Cdk1-Cyclin B1-mediated Phosphorylation of Tumor-associated Microtubule-associated Protein/Cytoskeleton-associated Protein 2 in Mitosis*

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Received for publication, January 13, 2009, and in revised form, March 19, 2009 Published, JBC Papers in Press, April 15, 2009, DOI 10.1074/jbc.M900257200

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During mitosis, establishment of structurally and functionally sound bipolar spindles is necessary for maintaining the fidelity of chromosome segregation. Tumor-associated microtubule-associated protein (TMAP), also known as cytoskeleton-associated protein 2 (CKAP2), is a mitotic spindle-associated protein whose level is frequently up-regulated in various malignancies. Previous reports have suggested that TMAP is a potential regulator of mitotic spindle assembly and dynamics and that it is required for chromosome segregation to occur properly. So far, there have been no reports on how its mitosis-related functions are regulated. Here, we report that TMAP is hyper-phosphorylated at the C terminus specifically during mitosis. At least four different residues (Thr-578, Thr-596, Thr-622, and Ser-627) were responsible for the mitosis-specific phosphorylation of TMAP. Among these, Thr-622 was specifically phosphorylated by Cdk1-cyclin B1 both in vitro and in vivo. Interestingly, compared with the wild type, a phosphorylation-deficient mutant form of TMAP, in which Thr-622 had been replaced with an alanine (T622A), induced a significant increase in the frequency of metaphase cells with abnormal bipolar spindles, which often displayed disorganized, asymmetrical, or narrow and elongated morphologies. Formation of these abnormal bipolar spindles subsequently resulted in misalignment of metaphase chromosomes and ultimately caused a delay in the entry into anaphase. Moreover, such defects resulting from the T622A mutation were associated with a decrease in the rate of protein turnover at spindle microtubules. These findings suggest that Cdk1-cyclin B1-mediated phosphorylation of TMAP is important for and contributes to proper regulation of microtubule dynamics and establishment of functional bipolar spindles during mitosis.

Tumor-associated microtubule-associated protein (TMAP), also known as cytoskeleton-associated protein 2 (CKAP2), LB-1, and se20-10, is frequently up-regulated in various malignancies, including gastric adenocarcinoma, diffuse B-cell lymphoma, and cutaneous T-cell lymphoma (1–3), and detected in various cancer cell lines (1, 4). Knockdown of TMAP significantly reduces the rate of cell growth (5, 6), indicating that it is essential for normal cell growth. However, the cellular functions of TMAP remain largely unknown. Recent findings indicate that TMAP plays an essential role in mitosis. Expression of TMAP changes in a cell cycle-dependent manner; its expression is relatively low during G1, starts to incline during G1/S transition, and peaks at G2/M phases of the cell cycle (5, 7). TMAP primarily localizes at mitotic spindle and spindle poles during mitosis (1, 4, 8, 9). During late stages of mitosis, however, TMAP localizes near the chromatin region and to the midbody microtubules (8). TMAP has microtubule-stabilizing properties (4, 8, 9), and its overexpression induces mitotic spindle defects, including monopolar spindle formation, and arrests cells at mitosis as a result (8). Similar to other mitotic regulators, TMAP is a substrate of the anaphase-promoting complex (8). TMAP is degraded during mitotic exit by the anaphase-promoting complex-Cdh1 in a KEN box-dependent manner. Results of the experiments using a nondegradable mutant of TMAP suggested that proper regulation of the TMAP protein level is functionally important for establishment of bipolar spindles and completion of cytokinesis. Recently, we also have shown that siRNA-mediated depletion of TMAP in mammalian cells results in chromosome missegregation, characterized by chromatin bridge formation and malformation of interphase nuclei, and such phenotype was associated with a reduction in the spindle assembly checkpoint activity (6). These findings suggest that TMAP is a potential regulator of mitotic spindle function and dynamics and that proper regulation of its protein level and functions is necessary for establishment of bipolar spindles as well as for maintaining the fidelity of the chromosome segregation process.

* This work was supported by the National R&D Program for Cancer Control, Ministry of Health and Welfare, Republic of Korea (Grant 0720370).

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3 The abbreviations used are: TMAP, tumor-associated microtubule-associated protein; CKAP2, cytoskeleton-associated protein 2; Cdk1, cyclin-dependent kinase 1; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; GSK-3β, glycogen synthase kinase 3β; WT, wildtype; KD, kinase-dead; FRAP, fluorescence recovery after photobleaching.
Cdk1-Cyclin B1-mediated Phosphorylation of TMAP/CKAP2

At the onset of mitosis, the microtubule network undergoes extensive rearrangements to form a unique bipolar structure, called the mitotic spindle. Multiple factors have been shown to associate with the mitotic spindle and regulate its function by influencing its assembly and dynamics (10, 11). Establishment of a functional bipolar mitotic spindle is critical for faithful segregation of sister chromatids and maintenance of genomic stability. In support of this notion, disruption or depletion of factors involved in regulation of the spindle microtubule dynamics or establishment of spindle bipolarity have been shown to induce spindle dysfunction and ultimately chromosome mis-segregation (12–14).

The cyclin-dependent kinase 1 (Cdk1) in complex with cyclin B1 (Cdk1-cyclin B1) is one of the key mitotic kinases. The kinase activity of Cdk1-cyclin B1 governs the entry into mitosis from G2 phase of the cell cycle (15, 16). Through mediating phosphorylation of a variety of substrates, Cdk1-cyclin B1 also plays an important role in multiple processes during mitosis, including chromosome condensation, nuclear envelope breakdown, centrosome separation, regulation of spindle microtubule dynamics, and metaphase to anaphase transition (17–20). In particular, a number of regulators of microtubules are among Cdk1-cyclin B1 substrates (21). For instance, phosphorylation of a kinesin-related motor protein, Eg5, by Cdk1-cyclin B1 is necessary for its centrosomal localization and ultimately for the centrosome separation process to occur properly (18). Also, Cdk1-cyclin B1-mediated phosphorylation of some of the effectors of microtubule dynamics has been shown to regulate their microtubule-stabilizing or -destabilizing activities during mitosis (22, 23). These suggest that the assembly and maintenance of bipolar spindles during mitosis are under regulation of Cdk1-cyclin B1.

We have recently reported that TMAP is phosphorylated specifically during mitosis (24), which led us to hypothesize that the mitotic functions of TMAP are regulated by timely phosphorylation. In the present study, we identified multiple, mitosis-specific phosphorylation sites on TMAP, one of which is phosphorylated by Cdk1-cyclin B1, and investigated the functional importance of Cdk1-cyclin B1-mediated phosphorylation of TMAP during mitosis.

MATERIALS AND METHODS

Cell Culture and Transfection—HeLa, HEK 293T, and HEK 293 cells were purchased from ATCC and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics, and were subcultured every 3 days. For transfection of plasmid DNA, Lipofectamine Plus (Invitrogen) reagent was used according to the manufacturer’s instructions. For transfection of double-stranded siRNA, DharmaFECT1 (Dharmacon) was used according to the manufacturer’s instructions.

Expression Constructs and siRNA—For generation and expression of various GST-fused deletion mutant TMAP constructs in mammalian cells, corresponding regions were PCR-amplified using human TMAP cDNA (transcript variant 1; NM_018204) as the template and subcloned into the pEBG-2T vector. Expression constructs for phosphorylation-deficient mutant forms of TMAP were generated using a QuikChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. For generation of pCMV6-HisX vector, HindIII-BamHI fragment (containing the 6× histidine and Xpress tag sequences) from pcDNA3.1/His C vector (Invitrogen) was inserted into HindIII and BamHI sites of the pCMV6 vector. The open reading frame of Cdk1 was PCR-amplified using HEK 293 cell cDNA with the following primers: 5′-AGAATTCATGGAAGATTATACCAAAATAGAG-3′ and 5′-AATTCTACGACATCTTTAATCTGATTTG-3′. The PCR product was digested with EcoRI and Xhol, subcloned into pCMV6-HisX vector, and sequence-verified. For generation of the kinase-dead (KD) mutant of Cdk1, Asp-146 was mutated to Asn (i.e. D146N) (25) as described above. The open reading frame of cyclin B1 was PCR-amplified using HEK 293 cell cDNA with the following primers: 5′-TCTAGATCCATGGCGCTCCGAGTCACCA-3′ and 5′-TATATCTGA-GTTACACCTTTGCCACAGCC-3′. The PCR product was digested with BamHI and Xhol, subcloned into pBluescript II KS(−) vector, and sequence-verified. For expression and purification of GST fusion proteins in mammalian cells, Cdk1 or cyclin B1 open reading frame was subcloned into the pEBG-2T vector. Characterization of human TMAP-specific siRNA has been previously described (5).

Antibodies—Generation and characterization of rabbit polyclonal antiserum to human TMAP has been previously described (1). Mouse monoclonal antibody against human TMAP was produced as previously described (24). Mouse monoclonal antibody against α-tubulin (clone B-5-1-2) was purchased from Sigma. Mouse monoclonal antibodies against GST (clone B-14) and cyclin B1 (clone GNS1) were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody against Cdk1 (clone CC43) was purchased from Celltech. Rabbit polyclonal antibodies against phospho-Histone H1 and phospho-Histone H3 were purchased from Upstate. Mouse monoclonal antibody against Xpress tag was purchased from Invitrogen. Mouse monoclonal antibody against CENP-A (clone 3-19) was purchased from Abcam. AlexaFluor 488-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG were purchased from Molecular Probes and Rockland, respectively.

Western Blot—Cells were harvested by boiling in 1X Laemmli buffer for 10 min. After determining the protein concentration using BCA protein assay, β-mercaptoethanol was added to each sample, which was then boiled for another 5 min. Protein samples (25 or 50 μg) were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). Following 30 min of incubation in the blocking solution (5% skim milk in TBST) at room temperature, the blot was incubated with an appropriate dilution of the primary antibody (in the blocking solution) for 1 h at room temperature or overnight at 4 °C. The blot was washed twice in TBST and incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (diluted in the blocking solution) for 1 h at room temperature. The antibody-antigen complex was then detected using SuperSignal West Pico solution (Pierce).

In Vivo 32P Labeling Assay—HEK 293T cells were transfected with a GST-fused TMAP C terminus (458–682 aa) construct (in pEBG-2T vector) expressing wild type or the indicated
mutant form. On the following day, cells were either left untreated (“asynchronous” sample) or treated with 1 μM nocodazole (“mitotic” sample) for ~14–16 h prior to the 32P labeling. For the 32P labeling, cells were incubated with fresh media (with or without nocodazole) containing 0.2 mCi of [32P]orthophosphate per milliliter of media for 4–5 h at 37 °C. Cells were then harvested, washed in PBS, and lysed in radioimmuno precipitation assay buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 1% Nonidet P-40, phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and 50 mM NaF). The GST fusion proteins were then purified from the resulting supernatant using glutathione-Sepharose 4B beads (Amersham Biosciences) according to the manufacturer’s instructions. Purified samples were separated by SDS-PAGE and analyzed by autoradiography and Western blotting for the level of 32P incorporation and for the level of total GST fusion protein, respectively.

Lambda Phosphatase Treatment—Asynchronous or nocodazole-arrested HeLa cells were lysed in radioimmune precipitation assay buffer (without 50 mM NaF) and centrifuged at 10,000 × g. The resulting lysate was untreated or treated with λ phosphatase (PerkinElmer Life Sciences) in the presence of 2 mM MnCl₂ for 1 h at 30 °C. The reaction was terminated by adding 5× Laemmli buffer. Samples were resolved by SDS-PAGE and immunoblotted using rabbit polyclonal TMAP antibody to observe the band shift. The mitotic band shift of Cdh1 protein was used as a positive control. In the case of measuring the level of TMAP phosphorylated at Thr-622 by Western blot, pT622-specific antibody was used.

Sequence Analysis—for identification of evolutionarily conserved residues, amino acid sequences of TMAP protein from the indicated species were aligned using ClustalX software (version 2.0.9).

In Vitro Kinase Assay—GST-Cdk1 (WT or KD)/HisX-cyclin B1 and GST-TMAP C-terminal (corresponding to 566–682 aa, WT, or TA mutants) were expressed by transient transfection and purified from HEK 293T cells using glutathione-Sepharose 4B beads (Amersham Biosciences) according to the manufacturer’s instructions. Histone H1, a positive substrate of Cdk1-Cyclin B1-mediated Phosphorylation of TMAP/CKAP2

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Generation of pT622-Specific Antibody—For production, purification, and characterization of the pT622-specific antibody, the following synthetic peptides were produced at Peptron (Daejon, Korea): unphosphorylated Thr-622 peptide, CFKELKFLTVPVRRSR-NH₂ and phosphorylated Thr-622 peptide (pT622), CFKELKFLTVPVRRSR-NH₂ and phosphorylated Thr-578 peptide, CDPTHEVKTPTNETTRT-NH₂. Rabbits were immunized with KLH-conjugated pT622 peptide mixed with adjuvant, TiterMax classic (TiterMax) by subcutaneous injections 4 times at a 3-week interval. pT622-specific antibodies were then affinity-purified. Affinity columns were produced by conjugating unphosphorylated peptide or phosphorylated peptide using SulfoLink Immobilization Kit (Pierce) according to the manufacturer’s instructions. First, crude serum was passed through the unphospho-peptide column to remove antibodies that recognize unphosphorylated epitope, and the resulting flow-through was passed through the phosho-peptide column to retain antibodies that are specific for pT622 epitope. The eluent from the second column was tested for specificity and used for the present study. For pre-absorbing the pT622-specific antibody with pT622 or pThr-578 peptide, 20 μl of affinity-purified antibody was incubated with or without 10 μg of indicated phosphate-peptide in 500 μl of 5% bovine serum albumin/PBS for 1 h at room temperature. The pre-absorbed antibody was then diluted at 1:500 and used for Western blotting.

Double Thymidine Block and Release—HeLa cells were seeded at a density of 6–7 × 10⁴ cells/35-mm dish, and on the following day treated with 2 mM thymidine for 19–20 h for the first block. Cells were washed twice with PBS, released into fresh media for 8–10 h, and incubated with 2 mM thymidine again for 16 h. For the release, cells were washed twice in PBS, released into fresh media, and harvested at indicated time points. Cells harvested at each time point were analyzed by both flow cytometry and Western blotting. For the flow cytometric analysis, cells were fixed in cold 70% ethanol overnight or longer. The fixed cells were spun down, washed in PBS containing 1% bovine serum albumin, and incubated in the propidium iodide solution containing 50 μg/ml propidium iodide, 0.1% sodium citrate, 0.3% Nonidet P-40, 50 μg/ml RNase A, and 1× PBS for 1 h at 37 °C. DNA profiles of propidium iodide-stained cells were obtained using a FACS Calibur system (BD Biosciences) and analyzed using CellQuest software, version 3.3 (BD Biosciences).

Immunofluorescence Staining—Cells grown on glass coverslips were fixed in 3.7% formaldehyde in PBS for 15 min at room temperature and permeabilized in 0.5% Triton X-100 in PBS for 10 min. Following incubation in the blocking solution (5% bovine serum albumin in PBS) for 30 min, the cells were incubated in primary antibody solution (diluted in the blocking solution) for 1 h at room temperature and washed twice in PBS. They were then incubated in a solution of secondary antibodies conjugated to fluorochromes (diluted in the blocking solution) for 1 h at room temperature and washed twice in PBS. They were finally counterstained with DAPI and mounted on glass slides using Gel/Mount (Biomeda). Primary antibody dilutions used for the immunofluorescence staining were: TMAP (1:600), pT622 (1:300), cyclin B1 (1:100), α-tubulin (1:5,000), and CENP-A (1:100). Fluorochrome-conjugated secondary antibodies were all used at dilutions of 1:400. Fluorescence images were viewed and acquired using an AxioPlan2 microscope (Carl Zeiss) equipped with an AxioCam charge-coupled device camera (Carl Zeiss).
with DMSO (a vehicle control), 20 μM purvalanol A, or 20 μM PD98059 for 1 h. For treatment with alsterpaullone and AR-A014418, nocodazole-arrested HeLa cells were incubated with DMSO (a vehicle control), 40 μM alsterpaullone, or 20 μM AR-A014418 for 15 min.

Quantification of Spindle Defects—HEK 293 cells grown on glass coverslips were transfected with the indicated construct (GFP, GFP-WT, or GFP-T622A), fixed on the following day, and immunofluorescently stained for α-tubulin to visualize microtubules/mitotic spindles. Stained cells were then observed under a microscope to identify GFP-positive cells at prometaphase or metaphase. Based on the spindle number and morphology, each cell was categorized into the following: normal bipolar, monopolar, abnormal bipolar, and multipolar. Over 200 prometaphase/metaphase cells were examined per slide, and four separate individual slides were examined for each construct. The differences in the mean values between GFP-WT and GFP-T622A were analyzed by Student’s t test to obtain the p values.

Time Lapse Video Microscopy—HEK 293 cells were transfected onto a 6-well plate with a GFP-WT or GFP-T622A mutant TMAP expression plasmid. 24 h after transfection, 2-channel time-lapse video microscopy was performed using a fully motorized Axiovert 200M microscope (Carl Zeiss), equipped with Axiocam HRm. Temperature and CO₂ control was maintained using the Incubator S-M and Heating insert M06 controlled by Tempcontrol 37-2 and CTI-Controller 3700. Both phase contrast and GFP fluorescence images were acquired for 48 h with a lapse time of 5 min using AxioVision 4.3 software. For routine and quantitative analyses, images were acquired using a 20× objective (LD Plan-Neofluar 20×/0.4 Corr Ph2, Carl Zeiss). For quantification of GFP fluorescence intensity, the acquired images were analyzed using ImageJ 1.40g (rsb.info.nih.gov/ij/). The GFP fluorescence intensity was measured from the area covering an entire mitotic cell and subtracted by the background fluorescence from a neighboring area.

FRAP—For fluorescence recovery after photobleaching (FRAP) HEK 293 cells grown on glass coverslip were transfected with GFP-WT or GFP-T622A mutant TMAP expression plasmid. On the following day, GFP-TMAP-expressing metaphase cells with structurally normal bipolar spindles were identified using an LSM 510 Meta/Duo confocal microscope (Carl Zeiss) under a 63× objective. For photobleaching of spindle microtubule-associated GFP-TMAP, a narrow region perpendicular to the spindle axis (across the spindle microtubules) was selected and photobleached by scanning the region 20 times with argon laser at full power. The fluorescence intensity of the photobleached region was continuously monitored at an interval of ~300 ms to measure the rate of recovery. For processing and quantitative analysis of the images, AxioVision 4.3 software (Carl Zeiss) was used.

RESULTS

TMAP Is Hyper-phosphorylated at the C Terminus during Mitosis—We have previously reported that the mitotic TMAP protein band becomes up-shifted on an SDS-PAGE gel, which suggested that TMAP is phosphorylated during mitosis (24). In support of this, we also have shown that TMAP is transiently phosphorylated at Thr-596 during early stages of mitosis using a monoclonal antibody specifically recognizing an unphosphorylated Thr-596-containing epitope near the C terminus (24). Based on these findings, we sought to identify functionally important, mitosis-specific phosphorylation sites on TMAP. Lambda phosphatase treatment of mitotic HeLa cell lysate abolished the up-shift of the TMAP protein band on a SDS-PAGE gel (Fig. 1A), indicating that the mitosis-specific shift was indeed phosphorylation-dependent. Next, to map the regions containing mitosis-specific phosphorylation sites, three different GST-fused deletion mutants of TMAP (corresponding to amino acids (aa) 1–263, 264–457, and 458–682) were introduced into HEK 293T cells and labeled with [³²P]orthophosphate, while the cells were either growing asynchronously or arrested at mitosis by nocodazole treatment. Each mutant was then purified, resolved by SDS-PAGE, and analyzed by autoradiography and Western blot to measure the relative level of its phosphorylation. All three regions were phosphorylated at variable levels. With 1–263 aa and 264–457 aa of TMAP, the level of phosphorylation did not increase in mitotic cells and rather decreased by ~20–30% compared with that of the asynchronous control. In contrast, the C-terminal portion (i.e. 458–682 aa) showed a dramatic increase in the level of phosphorylation in nocodazole-arrested mitotic cells (Fig. 1B), suggesting that the region harbors mitosis-specific phosphorylation sites.

We then selected candidate phosphorylation sites within this region by searching for evolutionarily conserved residues. Alignment of amino acid sequences corresponding to the C terminus of TMAP protein from multiple species revealed ~6 conserved serine and threonine residues (Fig. 1C). We first tested each of these sites if it makes a significant contribution to phosphorylation of TMAP in mitosis by mutating each residue to alanine and testing its effect on the mitotic band shift. Among these residues, mutation at Thr-578 or Ser-627 abolished or significantly diminished the mitotic band shift of TMAP protein (data not shown), suggesting that they are phosphorylated during mitosis. To further confirm this finding, we generated GST-fused C terminus of TMAP (i.e. 458–682 aa) constructs which express either wild-type (WT) or mutant proteins in which the candidate phosphorylation residues (i.e. Thr-578 and Ser-627) are mutated to alanines either individually or in combination. Each construct was expressed in HEK 293T cells and labeled with [³²P]orthophosphate while the cells were arrested at mitosis by nocodazole to measure the relative level of protein phosphorylation. Mutations at Thr-578 and Ser-627 indeed diminished the level of phosphorylation but, interestingly, failed to completely abolish the ³²P incorporation (Fig. 1D), which suggested that there are other additional phosphorylation sites. We then systemically mutated the remaining candidate residues in combination and measured the level of mitotic phosphorylation of each mutant to identify additional phosphorylation sites. Together with mutations at Thr-578 and Ser-627, additional mutations at Thr-595 and Ser-613 had no detectable effect on the level of phosphorylation in mitotic cells. In contrast, mutation at Thr-596 further reduced the level of ³²P incorporation, and additional mutation at Thr-622 reduced...
the level of phosphorylation to an almost undetectable level (Fig. 1D). This result indicated that Thr-578, Thr-596, Thr-622, and Ser-627 are the major mitotic phosphorylation sites located within the C terminus.

Thr-622 Is Phosphorylated by Cdk1-Cyclin B1 in Vitro—Sequence analysis revealed that three of the phosphorylation sites (i.e. Thr-578, Thr-596, and Thr-622) are followed by an evolutionarily conserved proline (Fig. 1C), which raised a possibility that these sites are phosphorylated by proline-directed kinase(s), such as Cdkks, MAPks, and GSK-3β (26). Because it is well known that Cdk1-cyclin B1 complex is one of the major mitotic kinases and regulates a number of mitotic processes, we first tested to see if Cdk1-cyclin B1 is able to directly phosphorylate TMAP in vitro. WT TMAP protein but failed to detect the T622A mutant form (Fig. 3A). Taken together, these results demonstrate the specificity of the antibody toward pT622 epitope.

To measure the cell cycle-dependent changes in Thr-622 phosphorylation, HeLa cells synchronized at the G1/S boundary by the double thymidine block were released and harvested at various time points. Cell lysates were then analyzed for the levels of total TMAP protein and pT622-TMAP by Western blot at the same time, the progression of the cell cycle was monitored by flow cytometry and by measuring the relative protein levels of cyclin B1 (a marker of G1/S phases) and phospho-histone H3 (a marker of mitosis). With progression of the cell cycle, the TMAP protein level gradually increased, peaking at G2/M phases of the cell cycle as previously reported (Fig. 3D).
(5). However, Thr-622 remained unphosphorylated during interphase. A sudden increase in Thr-622 phosphorylation was detected as cells started to enter mitosis. As cells exited mitosis, the level of Thr-622 phosphorylation declined as well (Fig. 3D). This indicates that phosphorylation of TMAP at Thr-622 indeed occurs in a mitosis-specific manner. To further confirm this finding and examine the subcellular localization of pT622-TMAP during mitosis, HEK 293 cells were immunofluorescently stained for both TMAP and pT622-TMAP. Consistent with the finding mentioned above, no interphase cells were stained with the pT622 antibody (Fig. 3E). pT622 staining began to appear during early prophase where chromosomes become condensed and TMAP protein starts to accumulate at the separating centrosomes. pT622 staining remained during prometaphase and metaphase, being localized to the spindle poles and microtubules, but started to decline during anaphase and was no longer detected at later stages of mitosis (Fig. 3E). Of note, the subcellular localization of pT622-TMAP was not different from that of total TMAP protein. Taken together, these results show that Thr-622 becomes phosphorylated specifically during early mitosis and de-phosphorylated as cells enter anaphase.

Cdk1-Cyclin B1 Phosphorylates TMAP in Vivo—We then tested whether TMAP is phosphorylated at Thr-622 by Cdk1-cyclin B1 also in vivo. Cdk1 and cyclin B1 were ectopically introduced, along with a C-terminal fragment of TMAP (corresponding to aa 566–682), into HEK 293T cells, and the level of Thr-622 phosphorylation was measured using the pT622-specific antibody. Overexpression of Cdk1 and cyclin B1 induced a marked increase in the level of Thr-622 phosphorylation of co-expressed C-terminal fragment of TMAP (Fig. 4A). In addition, short term treatment of nocodazole-arrested mitotic HeLa cells with Cdk inhibitors, purvalanol A and alsterpaullone, reduced the pT622 level as well as the level of phosphorylated histone H1, a previously known substrate of Cdk1-cyclin B1 (Fig. 4A). Because Cdk inhibitors used in the present study potentially inhibit other kinases, such as MEK1 and GSK-3β (27, 28), we also treated the cells with PD98059 (a MEK1 inhibitor) or AR-A014418 (a GSK-3β inhibitor), which produced no effect on the level of pT622 (Fig. 4B). These results strongly suggest that Cdk1-cyclin B1 is an in vivo TMAP kinase (at Thr-622) during mitosis. In support of this, TMAP and cyclin B1 also co-localized during the window of Thr-622 phosphorylation (i.e. at the centrosomes during early prophase and at spindle apparatus during metaphase) (Fig. 4C).

Expression of Phosphorylation-deficient Mutant of TMAP Induces Malformation of Bipolar Spindles and Delays Mitotic Progression—To study the functional significance of Cdk1-cyclin B1-mediated phosphorylation of TMAP at Thr-622 during mitosis, we introduced a WT or a phosphorylation-deficient mutant form of TMAP (i.e. T622A) in HEK 293 cells and observed for any changes in formation of mitotic spindles or in...
progression of mitosis. First, HEK 293 cells were transfected with GFP, GFP-WT, or GFP-T622A TMAP expression construct, fixed on the following day, and examined for the morphology of the mitotic spindles and for the alignment of metaphase chromosomes by staining for γ-tubulin (for microtubules and mitotic spindles) and for CENP-A (for centro-
reported (8), we focused our analysis on the cells that 1) expressed relatively low levels of the GFP fusion proteins; 2) forming cells to measure any differences in the kinetics of video microscopic analyses of GFP-WT or GFP-T622A-expressing similar levels of WT or T622A TMAP protein. In

Next, we examined if such increase in the frequency of abnormal bipolar spindles in T622A-expressing cells actually impairs the progression of mitosis. We carried out time lapse video microscopic analyses of GFP-WT or GFP-T622A-expressing cells to measure any differences in the kinetics of mitotic progression. Because expression of WT or T622A TMAP at an excessive level both induced formation of monopolar spindles and prolonged mitotic arrest as previously reported (8), we focused our analysis on the cells that 1) expressed relatively low levels of the GFP fusion proteins; 2) formed bipolar spindles; and 3) completed mitosis without cytokinesis defects. For the analysis, the GFP fluorescence intensity of the cells was measured to ensure that the cells were was fairly rapid, all reaching a plateau within 40–50 s. However, the fluorescence of GFP-T622A not only showed a relatively slower rate of decline, but also amounted to a lower level of the plateau, compared with the WT counterpart (Fig. 6), indicating that the rate of protein turnover at spindle microtubules is clearly decreased by the T622A mutation. This result suggests that the protein turnover of TMAP at spindle microtubules during early phases of mitosis is positively influenced by Cdk1-cyclin B1-mediated phosphorylation at Thr-622, which in turn, may be necessary to properly regulate the microtubule dynam-
ics and establishment of structurally sound bipolar spindles during mitosis.

**DISCUSSION**

TMAP is hyper-phosphorylated at the C terminus during mitosis. We identified the following four mitotic phosphorylation residues: Thr-578, Thr-596, Thr-622, and Thr-627. The findings of the present study are, in part, supported by previously published studies on proteomic analysis of (mitotic) phosphorylated proteins (29–31). The aforementioned studies reported the following residues of TMAP as phosphorylation sites: Ser-533, Thr-578, Thr-581, Ser-594, Thr-595, Thr-596, Tyr-598, Ser-601, Thr-611, Ser-613, and Thr-622. (The numbering of the residues is based on isoform 1 (682 aa) of TMAP. Of note, the isoform 1 (NP_060674) and isoform 2 (NP_001091995) of TMAP only differ by one amino acid.) Among these, Thr-578, Thr-596, and Thr-622 are included in our list of phosphorylated residues. However, in contrast to these reports, we found that Thr-595 and Ser-613 do not make significant contribution to phosphorylation of TMAP during mitosis. Also, none of these studies reported on phosphorylation of Ser-627. Although we have not comprehensively tested to see if other previously reported sites are also phosphorylated during mitosis, our results indicate that we identified major
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mitotic phosphorylation sites, because combination mutations, including all four residues (i.e. T578A, T596A, T622A, and S627A), reduced the level of mitotic TMAP phosphorylation to an almost undetectable level (see Fig. 1D). It is likely that the rest of the phosphorylation residues represent minor sites. So far, we have confirmed the present finding in HEK 293T, HeLa, human foreskin fibroblasts, and C2C12 (mouse myoblast) cells, suggesting that phosphorylation at these sites occurs during mitosis independently of the cell type. Moreover, based on the finding that the four residues we identified are among the most highly conserved residues in several vertebrate species (see Fig. 1C), they likely represent functionally important sites. This notion is supported by the results of the study using the phosphorylation-deficient mutant TMAP from the present study.

Cdk1-cyclin B1 phosphorylates TMAP both in vitro and in vivo. Because Thr-578, Thr-596, and Thr-622 are all followed by an evolutionarily conserved proline (see Fig. 1C), we initially hypothesized that they are phosphorylated by Cdk1-cyclin B1, which is the most well known proline-directed kinase at work during mitosis (26). However, in vitro kinase assays revealed that only Thr-622 is phosphorylated by Cdk1-cyclin B1. Consistent with this finding, only Thr-622 (T622TPVR625) perfectly conformed to the previously characterized consensus phosphorylation motif of cyclin-dependent kinases (i.e. (S/T)PX(K/R), where X denotes any amino acid) (32), whereas Thr-578 and Thr-596 are not followed by a Lys or Arg at the p + 3 position. While screening and epitope-mapping monoclonal antibodies raised against mouse TMAP, we have serendipitously found a monoclonal antibody, which loses its reactivity toward TMAP when it is phosphorylated at Thr-596 (24). Using the antibody, we demonstrated that Thr-596 begins to be phosphorylated during prophase and quickly becomes dephosphorylated as cells enter anaphase, confirming the present finding that Thr-596 phosphorylation occurs in a mitosis-specific manner. The timing of Thr-596 phosphorylation appears to coincide with that of Thr-622 phosphorylation, which follows the window of Cdk1-cyclin B1 activation (33). However, our results show that phosphorylation at Thr-596 does not appear to be directly regulated by Cdk1-cyclin B1. It is possible that Thr-578 and Thr-596 are phosphorylated by other proline-directed kinases, such as MAPks and GSK-3β. These kinases also have been shown to play important roles in mitotic entry and progression (34–36), implying that they mediate phosphorylation of functionally important substrates during mitosis. We are currently investigating whether these proline-directed kinases make significant contribution to mitosis-specific phosphorylation of Thr-578 and Thr-596. With respect to Ser-627, our preliminary data suggest that it is phosphorylated by another mitotic kinase,5 and we are currently investigating the mechanism and functional importance of its phosphorylation.

Cdk1-cyclin B1 plays an essential role in a number of early mitotic events. Particularly, Cdk1-cyclin B1 is clearly involved in establishment of bipolar spindles and regulation of spindle microtubule dynamics during mitosis, which is supported by the finding that a number of effectors of microtubules, including Ase1, Kar3, Slk19, and Stu2, are among substrates of Cdk1-cyclin B1 in yeast (21). Cdk1-cyclin B1 phosphorylates and mediates the microtubule localization of Eg5, a kinesin-related motor protein essential for separation of centrosomes, thus contributing to establishment of spindle bipolarity (18). Similarly, subcellular localization of other microtubule motors on the mitotic spindle requires Cdk1-cyclin B1-mediated phosphorylation (37, 38). Cdk1-cyclin B1 also regulates the spindle microtubule dynamics. For instance, addition of Cdk1 has been shown to increase the microtubule dynamics in cell-free Xenopus extract system (39, 40) and induce destabilization of microtubules in lysed mammalian cells (41), which suggest that the activity of Cdk1-cyclin B1 contributes to the dramatic increase in microtubule dynamics observed during early mitosis (42, 43).

In support of this, both positive and negative regulators of microtubule dynamics, such as MAP4, XMAP215, and Op18/stathmin, are directly phosphorylated by Cdk1-cyclin B1, which regulates their microtubule-binding or -stabilizing/de-stabilizing activities (22, 23, 44, 45). A recent report suggests that Cdk1-cyclin B1 more directly influences the microtubule dynamics by phosphorylating β-tubulin and impairing formation of microtubule polymers (46). Similarly, the microtubule-binding and/or -stabilizing properties of TMAP may be regulated by Cdk1-cyclin B1-mediated phosphorylation during early mitosis, which then contributes to regulation of microtubule dynamics and establishment of functional spindles.

We have previously shown that excessive levels of TMAP increase the microtubule stability and frequently cause defects in centrosome separation, which leads to formation of monopolar spindles and prolonged arrest in mitosis (8). Consistent with this, overexpression of WT TMAP alone induced spindle defects, predominated by formation of monopolar spindles, in the present study (see Fig. 5A). Expression of T622A

4 K. U. Hong, H. J. Kim, C. D. Bae, and J. Park, unpublished observations.

5 H. J. Kim, K. U. Hong, C. D. Bae, and J. Park, unpublished observations.
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mutant form of TMAP, however, induced a significantly higher rate of abnormal bipolar spindle formation (increased by ~3.2-fold), compared with the WT TMAP. Abnormal bipolar spindles formed in T622A mutant-expressing cells exhibited disorganized, asymmetrical, or narrow and elongated morphologies, and as a result, frequently accompanied misaligned chromosomes (see Fig. 5B). The spindle assembly checkpoint is known to monitor the status of the kinetochore-microtubule attachments as well as the tension generated between kinetochores and prevent the cells from entering anaphase until these requirements are met (47, 48). Thus, it is likely that the prolonged metaphase and delayed anaphase entry observed in T622A mutant-expressing cells are triggered by misaligned chromosomes and subsequent activation of the spindle assembly checkpoint. Most of the cells with abnormal bipolar spindles eventually pass metaphase and progress through mitosis, suggesting that they eventually form functional bipolar spindles and have their chromosomes properly aligned. These results suggest that Cdk1-cyclin B1-mediated phosphorylation of TMAP at Thr-622 is required for and contributes to proper establishment of functional bipolar spindles.

The mechanism by which T622A mutant interferes with the formation of functional bipolar spindles is currently unclear. One possibility is that the microtubule-stabilizing properties of TMAP are regulated by Thr-622 phosphorylation as discussed above. However, the microtubule association domain, which confers the microtubule-stabilizing effects of TMAP, is located toward the N terminus (4, 9). It is possible that phosphorylation at the C terminus may affect the properties of the microtubule association domain either allosterically or via intramolecular interactions, thus influencing its microtubule-binding properties (49). This notion is supported by one of our present findings that the turnover rate of TMAP protein associated with the spindle microtubules decreases with the T622A mutation (see Fig. 6). In other words, TMAP in its unphosphorylated form (as in T622A) may more tightly associate with the mitotic spindle and cause excessive stabilization of the spindle microtubules, which subsequently results in structural deformities of bipolar spindles observed in T622A-expressing cells. Thus, Thr-622 phosphorylation may contribute to proper regulation of microtubule dynamics during early phases of mitosis by influencing the dynamics of protein turnover of TMAP.

In the present study, we identified mitosis-specific phosphorylation sites on TMAP and explored the mechanism and functional significance of phosphorylation of TMAP at Thr-622. Our results suggest that regulation of TMAP via Cdk1-cyclin B1-mediated phosphorylation is important for establishment and/or maintenance of functional bipolar spindles during mitosis. Future studies will be focused on further investigating the role of phosphorylation in regulation of microtubule-binding and -stabilizing activities of TMAP and on contribution of mitotic phosphorylation to the role of TMAP in chromosome segregation. In addition, the mechanisms of phosphorylation of other residues identified in the present study will be explored.

Acknowledgment—We thank Dr. S. T. Kim for helpful discussions on the manuscript.

REFERENCES

33. JUNE 12, 2009 • VOLUME 284 • NUMBER 24
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22984–22992
Cdk1-Cyclin B1-mediated Phosphorylation of Tumor-associated Microtubule-associated Protein/Cytoskeleton-associated Protein 2 in Mitosis
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doi: 10.1074/jbc.M900257200 originally published online April 15, 2009

Access the most updated version of this article at doi: 10.1074/jbc.M900257200

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