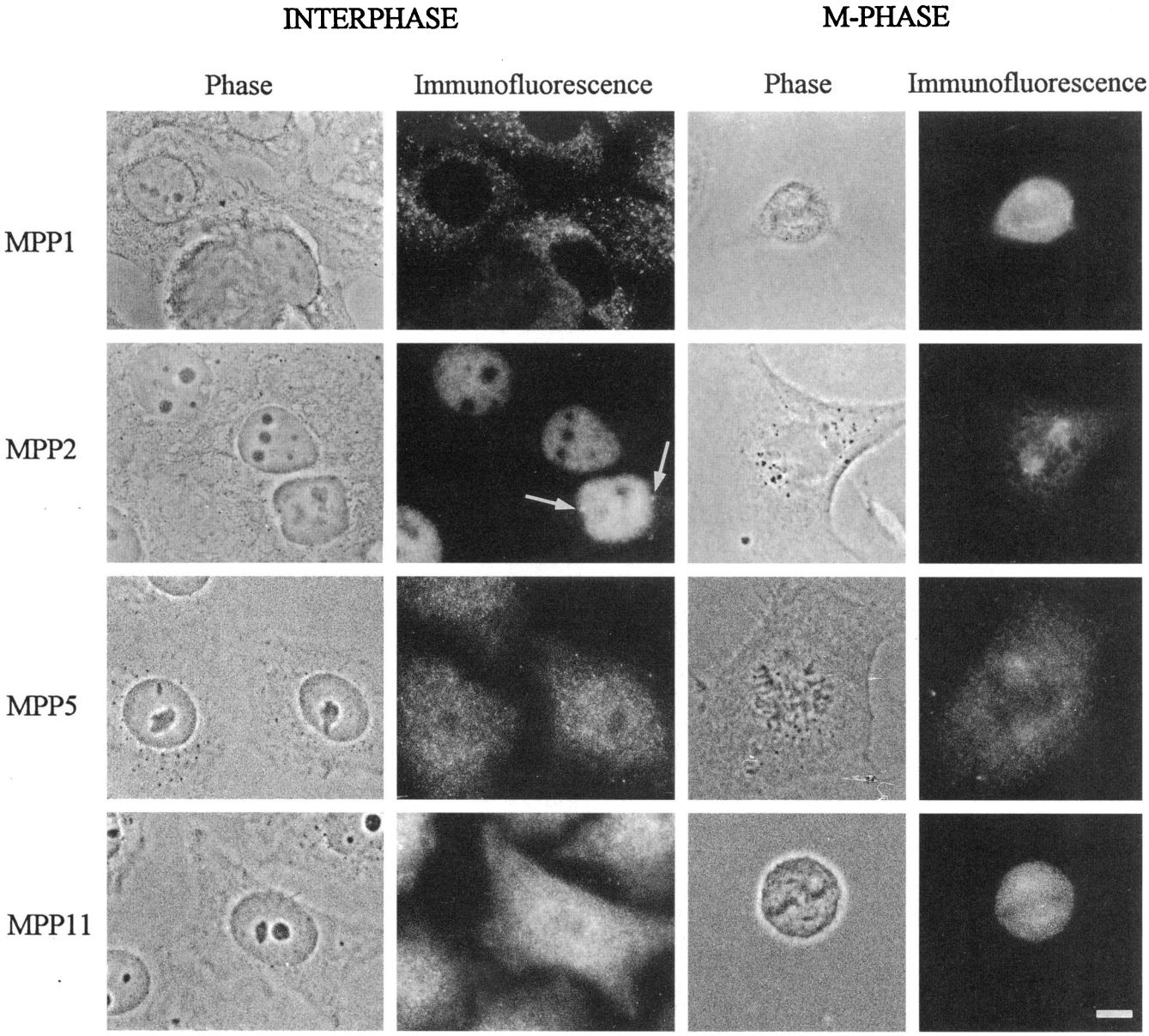

Figure 4. Immunofluorescent localizations of MPP1, MPP2, MPP5, and MPP11. Cells (HeLa or CV1) were grown on coverslips, fixed with formaldehyde, and stained as described in MATERIALS AND METHODS. Antiserum, Interphase (I) or M phase (M), and cell line: αMPP1, I, HeLa; αMPP1, M, CV1; αMPP2, I, CV1; αMPP2, M, CV1; αMPP5, I, HeLa; αMPP5, M, CV1; αMPP11, I, HeLa; and αMPP11, M, HeLa. The arrows in the αMPP2 interphase panel indicate αMPP2-staining centrosomes of a prophase cell. The location of the centrosomes was confirmed by double immunofluorescence staining with anti-tubulin. Bar, 2 μ.

In interphase, much of the HeLa cell MPP9 was localized to the Golgi complex (Figure 8). When compared with the localization of myc-tagged *N*-acetylglucosaminyl transferase I (NAGT I; Nilsson *et al.*, 1993), a transmembrane protein of the medial Golgi, MPP9 staining was found to overlap strongly with that of NAGT I. In cell fractionation experiments designed to confirm the localization of MPP9 to membranes, MPP9 was found in membrane fractions from which it was partially released by 0.5 M NaCl and totally removed by 0.1 M NaOH. These characteristics indicate that MPP9 is a peripheral membrane protein. There was also cytoplasmic background staining with anti-MPP9; this was probably due to the presence of some MPP9 in the cytosol as was observed in cell fractionation experiments. In mitosis, MPP9 was dispersed throughout the cell and did not localize to the Golgi fragments containing NAGT I (Figure 8).

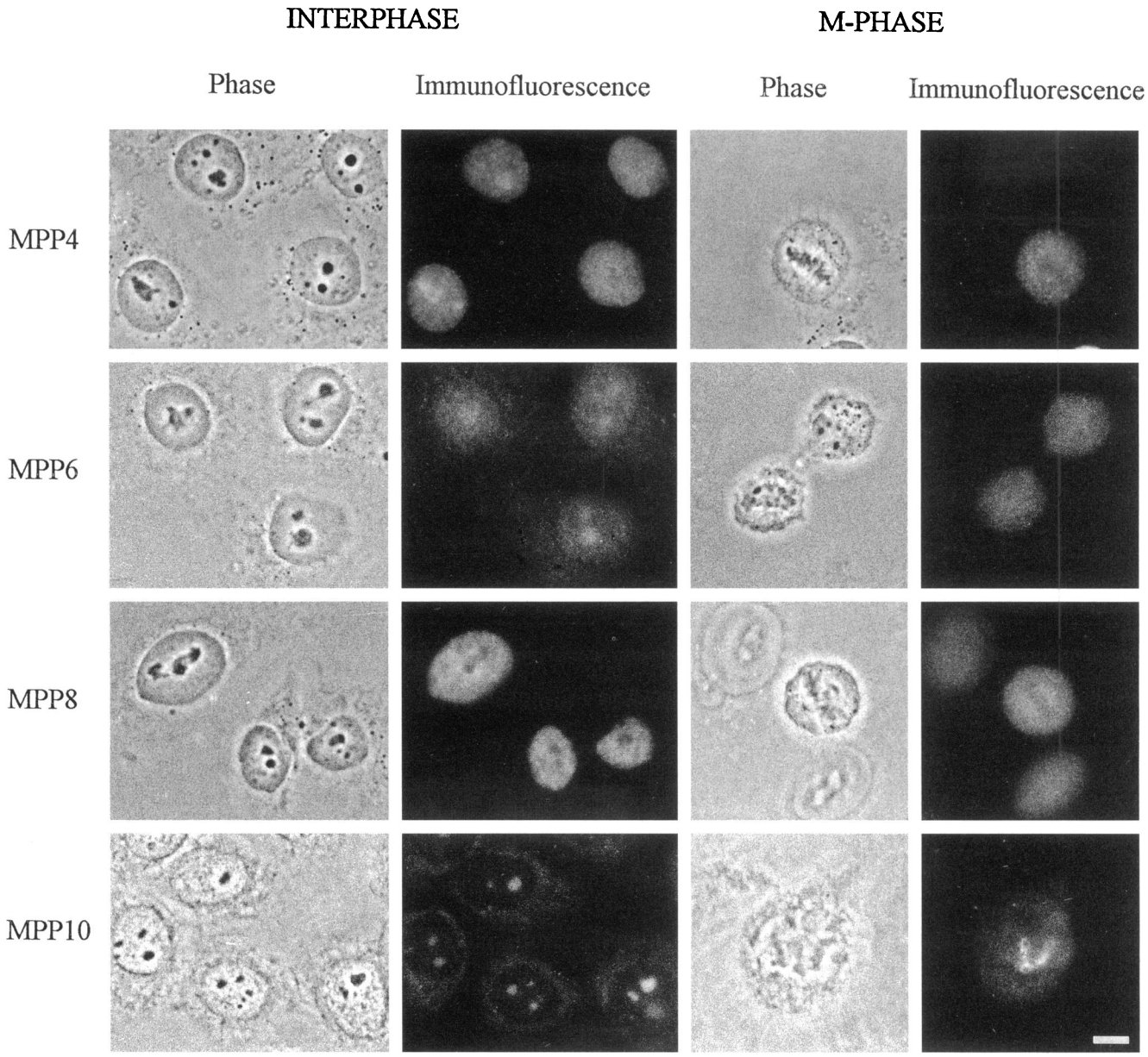

Figure 5. Immunofluorescent localizations of MPP4, MPP6, MPP8, and MPP10. HeLa cells were grown on coverslips, fixed with formaldehyde (Form) or methanol (Meth), and stained as described in MATERIALS AND METHODS. Antiserum, Interphase (I) or M phase (M), and fixation: αMPP4, I, Form; αMPP4, M, Form; αMPP6, I, Form; αMPP6, M, Form; αMPP8, I, Form; αMPP8, M, Form; αMPP10, I, Meth; and αMPP10, M, Meth. Bar, 2 μ.

DISCUSSION

Using our recently developed technique for isolation of proteins containing the phosphoepitope bound by MPM2, we have successfully isolated cDNAs for eight additional proteins (MPPs 4–11). After production of antibodies to protein fragments encoded by these cDNA clones and two previously isolated clones (for MPPs 1–2) (Westendorf *et al.*, 1994), we detect one or two major polypeptides for each MPP in HeLa cell lysates. Moreover, when each MPP protein is immunoprecipitated from extracts of M phase HeLa cells, the protein in the precipitate reacts with MPM2. This reactivity indicates that the MPPs are M phase phosphoproteins as well as MPM2-reactive phosphoproteins and demonstrates that our method is capable of isolating genuine M phase phosphoproteins.

Immunofluorescence microscopy indicates that each MPP has a characteristic pattern of localization. In

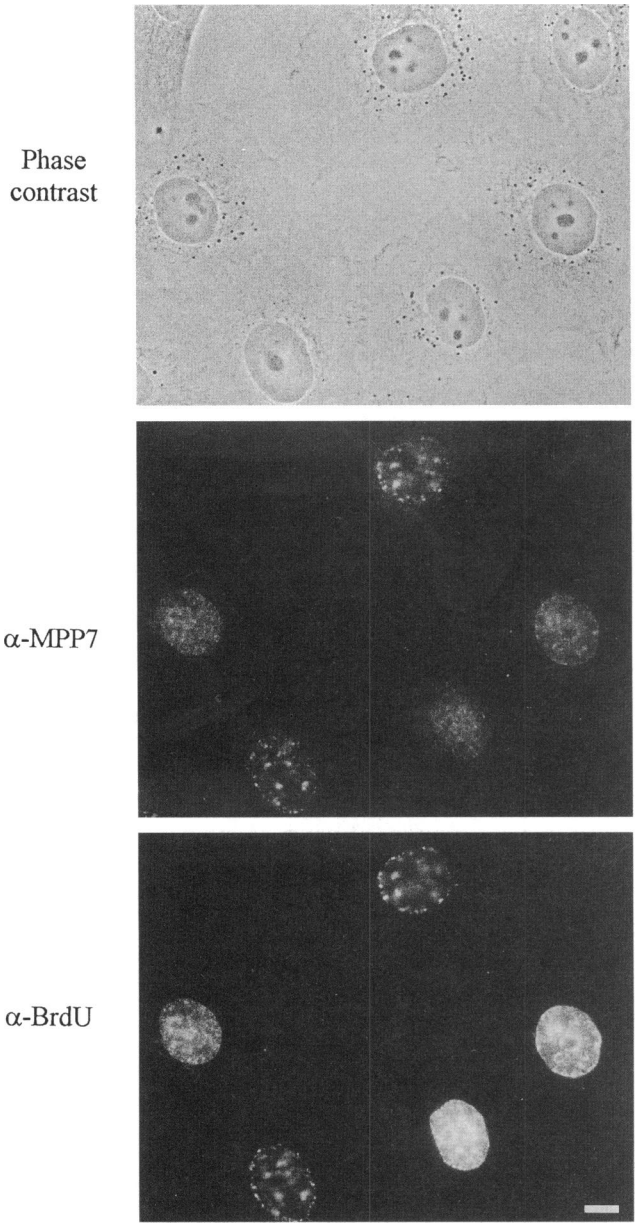

Figure 6. Immunofluorescent localization of MPP7 to centers of DNA replication. HeLa cells grown on coverslips were incubated with BrdU for 10 min and stained with anti-MPP7 and anti-BrdU as described in MATERIALS AND METHODS. Bar, 2 μ.

interphase, MPPs are present in the cytoplasm, the nucleus, the nucleolus, the centers of DNA replication, and the Golgi apparatus. In mitosis, as expected from the pattern of immunofluorescence seen with MPM2, most of the MPPs are spread throughout the cell, in some cases in punctate foci. Nonetheless, MPP2 may account for some of the MPM2 immunofluorescence associated with the centrosome and MPP10 for some of the chromosomal immunofluorescence. Therefore, the MPPs that we have isolated have the properties expected of MPM2-reactive M phase phosphoproteins, and their localization to various cell structures suggests that they will provide clues about how several cell structures and processes are regulated during mitosis.

Although the characterization of the MPPs has been performed almost entirely in HeLa cells, which were derived from a cervical carcinoma, and CV1 cells, a nontransformed kidney epithelial cell line, most of the MPPs are probably not confined to cells of epithelial origin. cDNAs encoding MPPs 2 and 4–11 were isolated from a cDNA library derived from MOLT4 lymphoblastic leukemia cells (Table 1). This limited survey of cell lines suggests that the MPPs are present in rapidly growing cells of various origins. Further characterization of the MPPs in other normal and transformed cells will be necessary to have a clear understanding of the relationship of each to cell growth and differentiation.

The localization of each MPP provides broad hints about its specific interphase function and how that might be altered in M phase. For instance, MPPs 4, 6, 8, and 10 are nuclear during interphase. Phosphorylation in M phase may turn off their nuclear functions to prevent inappropriate interactions with non-nuclear proteins. Alternatively, M phase phosphorylation might confer an M phase-specific function on these proteins.

Four of the MPPs—MPP1, MPP2, MPP5, and MPP11— are associated with punctate foci that are dispersed throughout the cell. Because these MPPs sometimes localize to centrosomes or the mitotic spindle, some of the structures seen as punctate foci may have a microtubule-related function. Currently, we are investigating the nature and activity of these structures.

The localizations of MPP7 and MPP9 point to functions within specific cell structures. MPP9, which localizes to the Golgi apparatus, may regulate Golgi structure or function. MPP7 is localized to centers of DNA replication during S phase and, therefore, is suggested to have a role in that process. Consistent with this localization, the sequence of MPP7 is identical to a subunit of a chromatin assembly factor essential for incorporation of histones into newly synthesized DNA (Kaufman *et al.*, 1995). Our data indicate further that this complex is appropriately localized to centers of DNA replication and is phosphorylated at mitosis.

While this manuscript was in preparation, a protein sequence 94% identical to MPP11 was deposited in the database. This protein, MIDA1, is related to the yeast Z-DNA binding protein zuotin and associates with the transcription factor Id (Shoji *et al.*, 1995). The migration of MIDA1 at 74 kDa on gels corresponds well with the predicted size of MIDA1 but contrasts with the migration of HeLa MPP11 on gels (130 kDa). This discrepancy could be due to an N terminal extension,

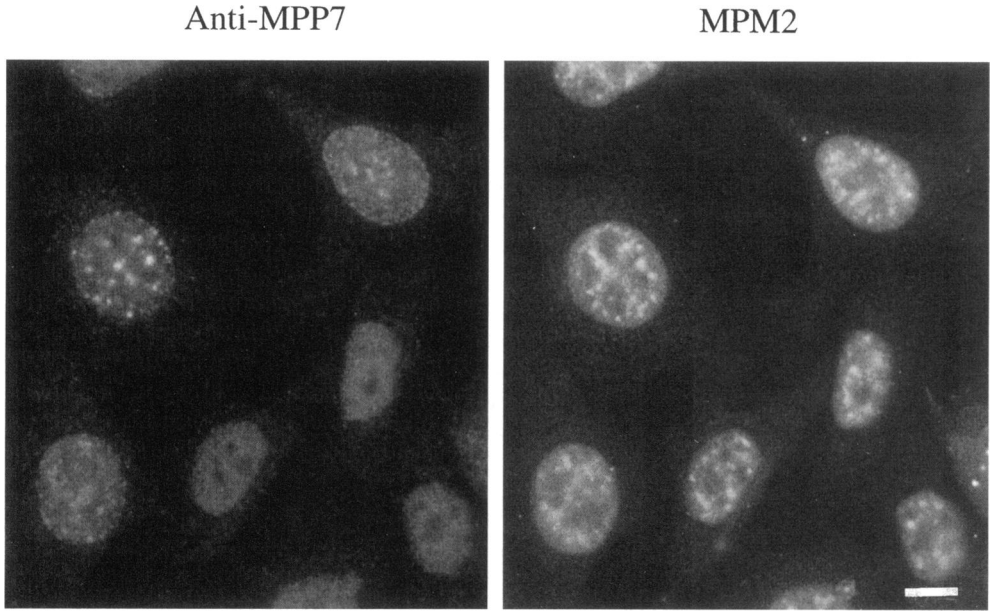

Figure 7. Comparison of MPP7 and MPM2 nuclear immunofluorescence. HeLa cells grown on coverslips were fixed with formaldehyde and stained with anti-MPP7 and MPM2 as described in MATERIALS AND METHODS. Bar, 2 μ.

anomalous migration, or phosphorylation of the human protein.

MPP4 is the human homologue of a previously described *Xenopus* double-stranded RNA binding protein whose function is unknown (Bass *et al.*, 1994). Our data indicate that this protein is also present in human cells, in which it is localized to the nucleus and undergoes phosphorylation in M phase.

Other than MPPs 4, 7, and 11, the sequences of the MPP cDNAs have not provided much information about the functions of the proteins. Further study of the MPPs will require full-length cDNA clones and their encoded protein sequences, which may also provide more clues about their functions.

We have previously defined the phosphorylation site for MPM2 to be a set of related phosphorylation sites, including F-phosphoT-P-L-Q (Westendorf *et al.*, 1994). The sequence of each of the MPPs except MPP8 contains at least one site corresponding to our previous description of potential MPM2 binding sites. MPP8 does, however, have four sequences with the central T/S-P of the MPM2 motif. Because many related peptide sequences are able to bind MPM2 and our previous selection of peptides capable of binding to MPM2 was not exhaustive, it is likely that at least one of the T/S-P sites of MPP8 will, when phosphorylated, bind to MPM2. Recently, a site for MPM2 binding that does not contain a proline has been described in MAP kinase (Taagepera *et al.*, 1994). A sequence corresponding to this site is not found in MPP8 or any of the MPPs. Interestingly, the corresponding site in MAP kinase of neuronal cells does contain a proline (Mohit *et al.*, 1995). If the secondary structure, possibly a beta turn, of this key regulatory site of MAP kinase is conserved, it may be that the MPM2-reactive site of MAP kinase is not really so different from the sites found in other MPM2-reactive proteins. Like the site in MAP kinase, MPM2-reactive phosphorylation sites of MPPs may be responsible for alterations of activity during M phase. Further biochemical studies will be needed to define the precise sites that are phosphorylated in these proteins during M phase and which of these are essential for MPM2 binding and, importantly, alterations of function.

In our screening efforts, we also isolated cDNAs for three known proteins. Interestingly, one of them is the casein kinase II β (regulatory) subunit, which previously has been shown to be phosphorylated during mitosis (Litchfield *et al.*, 1992). This further confirms the validity of our method for identification of mitotic phosphoproteins and indicates that casein kinase II may be part of the cascade of activation of protein kinases that occurs during mitosis. We do not know the significance of the isolations of cDNAs for ribosomal protein L18a or "laminin-binding protein," two proteins associated with ribosomes (Aoyama *et al.*, 1989; Auth and Brawerman, 1992). If these proteins are phosphorylated in vivo, phosphorylation could regulate the amount or kinds of proteins that are synthesized. As yet, we have no evidence that these two proteins are, indeed, phosphorylated in cells. The portion of the laminin-binding protein that is contained in our clones does not have a sequence corresponding to any previously defined M phase MPM2 binding site (Taagepera *et al.*, 1994; Westendorf *et al.*, 1994). Furthermore, it contains no central T/S-P motifs, but, like the MAP kinase site, the site in laminin-binding protein does not seem to be M phase-specific; laminin

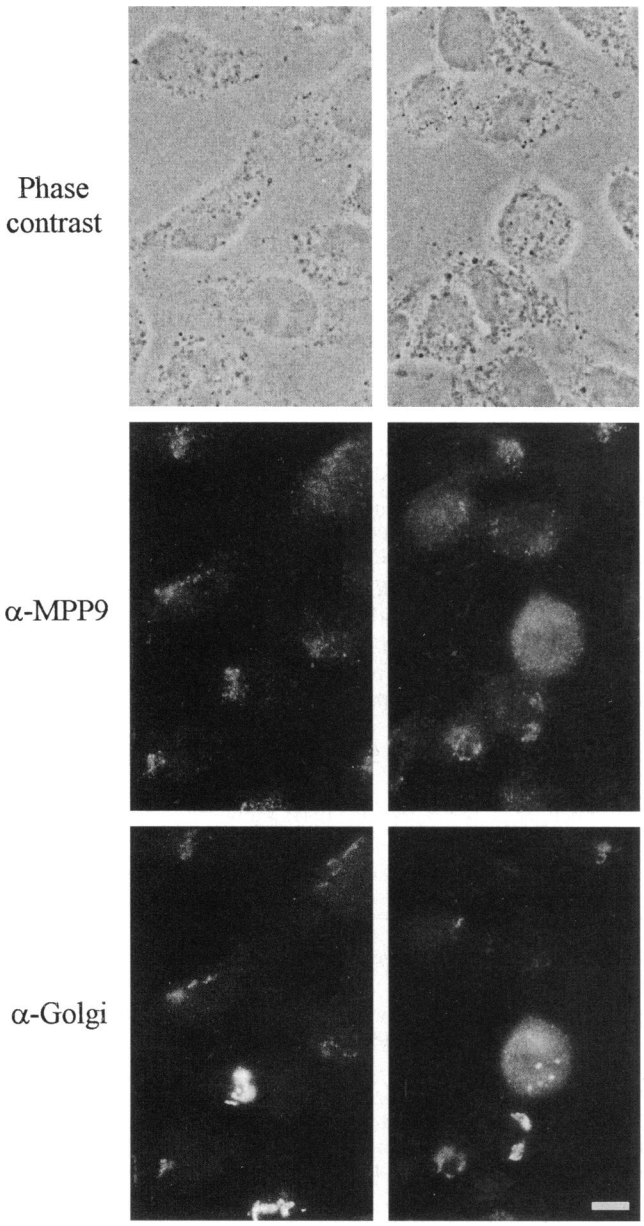

Figure 8. Immunofluorescent localization of MPP9 to the Golgi apparatus. HeLa mycNAGT I cells (Nilsson *et al.*, 1993) were grown on coverslips, fixed with formaldehyde, and stained with anti-MPP9 and anti-myc as described in MATERIALS AND METHODS. Bar, 2 μ.

binding protein fusion protein is phosphorylated to acquire MPM2 reactivity equally well by interphase and M phase extracts of HeLa cells (our unpublished observations). Perhaps the phosphorylation site of laminin-binding protein has a secondary structure similar to that of the proline-containing sites.

So far, four or five kinases have been shown to phosphorylate substrates on sites that, when phosphorylated, become MPM2 antigens. We have shown previously that MPP1 and MPP2 are phosphorylated primarily by different kinases of M phase extracts. For MPP1, the primary kinase seems to be $p34^{cdc2}$, whereas for MPP2, it is another, uncharacterized kinase. By phosphorylating *Xenopus* antigens on blots, Kuang and Ashorn (1993) have isolated two kinases, MAP kinase and a second, novel kinase, that yield MPM2 reactivity. One of these two kinases might be the major kinase that phosphorylates MPP2. In addition, working with MAP kinase as substrate, Tagepera *et al.* (1994) have shown that the activity of MAP kinase kinase can lead to MPM2 reactivity. This kinase may, however, phosphorylate only MAP kinase and no other substrates. The other MPPs, whose kinases have not yet been examined, will also be useful for examining the specificity of various M phase kinases and possibly purifying novel kinases. Likewise, the MPPs will be useful for identifying phosphatases involved in exit from M phase.

In conclusion, our results indicate that a broad range of M phase phosphoproteins can be identified by our expression cloning method. We are now in a position to study the functions of the MPPs that we have characterized in interphase and M phase and discover their roles in the process of cell division.

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