Journal of Cellular Biochemistry 94:967–981 (2005)

Anti-Phosphopeptide Antibody, P-STM as a Novel Tool for Detecting Mitotic Phosphoproteins: Identification of Lamins A and C as Two Major Targets

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Abstract A polyclonal, phospho-epitope-specific antibody (P-STM) was generated to detect the activated p21-activated kinase 2 (PAK2), based on the regulatory autophosphorylation site Thr^{402} of PAK2 [Yu et al., 1998]. In this report, we show that this antibody can also recognize many phosphoproteins in mitotic HeLa and A431 cells. Signal of these phosphoproteins emerged after treating the cells with nocodazole and okadaic acid, and was highly detected in G2-M phase transition of HeLa cells released from double thymidine block. Immunofluorescence analysis revealed that P-STM strongly stained HeLa cells at prometaphase and metaphase, but not at interphase and anaphase. Interestingly, this staining pattern was almost identical to that obtained by staining with MPM2, a monoclonal antibody known to react with phosphoproteins in mitotic HeLa cells. However, the phosphoproteins detected by the two antibodies are quite different. Two-dimensional gel electrophoresis (2DE) and tryptic peptide fingerprint analysis by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry were employed to identify lamins A and C as two of the mitotic cell-specific phosphoproteins recognized by P-STM. Lamins A and C immunoprecipitated from nocodazole-treated cells, but not from untreated cells showed strong reactivity to P-STM, and this reactivity lost completely after protein phosphatase 2A treatment. In summary, our results show that P-STM represents a novel tool for detecting mitotic phosphoproteins, which are different from those recognized by MPM2, and that lamins A and C are the two prominent mitotic phosphoproteins detected by P-STM. J. Cell. Biochem. 94: 967–981, 2005.

Key words: anti-phosphopeptide antibody; mitosis; phosphoproteins; lamins

Eukaryotic cell cycle progression is a complex process regulated by a precisely controlled mechanism in cells. Dramatic changes in cell shape and internal structure occur during this process. Biochemical and genetic studies have revealed that a family of cyclin-dependent kinases (cdks) plays critical roles in driving progression of cell cycle. $p34^{cdc2}$, the first cdk identified as one component of M-phase-promoting factor (MPF), is required for the induction of G_2/M transition [Draetta, 1994; King et al., 1994; Nurse, 1994; Sherr, 1994]. Many proteins

Abbreviations used: 2DE, two-dimensional gel electrophoresis; cdk, cyclin-dependent kinase; DAPI, 4′,6-diamidine-2′-phenylindole dihydrochloride; GTPase, guanosine triphosphatase; IEF, isoelectric focusing; IPG, immobilized pH gradient; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MAP, microtubule-associated protein; MAPK, mitogen-activated protein kinase; MPF, M-phase-promoting factor; MPP, M phase phosphoprotein; PAK2, p21-activated kinase 2; PP2Ac, the catalytic subunit of protein phosphatase 2A.

Grant sponsor: National Science Council of Taiwan, ROC; Grant numbers: NSC91-2320-B-182-038, NSC92-2320-B-182-047; Grant sponsor: Chang Gung University and Memorial Hospital, Taiwan, ROC; Grant number: CMRP1214.

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Received 1 September 2004; Accepted 30 September 2004
DOI 10.1002/jcb.20353

become phosphorylated at the onset of mitosis and are dephosphorylated at the end of mitosis [Engle et al., 1988]. To facilitate study of these events, investigators have generated some monoclonal antibodies with specificity towards mitotic-specific phosphoepitopes, such as MPM2 and 3F3/2 [Davis et al., 1983; Taagepera et al., 1993; Daum and Gorbsky, 1998]. Many of the MPM2-recognized phosphoproteins have been identified, including MAP4 [Vandre et al., 1991], topoisomerase IIα [Taagepera et al., 1993], Cdc25 [Kuang et al., 1994], MAPK [Taagepera et al., 1994], Wee1 [Mueller et al., 1995], Cdc27 [King et al., 1995], NIMA [Ye et al., 1995], RNA polymerase II [Albert et al., 1999], Ki-67 [Endl and Gerdes, 2000], and MPP1-11 [Westendorf et al., 1994; Matsumoto-Taniura et al., 1996; Abaza et al., 2003]. A set of related phosphorylation sites was unraveled as the MPM2-reactive epitopes, including F-phosphoTP-L-Q [Westendorf et al., 1994]. These phosphorylation events are believed to be functionally important for the mitotic process, because microinjection of MPM2 or 3F3/2 into living cells can block either entry into or exit from M phase [Davis and Rao, 1987; Kuang et al., 1989; Campbell and Gorbsky, 1995].

p21-activated kinases (PAKs) were initially discovered by Manser et al. [1994] as a set of 62–68 kDa protein kinases that can bind to small (21 kDa) guanosine triphosphatases (GTPases) Rac and Cdc42. At least three isoforms of PAK (PAK1-3) were found in mammalian tissues, and all have similar sequences containing an N-terminal regulatory region with a p21-binding site and a C-terminal kinase domain [for review, see Sells and Chernoff, 1997]. Recent studies have shown that PAKs, as an effector of the small GTPases, participate in regulating diverse cell functions, including cell morphogenesis, motility, survival, apoptosis, mitosis, and angiogenesis [for reviews, see Kumar and Vadlamudi, 2002; Bokoch, 2003]. The activity of PAKs is controlled by the binding of activated Rac or Cdc42 to their N-terminal regulatory region, after which PAKs are activated via an autophosphorylation/activation process [Manser et al., 1994; Martin et al., 1995]. PAK2, one of the PAK family members ubiquitously expressed in mammalian tissues, can also be activated by proteolytic removal of its N-terminal regulatory region [Benner et al., 1995; Jakobi et al., 1996; Rudel and Bokoch, 1997]. The C-terminal catalytic fragment released can then be activated by autophosphorylation at certain specific site(s) [Benner et al., 1995; Yu et al., 1998]. Recently, we determined Thr^{402} as the regulatory autophosphorylation site of the catalytic fragment of PAK2 and generated a phospho-epitope-specific antibody against the regulatory autophosphorylation site sequence of PAK [Yu et al., 1998]. This phospho-epitope-specific antibody, designated as P-STM, can selectively recognize the activated catalytic fragment of PAK2 that is autophosphorylated at Thr^{402} but not the non-phosphorylated/inactive enzyme [Yu et al., 1998]. To explore the utility of P-STM, a non-radioactive enzyme-linked immunosorbent assay for measurement of PAK activity has been successfully developed [Yu et al., 2001]. In this study, we further show that P-STM can also recognize many phosphoproteins in mitotic HeLa and A431 cells, and the pattern of P-STM-reactive phosphoproteins is quite different from that detected by MPM2. Through a proteomic approach, we identified lamins A and C as the two major antigens for P-STM in mitotic cells.

MATERIALS AND METHODS

Materials

Nocodazole, thymidine, phosphothreonine, phosphoserine, and goat anti-rabbit IgG antibody conjugated with alkaline phosphatase were purchased from Sigma (St. Louis, MO). Okadaic acid was from Boehringer Mannheim (Mannheim, Germany). 4′,6-Diamidine-2′-phenylindole dihyhrochloride (DAPI) was from Roche Applied Science (Mannheim, Germany). Monoclonal antibody MPM2 was from Upstate Biotechnology (Lake Placid, NY). Anti-lamin A/C antibody and anti-lamin A/C antibody conjugated with agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit IgG antibody conjugated with FITC or Rhodamine was from Jackson ImmunoResearch Laboratories (West Grove, PA). BCA protein assay reagent was from Pierce (Rockford, IL). Polyvinylidene fluoride (PVDF) membrane was from Millipore (Bedford, MA). CDP-Star® (a chemiluminescent substrate for alkaline phosphatase) was from Applied Biosystems (Bedford, MA). Silver nitrate and trifluoroacetic acid were from Merck (Whitehouse station, NJ). STM-23 peptide (EQSKRSTMVGTPYWM-APEVVTRK) and phospho-STM-11-C peptide ($SKRST_{(P)}$MVGTPYC) were synthesized from

Chiron Mimotopes (Victoria, Australia). [γ-^{32}P]ATP, ATP, IPG strips (pH 4–7), and protein A (or G)-Sepharose CL-4B were from Amershan Biosciences (Buckinghamshire, England). Sequencing grade trypsin was from Promega (Madison, WI). α-cyano-4-hydroxycinnamic acid was from Bruker Daltonics (Billerica, MA). Molecular weight marker proteins (Mark12) were from Invitrogen (Carlsbad, CA).

Purification of Enzymes

The catalytic fragment of PAK2 was isolated from pig liver to apparent homogeneity as described by Yu et al. [1998]. The catalytic subunit of protein phosphatase 2A (PP2Ac) was isolated from rabbit skeletal muscle [Yu, 1998].

Antibody Production

The phospho-specific antibody against the phosphorylated/activated PAK2 (P-STM) was produced in rabbits and purified as previously described [Yu et al., 1998]. The phospho-STM-11-C peptide ($SKRST_{(P)}MVGTPYC$) coupled to keyhole limpet haemocyanin was used as antigen to produce P-STM.

Cell Culture, Drug Treatments, and Synchronization

Human A431 and HeLa cells were cultured at 37°C in a 95% air/5% CO2 and water-saturated atmosphere in Dulbecco's modified Eagle's (DME) medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. To block cells at G_2/M phase, cells were incubated with nocodazole (1 μg/ml) for 12–16 hr. Synchronization of HeLa cells were carried out by the method of double thymidine block [Stein and Borun, 1972]. Briefly, cells were incubated with 2 mM thymidine for 12–16 h. After washing with culture medium, cells were incubated in fresh culture medium for 9 h. Thymidine was then added into the culture medium (to a final concentration 2 mM) and incubated for another 12 h. Cells blocked at G_1/S boundary were released by removing thymidine and incubating in fresh medium. Cells at various cell cycle stages were collected, washed twice with ice-cold PBS, and lysed in 600 μl of lysis buffer (20 mM Tris-HCl at pH 7.0, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 20 mM sodium pyrophosphate, and 1 mM sodium orthovanadate) on ice for 10 min. The cell lysates were collected and sonicated on ice by Sonicator (model W-380, Heat Systems-Ultrasonics) for 3×10 s at 50% power output followed by centrifugation at 15,000*g* for 20 min at 4°C. The resulting supernatants were used as the cell extracts.

Immunoblots

Immunoblotting was carried out as described previously [Yu et al., 1998]. Affinity-purified P-STM (4 μg/ml), commercial MPM2 (1 μg/ml) or anti-lamin A/C (0.2 μg/ml) antibody was used to probe proteins transferred from SDS-gel to PVDF membrane. Proteins of interest were detected using goat anti-rabbit or anti-mouse IgG antibody conjugated with alkaline phosphatase and CDP-Star® according to the procedure provided by the manufacturer. For reprobing, membranes were stripped with 2% SDS, 100 mM 2-mercaptoethanol, and 62.5 mM Tris at 50°C for 1 h with occasional agitation, washed three times in TTBS (20 mM Tris-HCl at pH 7.4, 0.5 M NaCl, and 0.05% Tween 20), and then reprobed with the appropriate antibody.

Immunofluorescent Cell Stain

A431 or HeLa cells were cultured on glass slides for 24 h. Cells grown on slides were fixed by formaldehyde, permeabilized by Triton X-100, blocked by bovine serum albumin (5 mg/ml in PBS), and incubated with P-STM (40 μg/ml) or MPM2 (10 μg/ml) at room temperature for 3 h. After washing three times with PBS, cells were incubated with second antibody conjugated with FITC or Rhodamine (1:100) at room temperature for 1 h and then observed under a fluorescence microscope (Axioplan II, Zeiss, Germany). Cells were simultaneously stained with DAPI to locate the nuclear chromosomes.

Two-Dimensional Gel Electrophoresis (2DE)

Cells were solubilized in appropriate volume of 2DE lysis buffer (8 M urea, 2% CHAPS, 40 mM Tris base, 1 mM benzamidine, and 1mM phenylmethylsulfonyl fluoride). One hundred microliters of cell extracts (200 μg protein) were mixed with 150 μl of rehydration buffer (8 M urea, 2% CHAPS, 20 mM dithiothreitol, 0.5% IPG buffer, and 0.01% bromophenyl blue) and separated in the first-dimension by isoelectric point in 13-cm IPG strips (pH 4–7) using Ettan

IPGphor IEF system (Amersham Biosciences) at 20°C under the following condition: 50 V for 12 h, 100 V for 0.5 h, 150 V for 0.5 h, 250 V for 0.5 h, 500 V for 0.5 h, 1,000 V for 0.5 h, 4,000 V for 0.5 h, then 4,000–8,000 V for about 12 h with a total of 45,000 V-h. After IEF, the strips were incubated in SDS equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% dithiothreitol and 0.01% bromophenyl blue) at room temperature for 15 min, and then in 2.5% iodoacetamide (in SDS equilibration buffer) for another 15 min. The strips were then subjected to second-dimension separation by molecular weight in 10% SDS-gels. After gel electrophoresis, proteins were silver-stained for protein identification or transferred to PVDF membrane for immunoblot.

In-Gel Digestion of Proteins and Mass Spectrometric Analysis

The following procedures were adapted from Shevchenko et al. [1996]. Briefly, the protein spots picked up from gels were washed twice with 50% acetonitrile containing 25 mM NH_4HCO_3 for 15 min, and then with acetonitrile several times. After drying, the gel pieces were subjected to reduction and alkylation by dithiotreitol/iodoacetamide in 25 mM NH_4HCO_3, followed by in-gel digestion with freshly prepared enzyme solution (5 ng/μl of trypsin in 25 mM NH_4HCO_3) at 37°C for overnight. The resulting tryptic peptides were acidified with 0.5% trifluoroacetic acid and loaded onto an MTP AnchorChipTM 600/384 TF (Bruker-Daltonik GmbH, Bremen, Germany). α-Cyano-4-hydroxycinnamic acid was used as the matrix. Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry analysis was performed on an Ultraflex™ MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). Monoisotopic peptide masses were assigned and used for database searches with the MASCOT search engine (http://www.matrixscience.com) (Matrix Science, London, UK). All human proteins present in the Mass Spectrometry Protein Sequence Database (MSDB) were used, without any pI or Mr restrictions. The peptide mass error was limited to 50 ppm, and one possible missed cleavage was accepted. The trypsin autolytic fragment peaks (842.56 and 2211.11), angiotensin II (1046.54), and ACTH (2465.19) were used as molecular weight standards for mass calibration.

Immunoprecipitation

Before immunoprecipitation, protein concentration of the cell extracts was first adjusted to equal amount with lysis buffer. For immunoprecipitation, 1 ml of cell extracts (2 mg protein) was incubated with 15 μl of agarose conjugated with anti-lamin A/C antibody (2 mg/ml) at 4°C for 1.5 h with shaking. The immunocomplexes were collected by centrifugation, washed three times with 1 ml of solution A (20 mM Tris-HCl at pH 7.0 and 0.5 mM dithiothreitol) containing 0.5 M NaCl, and resuspended in 40 μl of solution A.

Dephosphorylation of Immunoprecipitated Lamin A/C by Protein Phosphatase

Immunoprecipitated lamin A/C from nocodazole-treated HeLa cells was incubated with the purified PP2Ac (5 μg/ml) in a 20-μl reaction mixture containing 20 mM Tris-HCl at pH 7.0, 0.5 mM dithiothreitol and 10 mM $MnCl_2$ at 30°C for 30 min. The reaction was stopped by the addition of 2× Laemmli sample buffer, and the samples were resolved in 10% SDS-gels and electroblotted onto PVDF membrane. The membrane was then probed with anti-lamin A/C antibody or P-STM.

RESULTS

Recognition of Mitotic Cell-Specific Phosphoproteins by P-STM

When A431 cells were treated with a serine/threonine protein phosphatase inhibitor, okadaic acid [Bialojan and Takai, 1988] or with a microtubule inhibitor, nocodazole [De Brabander et al., 1976] and then analyzed by immunoblotting, a variety of proteins ranged from 33 to >200 kDa could be recognized by P-STM (Fig. 1A). Because these signals emerged in cells after inhibition of serine/threonine protein phosphatases and also after cell cycle blockage at G_2/M phase, the P-STM-reactive antigens might represent mitotic phosphoproteins. This notion is supported by the observation that A431 cells at prometaphase were strongly stained with P-STM (Fig. 1B, arrows). To examine whether these P-STM-positive signals can be dynamically detected during cell cycle progression, HeLa cells were synchronized by double thymidine block, and cell extracts from cells released for various

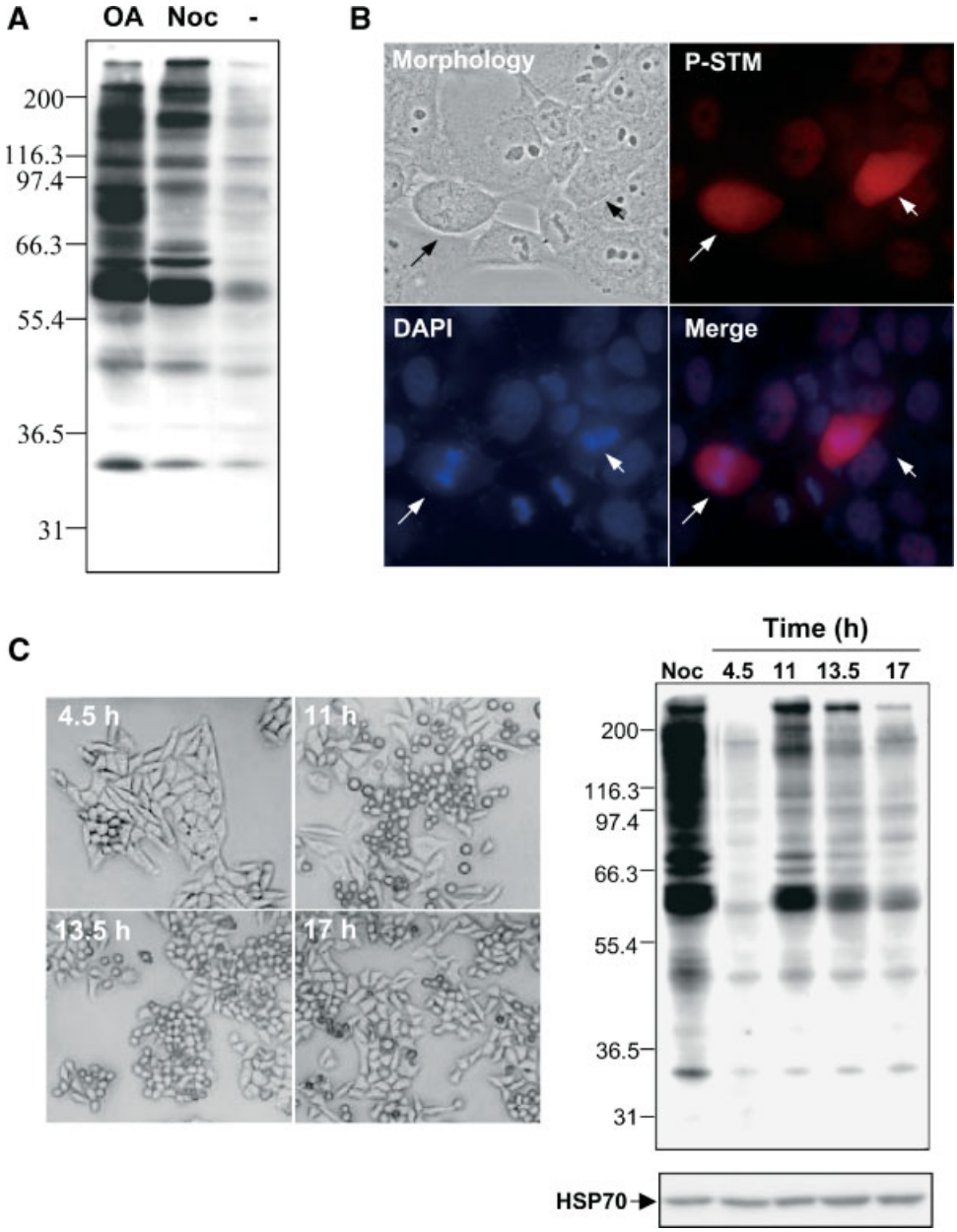


Fig. 1. Recognition of mitotic cell-specific phosphoproteins by P-STM. **A**: A431 cells were left untreated (–) or treated with okadaic acid (OA) (400 nM, 2 h) or nocodazole (Noc) (1 μg/ml, 14 h). Cell extracts (60 μg) were resolved in 10% SDS–PAGE, transferred to PVDF membrane, and then probed with P-STM. **B**: A431 cells grown on glass slides were immunostained with P-STM. Cells were simultaneously stained with DAPI to locate the nuclear chromosomes. Cells at the prometaphase were denoted by arrows. **C**: HeLa cells were synchronized by double thymidine block, and then released from the second thymidine block for 4.5 h (S phase), 11 h (G2 phase), 13.5 h (M phase), and 17 h (G1 phase), respectively. Morphology of cells at different stages of cell cycle is shown at left panel. Cells at different stages were collected, and extracts (60 μg) were subjected to immunoblot with P-STM (right panel). The level of heat shock protein 70 (HSP70) in different cell cycle stages was used as loading control.

time periods were analyzed by immunoblot with P-STM. As shown in Figure 1C, nocodazole treatment also induced strong P-STM-positive signals in HeLa cells, and these signals indeed dynamically fluctuated during cell cycle progression, with peak intensity at 11–13.5 h post release from thymidine block, which theoretically represents the cell cycle stage at G_2/M phase [Stein and Borun, 1972].

Both P-STM and MPM2 Stain Mitotic Cells but Recognize Different Proteins

It is well known that MPM2, a monoclonal antibody raised against extracts of mitotic HeLa

cells, can recognize a subset of mitotic phosphoproteins through a phosphoepitope containing phospho-T/SP [Davis et al., 1983; Westendorf et al., 1994]. We therefore compared the cell staining and immunoblotting patterns using the two antibodies. As shown in Figure 2A, P-STM strongly stained HeLa cells at prometaphase (arrow 1) and metaphase (arrow 2), but not anaphase (arrow 3) and interphase, which were revealed by DAPI staining of nuclear chromosomes. This staining pattern was almost identical to that obtained by staining with

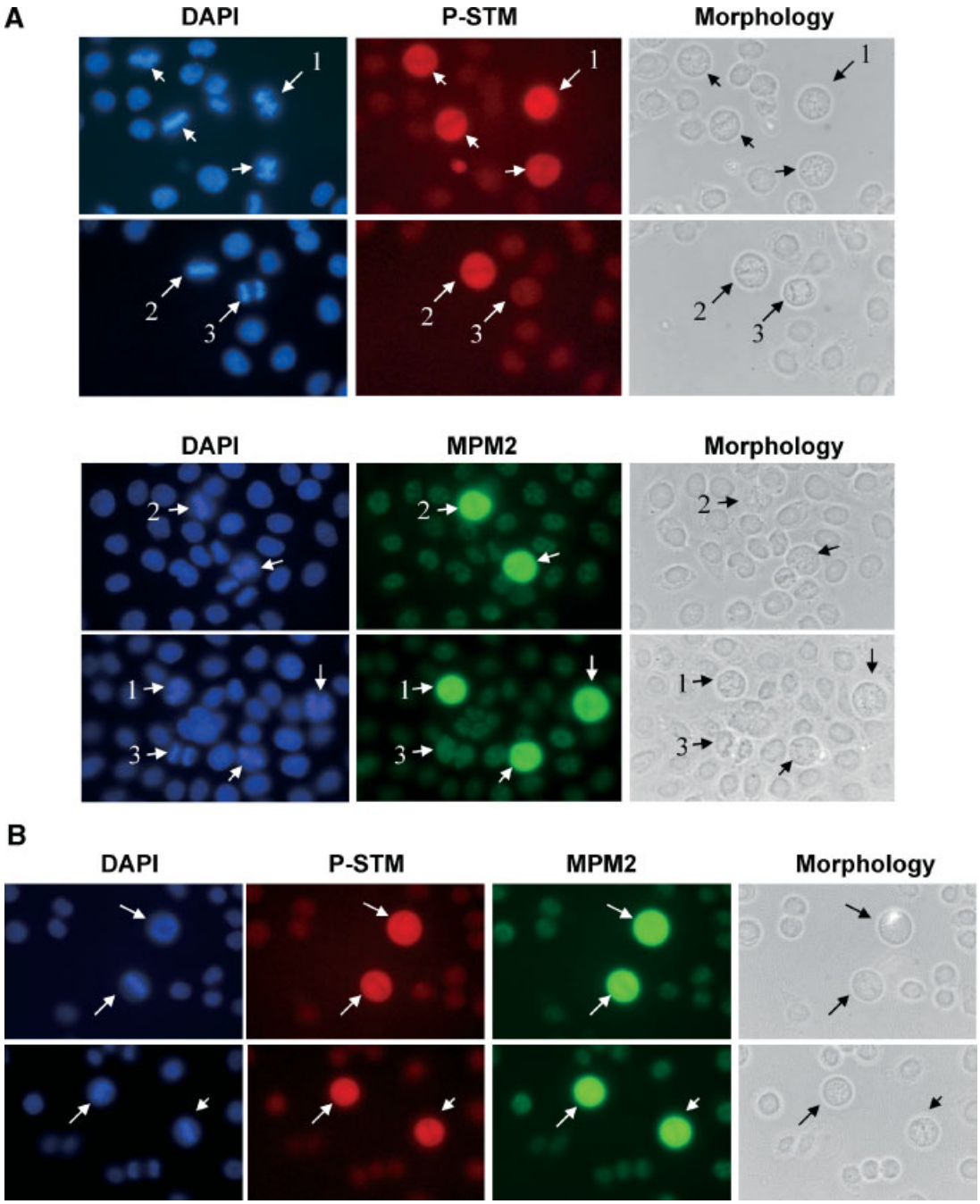


Fig. 2. Cell staining by P-STM and MPM2. **(A)** HeLa cells were cultured on glass slides in regular growth medium for 24 h. Cells on the slides were then fixed and immunostained with P-STM or MPM2, respectively. Cells were simultaneously stained with DAPI to locate the nuclear chromosomes. Cells with prometaphase (1), metaphase (2), and anaphase (3) chromosomes are denoted. **B, C**: HeLa (B) or A431 (C) cells were doubly stained with both antibodies.

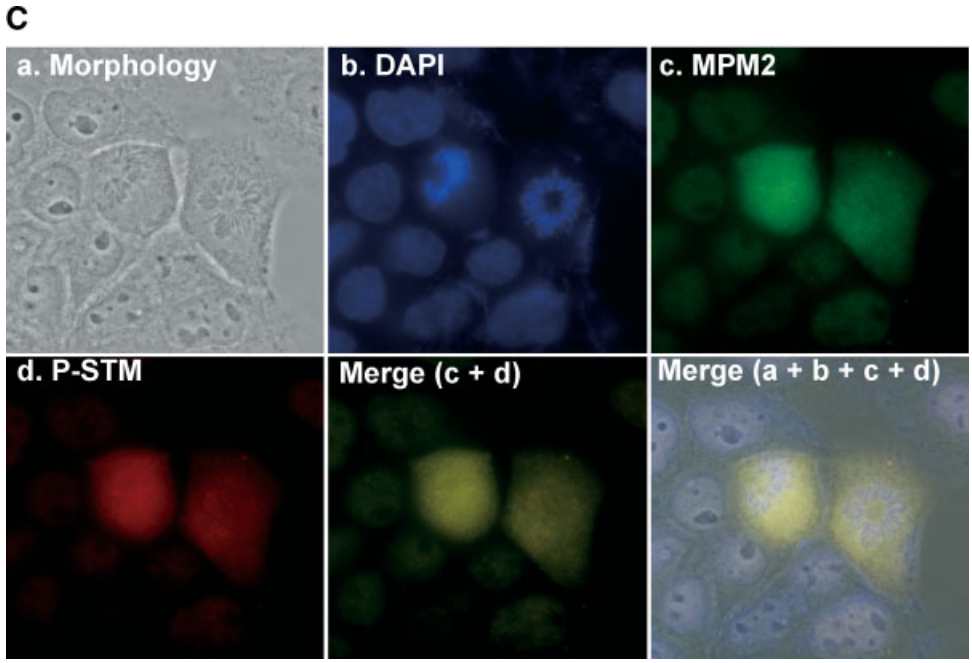


Fig. 2. (*Continued*)

MPM2 (Fig. 2A). When HeLa cells were doubly stained by both antibodies, mitotic cells at prometaphase and metaphase show similar reactivity to both antibodies (Fig. 2B). Similar observation could also be obtained when A431 cells were doubly stained (Fig. 2C).

Although both antibodies can stain mitotic cells, the proteins that they recognize in mitotic cells might not be the same. Immunoblot analysis of the proteins extracted from nocodazole-treated HeLa cells showed that the recognition pattern by P-STM was significant different from that detected by MPM2 (Fig. 3A). It is apparent that more than 10 protein bands could be differentially detected in mitotic cells by P-STM when compared to those detected by MPM2 (Fig. 3A, asterisks).

To further analyze the immuno-specificity of P-STM, extracts of nocodazole-treated HeLa cells were immunoblotted by P-STM in the presence of various agents. As shown in Figure 3B, phospho-STM-11-C peptide, the antigen through which P-STM was generated [Yu et al., 1998], completely blocked those P-STM-detectable signals, whereas STM-23 peptide, which contains the amino acid sequence of phospho-STM-11-C peptide but without phosphate group, had little effect on the immunoreactivity of P-STM. Moreover, phosphoamino acids including phospho-Thr and phospho-Ser also did not interfere the binding of P-STM to its target proteins (Fig. 3B). When similar experiments were performed to examine the immunospecificity of MPM2, none of these agents could affect the immuno-reactivity of MPM2 (Fig. 3C). The results clearly show that P-STM has a unique immuno-specificity that is against on the whole segment of the phosphopeptide antigen including the phosphate group introduced, but not on the peptide backbone or the phosphate group alone. The results also implicate that the proteins recognized by P-STM in mitotic cells should have similar structure characteristics to that of phospho-STM-11-C peptide.

Identification of Two P-STM-Recognized Proteins in Mitotic HeLa Cells by 2DE and Mass Spectrometry

An ideal way to identify the P-STM-recognized mitotic proteins is to immuno-purify its antigens from cell extracts. However, we found that P-STM could not immunoprecipitate its antigens (data not shown). We, therefore, adapted an alternative approach to address this issue: compare the 2DE protein staining and immunoblot patterns of mitotic cell extracts and analyze the matched spots by MALDI-TOF mass spectrometry. By this way, at least four groups of protein spots (groups 1–4) with

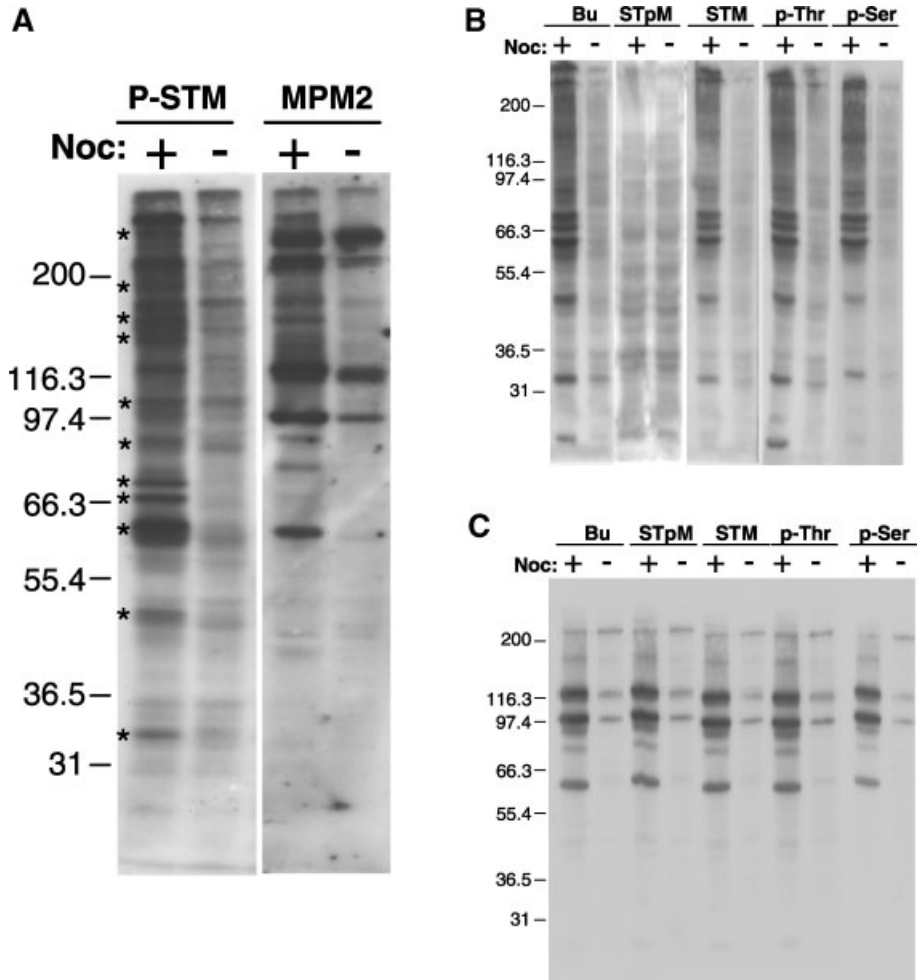


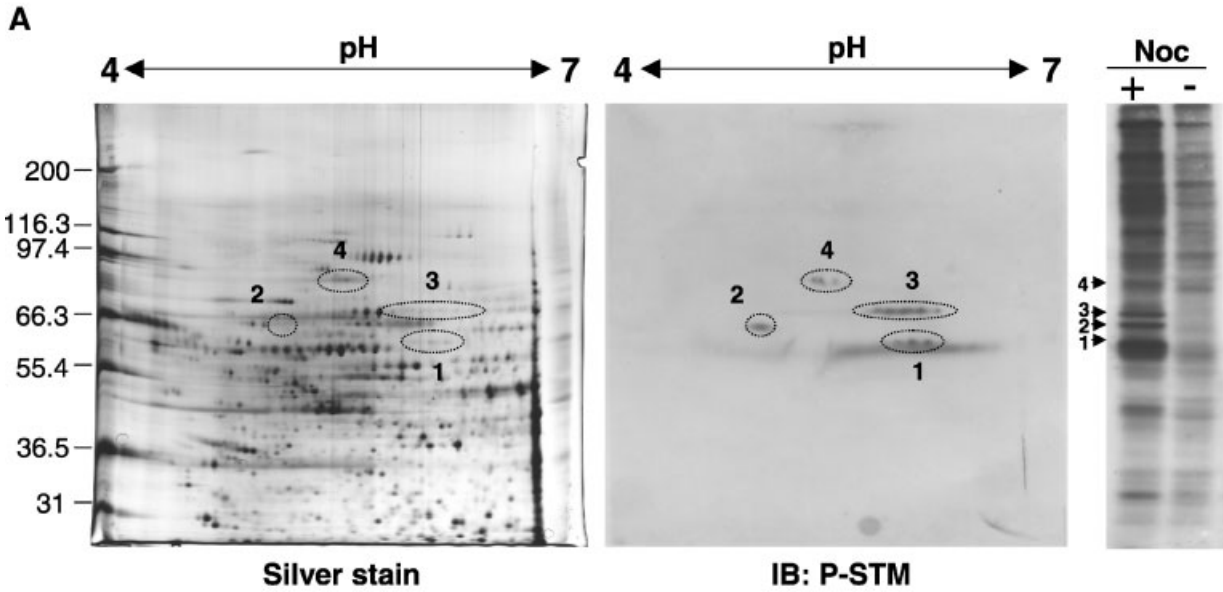
Fig. 3. Immunospecificity between P-STM and MPM2. **A**: HeLa cells were left untreated (−) or treated with nocodazole (+) (1 μg/ml) for 14 h. Cell extracts (100 μg) were resolved in 8% SDS–PAGE and transferred to PVDF membranes. The membranes were then probed with P-STM and MPM2, respectively. Asterisks denote protein bands specifically detected by P-STM. **B, C**: P-STM (B) or MPM2 (C) was incubated with TTBS buffer (Bu) or buffer containing phospho-STM-11-C peptide (STpM, 50 μg/ml), STM-23 peptide (STM, 50 μg/ml), phospho-Thr (p-Thr, 50 μg/ml) or phospho-Ser (p-Ser, 50 μg/ml) at room temperature for 1 h, and then used to probe HeLa cell extracts as described in (A).

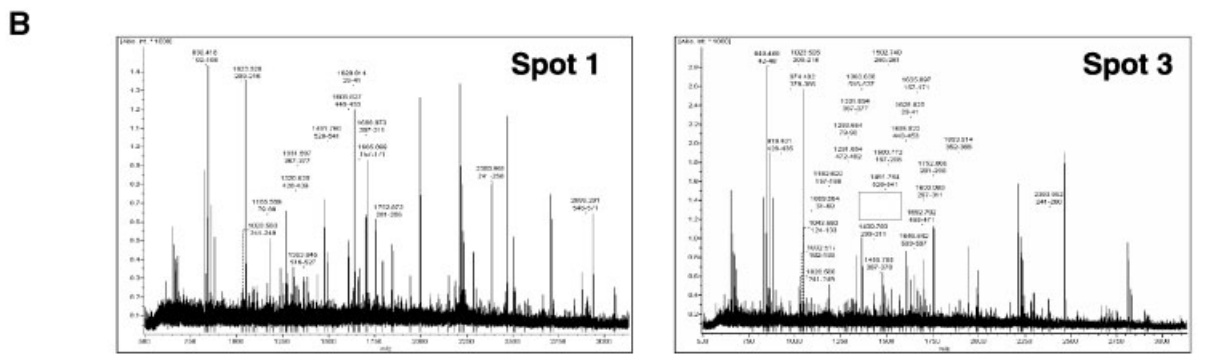
apparent molecular weights from ~60–90 kDa could be detected by P-STM when extracts of nocodazole-treated HeLa cells were resolved by 2DE at pH range 4–7 (Fig. 4A). These four groups of proteins were then picked up from the silver-stained gel, in-gel digested with trypsin, and subjected to MALDI-TOF mass spectrometric analysis. Results showed that 15 peptide masses theoretically derived from tryptic-digested lamin C could be detected in group 1 (sequence coverage 33%), and 27 peptide masses theoretically derived from tryptic-digested lamin A could be found in group 3 (sequence coverage 40%) (Fig. 4B). The mass signals in groups 2 and 4 were relatively weak, and the identities of proteins in these spots required further investigation.

To confirm the results from mass spectrometry, immunoblot analysis of extracts of HeLa cells treated without or with nocodazole by P-STM and anti-lamin A/C antibody was performed. In nocodazole-treated HeLa cell extracts two prominent P-STM-detectable bands co-migrated exactly with lamins A and C in one-dimensional SDS–PAGE (Fig. 4C), as well as in 2DE (Fig. 4D). The results strongly suggest that lamins A and C represent two major targets for P-STM in mitotic cells.

Lamins A and C Isolated From Mitotic HeLa Cells Contain the Phospho-Epitope to P-STM

To further validate lamins A and C as P-STM-recognizable phosphoproteins in mitotic cells, lamins A and C were directly immunoprecipi-





Lamin C sequence

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  1 METPSQRRAT RSGAQASSTP LSPTRITRLQ EKEDLQELND RLAVYIDRVR SLETENAGLR LRITESEEVV
 71 SREVSGIKAA YEAELGDARK TLDSVAKERA RLQLELSKVR EEFKELKARN TKKEGDLIAA QARLKDLEAL
141 LNSKEAALST ALSEKRTLEG ELHDLRGQVA KLEAALGEAK KQLQDEMLRR VDAENRLQTM KEELDFQKNI
211 YSEELRETKR RHETRLVEID NGKQREFESR LADALQELRA QHEDQVEQYK KELEKTYSAK LDNARQSAER
281 NSNLVGAAHE ELQQSRIRID SLSAQLSQLQ KQLAAKEAKL RDLEDSLARE RDTSRRLLAE KEREMAEMRA
351 RMQQQLDEYQ ELLDIKLALD MEIHAYRKLL EGEEERLRLS PSPTSQRSRG RASSHSSQTQ GGGSVTKKRK
421 LESTESRSSF SQHARTSGRV AVEEVDEEGK FVRLRNKSNE DQSMGNWQIK RQNGDDPLLT YRFPPKFTLK
491 AGQVVTIWAA GAGATHSPPT DLVWKAQNTW GCGNSLRTAL INSTGEEVAM RKLVRSVTVV EDDEDEDGDD
561 LLHHHHVSGS RR
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Lamin A sequence

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  1 METPSQRRAT RSGAQASSTP LSPTRITRLQ EKEDLQELND RLAVYIDRVR SLETENAGLR LRITESEEVV
 71 SREVSGIKAA YEAELGDARK TLDSVAKERA RLQLELSKVR EEFKELKARN TKKEGDLIAA QARLKDLEAL
141 LNSKEAALST ALSEKRTLEG ELHDLRGQVA KLEAALGEAK KQLQDEMLRR VDAENRLQTM KEELDFQKNI
211 YSEELRETKR RHETRLVEID NGKQREFESR LADALQELRA QHEDQVEQYK KELEKTYSAK LDNARQSAER
281 NSNLVGAAHE ELQQSRIRID SLSAQLSQLQ KQLAAKEAKL RDLEDSLARE RDTSRRLLAE KEREMAEMRA
351 RMQQQLDEYQ ELLDIKLALD MEIHAYRKLL EGEEERLRLS PSPTSQRSRG RASSHSSQTQ GGGSVTKKRK
421 LESTESRSSF SQHARTSGRV AVEEVDEEGK FVRLRNKSNE DQSMGNWQIK RQNGDDPLLT YRFPPKFTLK
491 AGQVVTIWAA GAGATHSPPT DLVWKAQNTW GCGNSLRTAL INSTGEEVAM RKLVRSVTVV EDDEDEDGDD
561 LLHHHHGSHC SSSGDPAEYN LRSRTVLCGT CGQPADKASA SGSGAQVGGP ISSGSSASSV TVTRSYRSVG
631 GSGGGSFGDN LVTRSYLLGN SSPRTQSPQN CSIM
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Fig. 4. Identification of lamins A and C as two candidates of P-STM-recognized proteins in mitotic HeLa cells. **A**: Extracts (200 μg) of nocodazole-treated HeLa cells were resolved by 2DE in duplicate gels. One gel was silver-stained, and the other was transferred to PVDF membrane and probed with P-STM. The immunoblot pattern of extracts from HeLa cells treated with (+) and without (−) nocodazole was shown for comparison. **B**: Protein spots (groups 1–4) were picked up, in-gel digested with trypsin, and analyzed by MALDI-TOF mass spectrometry. The results for spot groups 1 and 3 are shown. The matched peptides from mass spectrometric analysis of spots 1 (for lamin C) and 3 (for lamin A) to the amino acid sequence of human lamin A/C are in bold. **C**: Extracts (100 μg) of HeLa cells treated without (−) or with (+) nocodazole were resolved by 10% SDS–PAGE and subjected to immunoblot analysis by P-STM and lamin A/C antibody, respectively. **D**: Extracts (200 μg) of nocodazole-treated HeLa cells were resolved by 2DE, transferred to PVDF membrane, and probed sequentially with the anti-lamin A/C antibody and P-STM. The two immunoblot images were differentially colorized using the ImageMaster 2D Platinum software (Amershan Biosciences) and the same area of the two colorized images were merged. The comigrated spots are denoted by arrow heads.

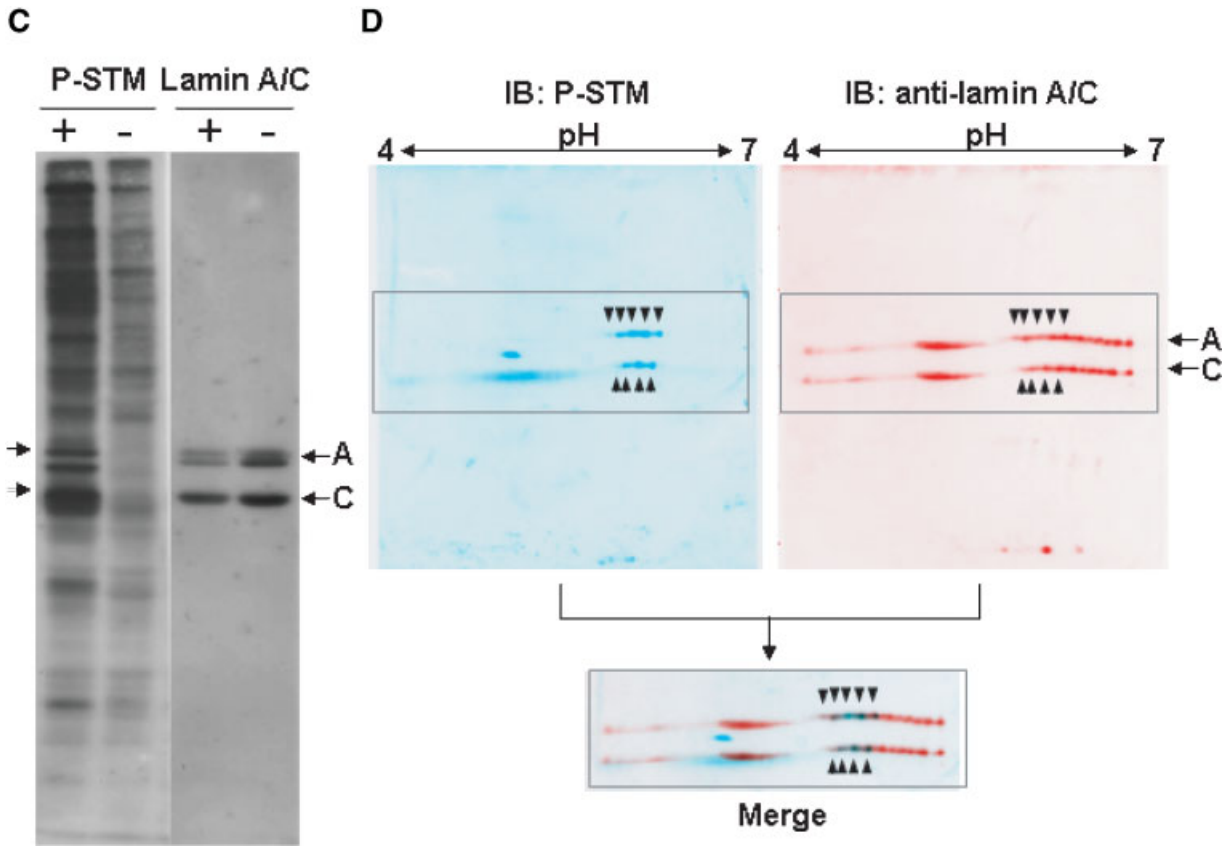

Fig. 4. (*Continued*)

tated from HeLa cells treated with and without nocodazole, and their reactivity to P-STM was analyzed. Both lamins A and C could be efficiently immunoprecipitated from extracts of HeLa cells treated with and without nocodazole (Fig. 5A, left panel). Under this circumstance, only those immunoprecipitated from nocodazole-treated cells showed strong reactivity to P-STM (Fig. 5A, middle panel). Interestingly, MPM2 did not recognize the two lamins immunoprecipitated from nocodazole-treated cells (Fig. 5A, right panel). To demonstrate that the reactivity of lamins A and C to P-STM is indeed derived from a phospho-epitope, lamins A and C immunoprecipitated from nocodazole-treated cells were dephosphorylated by the catalytic subunit of protein phosphatase 2A (PP2Ac) and the dephosphorylated products were examined by immunoblotting again. As shown in Figure 5B, phosphatase treatment did not alter the protein level of lamins A and C immunoprecipitated from nocodazole-treated cells, but indeed caused a complete loss of their reactivity to P-STM.

DISCUSSION

The phospho-epitope-specific antibodies highly selective in distinguishing phosphorylated and non-phosphorylated forms of a variety of protein kinases and their substrates have been shown to be valuable tools for measuring kinase activity and substrate phosphorylation in a variety of physiological settings. Many anti-phosphopeptide antibodies have been successfully generated to selectively recognize target proteins in their phosphorylated state by various laboratories and biotech companies [Epstein et al., 1992; Coghlan et al., 1994; Takai et al., 1996; Weng et al., 1998; D'Angelo et al., 1999; Goueli and Jarvis, 2001; Flemmer et al., 2002; Mandell, 2003; New England Biolabs]. To our knowledge, there are two phospho-epitope-specific antibodies reported to be valuable agents to detect mitotic phosphoproteins, namely MPM2 and 3F3/2. Both are monoclonal antibodies, which were raised against total lysates of mitotic HeLa cells (for MPM2) or extracts of *Xenopus* egg pretreated with ATPγS

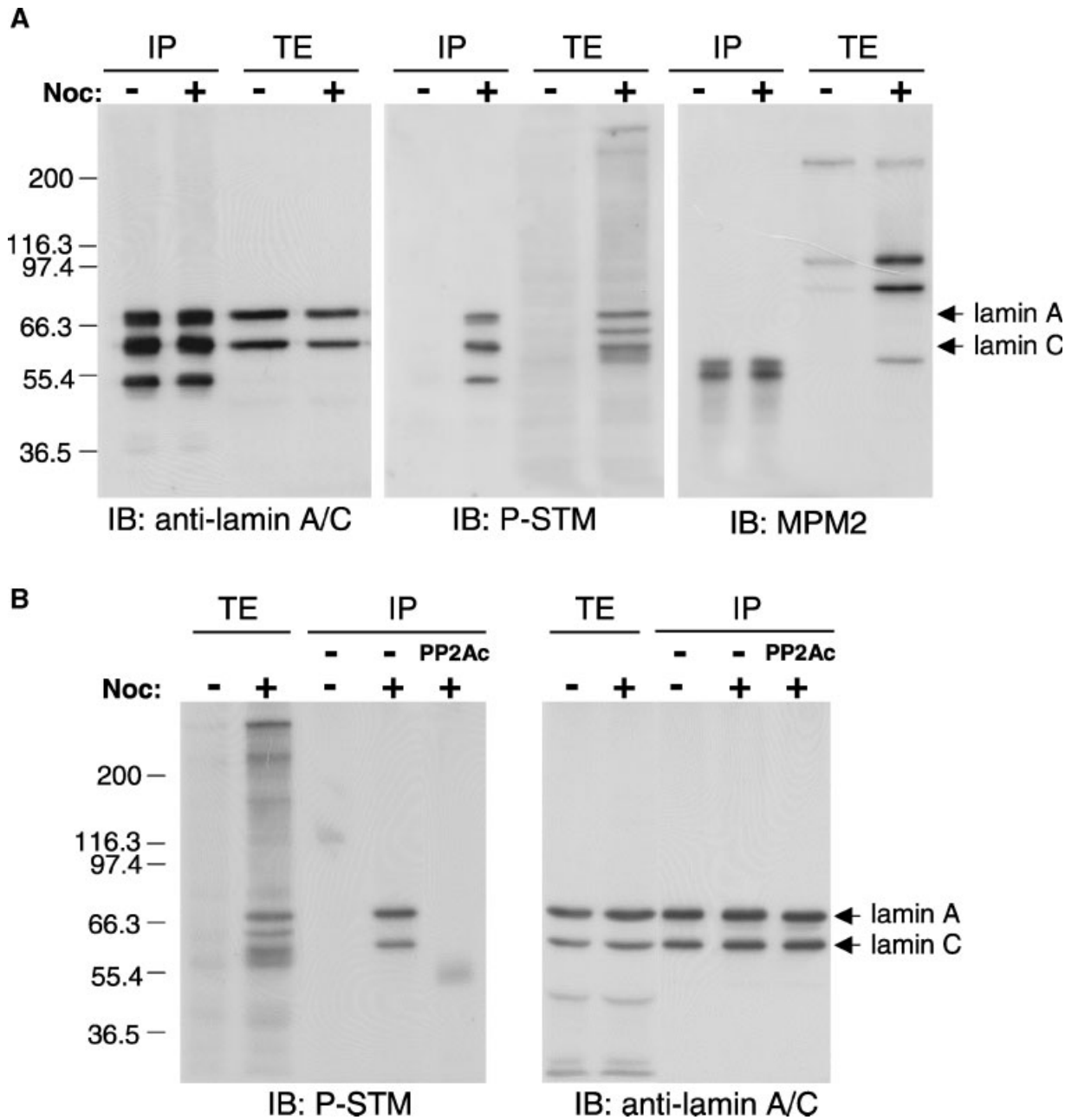

Fig. 5. Lamins A and C isolated from nocodazole-treated HeLa cells contain the phospho-epitope to P-STM. **A**: Lamins A and C were immunoprecipitated from extracts (1 mg) of HeLa cells treated without (–) or with (+) nocodazole as described in Materials and Methods. The immunoprecipitates were resolved by 8% SDS–PAGE and subjected to immunoblot analysis by anti-lamin A/C antibody, P-STM, and MPM-2, respectively. Total cell extracts (TE) were used as controls. **B**: Immunoprecipitated lamins A and C from nocodazole (Noc)-treated HeLa cells were incubated without and with the catalytic subunit of protein phosphatase 2A (PP2Ac) for 30 min, and the reaction mixtures were resolved by 8% SDS–PAGE and subjected to immunoblot analysis by anti-lamin A/C antibody and P-STM.

(for 3F3/2). In contrast to MPM2, which was shown to be able to detect more than 40 mitotic phosphoproteins, 3F3/2 can only recognize a small number of antigens in mitotic cells [Davis et al., 1983; Daum and Gorbsky, 1998]. This indicates that MPM2 has a broader reactivity against phosphorylated motifs, by which identification of multiple phosphoproteins in mitotic cells can be possible [Westendorf et al., 1994; Matsumoto-Taniura et al., 1996]. In the present study, we show that the anti-phosphopeptide antibody P-STM, originally produced to react

with the regulatory autophosphorylation site sequence of PAK2 [Yu et al., 1998], also has a broader immunoreactivity against phosphorylated motifs that occur during mitosis, as evident by immunostaining and immunoblotting of mitotic cells (Figs. 1 and 2). Approximately 20 protein bands could be detected by P-STM in the nocodazole-treated A431 or HeLa cells (Figs. 1 and 3). We also found that the pattern of P-STM-reactive antigens in extracts of mitotic cells was quite different from that detected by the commercially available MPM2 (Fig. 3A). On the basis of the apparent molecular weights observed on one-dimensional SDS–PAGE and immunoblot, it was estimated that more than 10 proteins were specifically recognized by P-STM, which were not MPM2-reactive (Fig. 3A). Therefore, P-STM represents the first anti-peptide antibody that could be a novel agent to detect mitotic phosphoproteins.

Through a proteomic approach, we have identified potential antigens for P-STM in mitotic cells. Under our assay condition, four prominent groups of P-STM-reactive proteins could be clearly detected after separation by 2DE (Fig. 4A). By MALDI-TOF mass spectrometric analysis, we identified lamins A and C as the two potential P-STM-reactive antigens in mitotic cells (Fig. 4B). Evidence for further confirmation came from the following two experiments. One is the co-migration of lamins A and C signals with the P-STM-positive ones in immunoblot images of mitotic cell extracts after 2DE separation (Fig. 4D); the other is the strong reactivity of lamins A and C immunoprecipitated from mitotic cells, but not from interphase cells, to P-STM, which lost completely after phosphatase treatment (Fig. 5). In Figure 4D, it was noticed that both lamins A and C existed as a series of discrete spots (>10) in mitotic cell extracts, and the P-STM-positive signals (five and four spots for lamins A and C, respectively) co-migrated only with the lamins A and C molecules located at more acidic region, which is consistent with the fact that phosphorylation reduces the isoelectric points of target proteins.

Lamins A and C, together with lamin B, are the major components of nuclear lamina that form 10 nm, orthogonally arranged filaments at the inner membrane of nuclear envelope [Fisher et al., 1986; McKeon et al., 1986]. Lamins A and C are alternative splicing forms encoded by one single gene; lamin A has an extra 98 amino acid residues than lamin C at its C-terminus [Fisher et al., 1986; McKeon et al., 1986]. It has been long recognized that lamins are reversibly depolymerized and hyperphosphorylated during mitosis. [Gerace and Blobel, 1980; Ottaviano and Gerace, 1985; Dessev et al., 1988]. Multiple phosphorylation sites of lamin C in response to MPF in vitro have been determined, and this phosphorylation resulted in disassembly of lamin filaments [Ward and Kirschner, 1990]. The essentiality of lamin phosphorylation cycle in mitosis has been demonstrated by experiments that mutation of two phosphorylation sites, Ser-22 (in QASSTPLS^{22}PTRIT) and Ser-392 (in RLRLSPS^{392}PTSQR) to non-phosphorylatable residue Ala in lamin A could significantly prevent its disassembly in cells undergoing mitosis, which subsequently blocked normal cell cycle progression [Heald and McKeon, 1990]. Although the two phosphorylation sites in lamin A are all followed by a proline residue, which resemble to some extent to the MPM2-reactive phosphorylated epitope LT*PLK or FT*PLQ (where the asterisk indicates the phosphorylation site) [Westendorf et al., 1994], we found that hyperphosphorylated lamins A and C in mitotic cells were recognized by P-STM, but not reactive to MPM2 (Figs. 3A and 5A). To our knowledge, P-STM appears to be the first phospho-specific antibody that can detect lamins A and C in their hyperphosphorylated forms during mitosis. This finding indicates P-STM, like MPM2, as a valuable tool for the identification of phosphoproteins involved in the regulation of mitotic process. The identities of the other P-STM-reactive phosphoproteins in mitotic cells are currently investigated in this laboratory. Moreover, although several protein kinases, including p34^{cdc2}/cyclin B and protein kinase C, could phosphorylate lamin A/C on multiple sites during mitosis [Ward and Kirschner, 1990; Eggert et al., 1991; Haas and Jost, 1993], it is not easy to assess the dynamic change of specific phosphorylation site on lamin A/C during cell cycle. Since P-STM can recognize the hyperphosphorylated forms of lamins A and C (Fig. 4D), determination of the specific P-STM-recognizable phosphorylated epitope(s) on lamins A and C would provide a solid basis for further application of P-STM to monitor the phosphorylation levels of specific site on lamin A/C during cell cycle progression. Because no obvious homology exists between the amino acid sequence of lamin A/C and the peptide antigen (SKRSpTMVGTPY) used to

raise P-STM in rabbits (see Fig. 4B), further identification of the P-STM-recognizable phosphorylated epitope(s) on lamins A and C should also provide information to elucidate the common characteristics of the epitope structures for P-STM.

Anti-phosphopeptide antibody, like P-STM described here, with a broader immunoreactivity to structurally similar phosphorylated motifs may not be unusual. Recently, investigators have generated some phospho-epitope-specific antibodies based on the consensus phosphorylation site motif of certain serine/threonine kinases, such as PKB/Akt and PKC, and used these antibodies to identify potential physiological substrates for the two kinases [Kane et al., 2002; Manning et al., 2002; Zhang et al., 2002; Astoul et al., 2003]. Among these studies, Kane et al. [2002] and Manning et al. [2002] reported the use of polyclonal antibodies against a phosphopeptide library with fixed residues corresponding to PKB/Akt substrate motif RXRXXT*/S* to detect a subset of phosphoproteins in cells when stimulated with signals known to activate PKB/Akt, and to identify AS160 (a protein containing Rab GTPase-activating domain) and tuberin as new substrates for PKB/Akt. Another example [Astoul et al., 2003] employed a polyclonal antiserum raised against the phosphopeptide RARTSpSFAEP corresponding to Ser-21 in glycogen synthase kinase 3α, a known substrate for PKB/Akt to detect several phosphoproteins in T-cells when their antigen receptor were activated. They identified a 55-kDa protein, SLY as one of the target phosphoproteins and determined SLY Ser-27 sequence, LQRSSpSFK as the recognition motif for this antiserum. However, phosphorylation of Ser-27 of SLY is likely attributed to activation of PKC, but not PKB/Akt in T-cells [Astoul et al., 2003], indicating that although SLY was identified in activated T-cells using the PKB/Akt substrate antiserum, it is not necessary for PKB/Akt to perform this phosphorylation in vivo.

In conclusion, our results demonstrate P-STM as a novel tool for detecting mitotic phosphoproteins, and lamins A and C represent the two prominent P-STM-reactive mitotic phosphoproteins. Using this antibody, investigators may have the chance to identify new mitotic phosphoproteins (i.e., proteins whose phosphorylation state is regulated in a cell cycle-dependent manner but have not been reported previously). The identification of new mitotic phosphoproteins could be helpful to understand phosphorylation-dephosphorylation cycles of mitotic cells.

ACKNOWLEDGMENTS

We greatly appreciate Dr. Ying-Hsiung Lee for his efforts in developing the Chang Gung Proteomics Core Laboratory. We also thank Dr. Chen-Kung Chou for helpful discussion and suggestions.

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