# Cell Cycle-Dependent Phosphorylation of MAN1[†]

Yasuhiro Hirano,*[,‡] Yuka Iwase,[§] Kohei Ishii,[§] Masahiro Kumeta,[‡] Tsuneyoshi Horigome,[§,||] and Kunio Takeyasu[‡,||]

*Graduate School of Biostudies, Kyoto University, Yoshida-konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan, and Course of Fundamental Science, Graduate School of Science and Technology, Niigata University, Igarashi-2, Nishi-ku, Niigata 950-2181, Japan*

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ABSTRACT: The LEM (LAP2$\beta$, Emerin, and MAN1) proteins are essential for nuclear membrane targeting to chromatin via an association with barrier-to-autointegration factor (BAF). Herein, we focused on the mitotic phosphorylation of MAN1 and its biological role. MAN1 was phosphorylated in a cell cycle-dependent manner in the *Xenopus* egg cell-free system, and the mitotic phosphorylation at the N-terminal region of MAN1 suppressed the binding of MAN1 to BAF. Titansphere column chromatography followed by MS/MS sequencing identified at least three M-phase-specific phosphorylation sites, Thr-209, Ser-351, and Ser-402, and one cell cycle-independent phosphorylation site, Ser-463. An in vitro BAF binding assay involving mutants S402A and S402E suggested that the phosphorylation of Ser-402 was important for regulation of the binding of MAN1 to BAF.

The nuclear envelope (NE)[1] is a highly organized structure that consists of a double nuclear membrane (outer and inner nuclear membranes), nuclear pore complexes, and nuclear lamina (*1*−*4*). The NE undergoes a dynamic turnover during cell division; it breaks down during prophase and reassembles during telophase. The NE plays an important role not only as a barrier that protects the genome but also as a regulator of chromatin functions. The inner nuclear membrane (INM) contains many INM-specific proteins, such as the lamin B receptor (LBR), lamina-associated polypeptides (LAPs), emerin, MAN1 (also termed LEMD3), and SUN proteins (*5*−*10*). LAPs, emerin, and MAN1 are termed LEM proteins, since these proteins share a conserved LEM domain. These INM-specific proteins bind directly or indirectly to DNA and/or chromatin and attach the chromatin to the NE.

It has been thought that LBR and LEM proteins are essential for the nuclear membrane targeting step, which is the initial step of the NE reassembly at telophase, because nuclear membrane targeting is inhibited by (i) the depletion and neutralization of LBR by its antibody (*11, 12*) and (ii) the addition of peptides corresponding to the RS domain of LBR (*13*) or the LEM domain of LAP2$\beta$ into the *Xenopus* egg cell-free system (*14*).

Interestingly, LBR and LEM proteins associate on the chromatin surface in different ways. LBR binds directly to the chromatin, whereas the LEM protein−chromatin interaction is bridged by the barrier-to-autointegration factor (BAF; a 10 kDa LEM domain-specific binding protein). LEM proteins, but not LBR, transiently localize to a specific region of the chromatin, the core region, which is the microtubule-attaching region, 6−8 min after the metaphase−anaphase transition in HeLa cells (*15, 16*). BAF is required for the accumulation of LEM proteins onto the core region (*15, 17*) and directly binds to LEM proteins in vitro (*18*−*21*). Therefore, the binding mechanism for LEM proteins and BAF is important for determining the NE reassembly mechanism.

Previous studies suggested that the mitotic phosphorylation on Ser-175 of emerin, which is outside of the LEM domain, negatively regulates emerin−BAF binding (*22*), and the mitotic phosphorylation on Ser-4 of BAF by vaccinia-related kinase 1 (VRK1) also negatively regulates BAF−DNA and −LEM domain binding (*23*−*25*). Although these studies suggest that the mitotic phosphorylations of LEM proteins may be one of the key factors regulating the binding between LEM proteins and BAF, the detailed phosphorylation mechanisms of LEM proteins are still obscure. In this study, we focused on MAN1 protein, which is one of the LEM proteins, because emerin and MAN1 have overlapping function(s) essential for chromosome segregation and cell division in *Caenorhabditis elegans* embryos (*26*), and these proteins are highly conserved in metazoans (*8, 27*). MAN1 was first identified as an autoimmune antigen in collagen vascular disease (*28*). Human MAN1 consists of 911 amino acids and has two transmembrane domains that expose both the N-

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* To whom correspondence should be addressed: Laboratory of Plasma Membrane and Nuclear Signaling, Graduate School of Biostudies, Kyoto University, Yoshida-konoe-cho, Sakyo-ku, Kyoto 606-8501, Japan. Telephone and fax: +81-75-753-7905. E-mail: hirano@lif.kyoto-u.ac.jp.

[‡] Kyoto University.

[§] Niigata University.

[||] These authors contributed equally to this work.

[1] Abbreviations: GST, glutathione *S*-transferase; BAF, barrier-to-autointegration factor; LAP, lamina-associated polypeptide; LBR, lamin B receptor; MC, M-phase *Xenopus* egg cytosol fraction; SC, S-phase *Xenopus* egg cytosol fraction; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; NE, nuclear envelope; CHCA, α-cyano-4-hydroxycinnamic acid; TFA, trifluoroacetic acid; HF, hydrofluoric acid.

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and C-terminal domains to the nucleoplasmic side. Since MAN1 is a phosphoprotein (*29*) that binds to BAF (*21, 26*), the binding of MAN1 to BAF may be regulated by the cell cycle-dependent phosphorylation of MAN1, as is the case for emerin. Therefore, we examined the regulatory mechanism of the binding of MAN1 to BAF, and we found that mitotic phosphorylation outside of the LEM domain was responsible for the disassembly of MAN1 from the MAN1−BAF complex. This general regulatory mechanism by phosphorylation may be shared across the LEM proteins.

## MATERIALS AND METHODS

*Cloning and Expression of GST-Fused MAN1 Proteins.* Full-length human MAN1 was amplified by PCR. The PCR was performed with a Quick-Clone cDNA (Clontech) using primers 5′-GGAATTCCATGGCGGCGGCAGCAGCTTCG-3′ and 5′-CCGCTCGAGGGAACTTCCTTGAGAATTG-GTTA-3′, and the product was directly inserted into the pT7Bule-2 vector (Novagen). The DNA sequence of the insert was confirmed by five independent clones. Compared to the database sequence, the bases corresponding to positions 569, 942, and 1878 were mutated from G to A, A to T, and T to G, respectively, at the DNA level. Only the mutation at position 569 causes an amino acid substitution; this substitution is a change from R to Q at residue 190. Since all five clones contained these mutations, we decided to use this MAN1 sequence in this report. MAN1-N (amino acid residues 1−471), MAN1-C (amino acid residues 651−910), and MAN1-NΔLEM (amino acid residues 50−471) were also amplified by PCR, digested with EcoRI and XhoI, and then inserted into the pGEX vector (Novagen) at the EcoRI and XhoI sites. Point mutants that caused phosphorylation sites to be replaced with alanine were generated by using the Genetailor mutagenesis kit (Invitrogen), according to the manufacturer's protocol. His-tagged BAF was prepared as described previously (*22*). GST-fused MAN1 proteins were expressed in *Escherichia coli* XL1-blue cells and purified according to a standard protocol.

To construct a FLAG-fused N-terminal region of MAN1 corresponding to amino acids 1−554, these plasmids with (S402A and S402E) or without (WT) mutation were digested with XmnI and BamHI. After the 5′ overhangs were filled in with Klenow fragment, these plasmids were recombined with ligase. WT, S402A, and S402E were digested with EcoRI and BamHI and cloned into the ×3 FLAG-CMV-10 vector (Sigma).

*Preparation of Xenopus Egg Cytosol Fractions. Xenopus* eggs were collected, degelled, and then lysed to prepare S-phase and M-phase cytosol fractions as described previously (*30*).

*Phosphorylation Assay of MAN1 Fragments with a Xenopus Egg Cytosol Fraction.* Approximately 3 μg of GST-fused MAN1 bound to glutathione-Sepharose beads was incubated with S-phase and M-phase *Xenopus* egg cytosol fractions at 23 °C for 1 h. After being washed twice with extraction buffer [50 mM HEPES-KOH (pH 7.7), 250 mM sucrose, 50 mM KCl, and 2.5 mM $MgCl_2$], the proteins were separated by SDS−PAGE. Phosphorylation of the protein was detected with ProQ diamond stain (Molecular Probes) according to the manufacturer's instructions. After ProQ diamond staining, the gel was stained with CBB-R250.

*In Vitro Binding Assay of MAN1 and BAF.* Beads bearing approximately 2 μg of MAN1-N, MAN1-C, or point mutants were preincubated with a *Xenopus* egg cytosol fraction or extraction buffer and were then washed twice with binding buffer [20 mM Tris-HCl (pH 7.6), 134 mM NaCl, and 0.1% Tween 20]. *E. coli* cells, BL21(DE3), expressing His-tagged BAF were sonicated vigorously and centrifuged at 12000*g* for 10 min. The supernatant was incubated with the *Xenopus* egg cytosol-treated beads bearing MAN1 at 4 °C for 3 h. The beads were washed three times with binding buffer, subjected to SDS−PAGE, and then transferred to a PVDF membrane. BAF bound to beads was detected with an anti-His tag antibody and chemical luminescence (Millipore).

*Identification of the Phosphorylation Sites of MAN1.* This experiment was performed according to the method described previously with some modifications (*22*). Beads bearing approximately 50 μg of GST-fused MAN1-N and MAN1-NΔLEM were treated with the *Xenopus* egg S-phase or M-phase cytosol fraction (as described above). The treated beads were separated via 10% SDS−PAGE and visualized by CBB-R250 staining. Full-length GST−MAN1-N, GST−MAN1-C, and GST−MAN1-NΔLEM bands were excised from the gel and then in-gel digested with trypsin in 50 mM $NH_4HCO_3$ at 37 °C for 16 h. The obtained peptides were dried, dissolved in solvent A [Milli Q grade water containing 0.1% (v/v) TFA], and then applied to a Titansphere column (4.0 mm inside diameter × 10 mm column; GL Science Co.) equilibrated with solvent A at a flow rate of 0.1 mL/min for 30 min. Phosphopeptides trapped on the Titansphere column were eluted with solvent C [0.5 M $H_3PO_4$-NaOH (pH 8.0)] at a flow rate of 0.5 mL/min for 15 min. The eluted phosphopeptides were directly applied to a reversed-phase TSK-GEL C18 column (4.6 mm inside diameter × 150 mm column; TOSOH), briefly washed with solvent A, and then eluted with a 90 min linear gradient, from 0 to 45%, of solvent B [acetonitrile containing 0.082% (v/v) TFA] at a flow rate of 1 mL/min. The isolated phosphopeptides were analyzed with an AXIMA-CFR MALDI-TOF MS instrument (Shimadzu Co.) by using CHCA as a matrix. To confirm the phosphorylation of isolated peptides, we used the HF dephosphorylation method (*31*). MS/MS sequencing was performed with QSTAR Pulsar *i* (Applied Biosystems).

*Cell Culture and Two-Dimensional PAGE.* HeLa S3 cells were cultured in complete DMEM (Sigma) supplemented with 10% fetal bovine serum (Hyclone) in 5% $CO_2$ at 37 °C. Flag-tagged WT and S402A were transiently expressed in HeLa cells. The HeLa cells were cultured for 10 h at 37 °C in the presence or absence of 2 μM nocodazole to arrest the cells in the M phase. The M-phase cells and asynchronous cells were harvested by mechanical washing and by scraping the dish, respectively. The cells were resolved in lysis buffer [20 mM Hepes-KOH (pH 7.4), 120 mM NaCl, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, 1 mM PMSF, and protease inhibitor cocktail (Nacalai Tesque)] containing phosphatase inhibitor cocktail (Nacalai Tesque). After a brief sonication, Triton X-100 was added to a final concentration of 1% and incubated for 20 min on ice. The supernatant was collected by centrifugation at 2000*g* for 20 min. Proteins included in the supernatant were concentrated by acetone precipitation and resolved in IPG swelling buffer (9 M urea, 2% CHAPS, and 1% ampholytes). As a first dimension, isoelectric focusing electrophoresis (IPG strip, pI 4−7, 13 cm) was

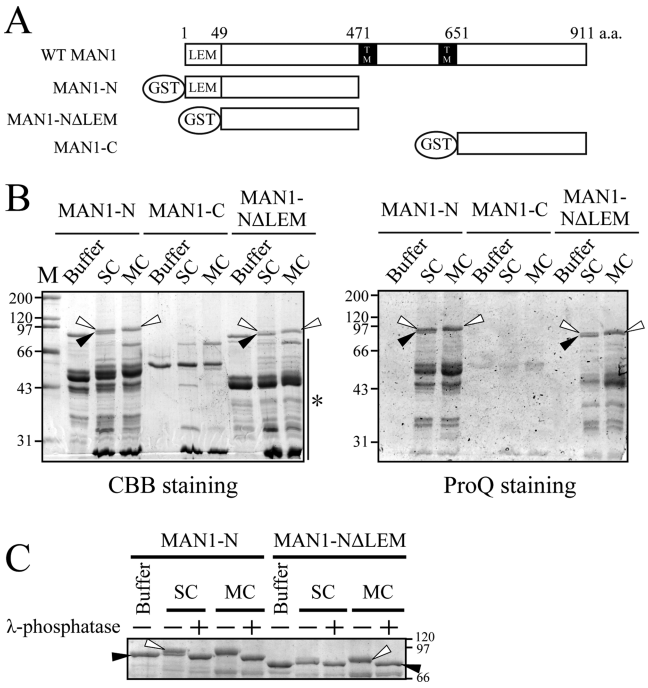


FIGURE 1: MAN1 is a phosphoprotein throughout the cell cycle. (A) Schematic diagram of MAN1 expressed as GST fusion proteins. (B) SDS−PAGE of GST fusion proteins. GST−MAN1-N (MAN1-N), GST−MAN-C (MAN1-C), and GST−MAN1-NΔLEM (MAN1-NΔLEM) were expressed in *E. coli* and then purified with glutathione-Sepharose beads. Beads bearing MAN1-N, MAN1-NΔLEM, and MAN1-C were treated with buffer alone or with S-phase or M-phase *Xenopus* egg extract for 1 h at 23 °C. Treated beads were washed, subjected to SDS−PAGE on a 10% gel, and then stained with CBB (left panel) and ProQ diamond (right panel). Empty and filled arrowheads indicate the phosphorylated bands. The asterisk indicates degraded MAN1. The values at the left are the relative molecular masses of the marker proteins. (C) Dephosphorylation assay. Beads bearing MAN1-N and MAN1-NΔLEM were pretreated with buffer alone or with SC or MC. After being washed, these beads were treated with or without 2 units of λ-phosphatase for 1 h at 23 °C. Thus, treated beads were subjected to SDS−PAGE and stained with CBB. Empty and filled arrowheads indicate the phosphorylated bands corresponding to panel B.

performed by using IPGphor (GE healthcare) according to the manufacturer's protocol. As a second dimension, strips were subjected to SDS−PAGE. Finally, separated Flag-tagged WT and S402A were detected by Western blotting by using anti-FLAG M2 monoclonal antibody (Sigma) as a probe.

## RESULTS

*Cell Cycle-Dependent Phosphorylation of MAN1 Regulates the Binding of MAN1 to BAF.* To explore the binding regulation mechanism between MAN1 and BAF, we expressed the N-terminal region (MAN1-N, corresponding to amino acids 1−471), the C-terminal region (MAN1-C, corresponding to amino acids 651−911), and the LEM domain-deleted N-terminal region (MAN1-NΔLEM, corresponding to amino acids 50−471) of MAN1 as GST fusion proteins in *E. coli* (Figure 1A). These proteins were purified and subjected to SDS−PAGE before and after being treated with the S-phase (SC) and M-phase (MC) *Xenopus* egg cytosol fractions. The proteins were stained with CBB (Figure 1B, left panel) or ProQ diamond, which specifically stains phosphoproteins (Figure 1B, right panel). Bands

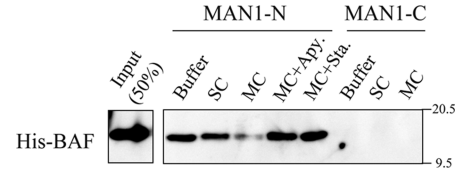


FIGURE 2: Phosphorylation of MAN1-N by the M-phase cytosol fraction suppressed the binding of MAN1-N to BAF. Beads bearing MAN1-N and MAN1-C (∼3 μg) were treated with buffer, SC, MC, MC pretreated with apyrase (MC+Apy.), or MC pretreated with staurosporine (MC+Sta.). After being washed, the beads were treated with His-tagged BAF for 3 h at 4 °C. The treated beads were subjected to 12% SDS−PAGE, transferred to a PVDF membrane, and then incubated with an anti-His tag antibody. Fifty percent of the loading amount of His-tagged BAF in the reaction mixture was also reacted with an anti His-tag antibody (Load). Bound antibodies were detected by enhanced chemiluminescence. The molecular masses of marker proteins are indicated at the right.

denoted with asterisks were degraded bands, as reported by Pan et al. (*32*). The mobilities of MAN1-N and MAN1-NΔLEM on SDS−PAGE were decreased by both SC and MC treatments. Doublet bands (Figure 1B, filled and empty arrowheads) could be observed after the SC treatment. After the MC treatment, the bottom band (Figure 1B, filled arrowhead) shifted to the upper band (Figure 1B, empty arrowhead). In contrast to MAN1-N, MAN1-C did not show a cell cycle-dependent mobility shift. ProQ staining showed that MAN1-N and MAN1-NΔLEM were phosphorylated throughout the cell cycle, whereas MAN1-C was specifically phosphorylated in the M phase at a very low level. These results were verified by a dephosphorylation assay (Figure 1C). Namely, the mobility changes of MAN1-N and MAN1-NΔLEM, which appeared after SC or MC treatment (Figure 1C, empty arrowhead), were canceled by following phosphatase treatment (Figure 1C, filled arrowhead). Thus, we concluded that MAN1 is a cell cycle-dependent phosphoprotein, especially hyperphosphorylated in the M phase, and that the mobility changes of MAN1-N and MAN1-NΔLEM resulted from cell cycle-dependent phosphorylation.

The effect of the cell cycle-dependent phosphorylation of MAN1 on its BAF binding activity was examined by an in vitro pull-down assay (Figure 2). MAN1-N bound BAF under a buffer only and after the SC treatments (Figure 2, Buffer and SC), whereas the binding activity of MAN1-N with BAF was greatly suppressed by the MC treatment (Figure 2, MC). Furthermore, pretreatments of MC with apryrase and staurosporine, which are broad kinase inhibitors, canceled the suppression of the binding of MAN1-N to BAF in MC (Figure 2, MC+Apy. and MC+Sta.). These results indicate that the mitotic phosphorylation of MAN1-N suppresses the binding of MAN1-N to BAF. However, we could not detect the binding of MAN1-C to BAF under any conditions in this system (Figure 2, MAN1-C). Therefore, we focused on the mitotic phosphorylation of MAN1-N in the following experiments.

*Identification of the Mitotic Phosphorylation at Ser-402.* To identify the M-phase-specific phosphorylation site(s) of MAN1-N, we used a phosphopeptide-specific isolation system with a Titansphere column (for the detailed procedure, see Materials and Methods and our previous paper). The full-length bands of SC- and MC-treated MAN1-N digested with trypsin and phosphopeptides were isolated (Figure 3A, SC and MC). As in Figure 3A, three peaks were specific in MC-

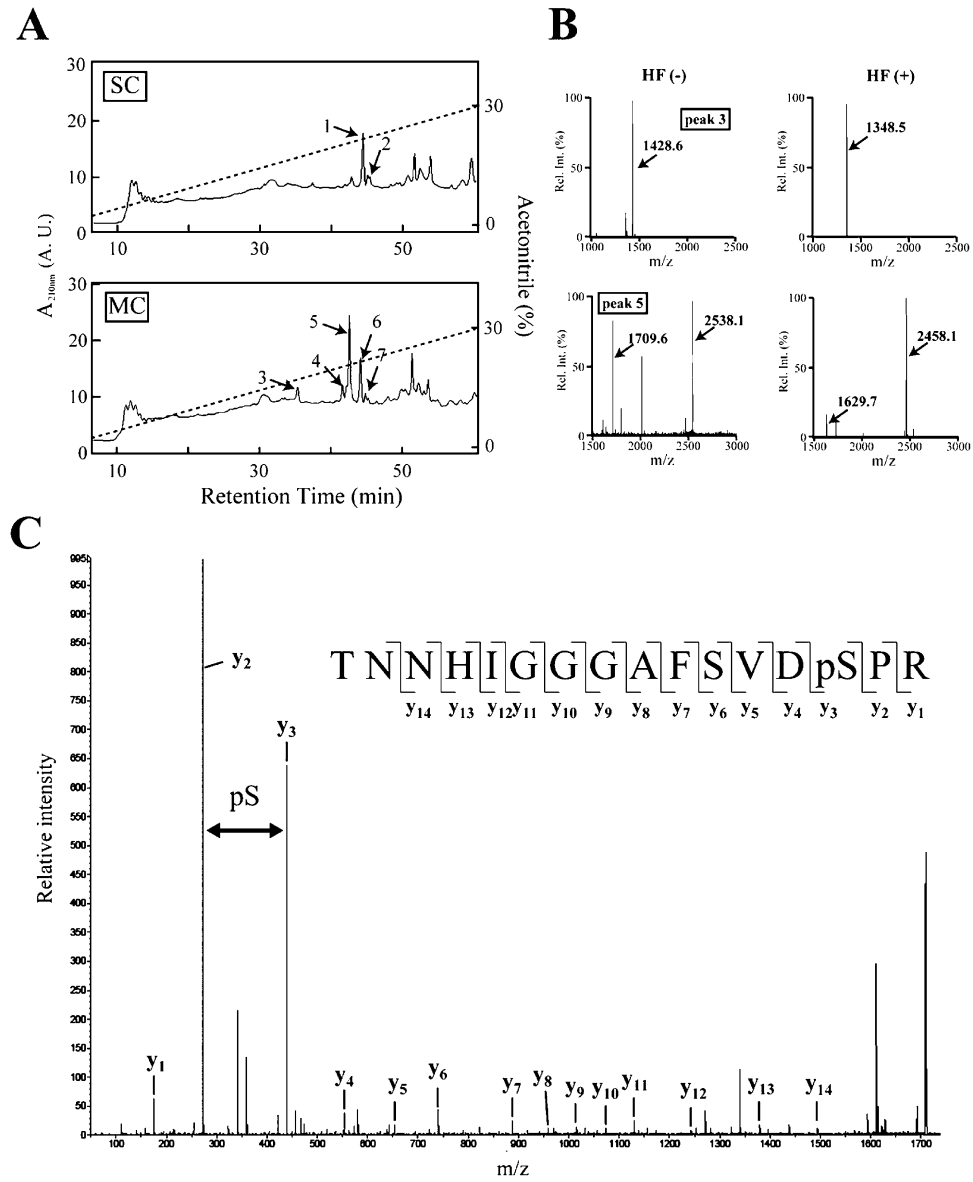


FIGURE 3: Identification of the phosphorylated peptides derived from MAN1-N. (A) Separation of phosphopeptides derived from MAN1-N. Approximately 50 $\mu$g of MAN1-N treated with SC and MC (as shown in Figure 2) was trypsinized (SC and MC, respectively). The resulting peptides were applied to a Titansphere column and eluted with 0.5 M phosphate buffer (pH 8.0). The retained tryptic phosphopeptides were separated by subsequent C18 chromatography (see Materials and Methods). The collected fractions were analyzed by MALDI-TOF MS. The numbered peak fractions contained phosphopeptides. (B) Detection of phosphopeptides of MAN1-N via MALDI-TOF MS. One-eighth of the obtained peptides in panel A was analyzed by MALDI-TOF MS with CHCA as a matrix [HF(−)]. For dephosphorylation, the same amount of these phosphopeptide fractions was dried, dissolved in 46% hydrofluoric acid, and incubated at room temperature for 1.5 h. The treated peptides were dried again and then analyzed via MALDI-TOF MS. Values shown in the figures indicate the monoisotopic peptide masses. (C) Identification of phosphorylation sites. A mass spectrum of the 1709.6 Da phosphopeptide (residues 389−404, monophosphorylated) was obtained by MALDI MS/MS. The prominent fragment ion series is made up of the y ions.

treated MAN1-N (Figure 3A, peaks 3−5), and two peaks were common in both SC- and MC-treated MAN1-N (Figure 3A, peaks 1 and 2 in the SC pattern and peaks 6 and 7 in the MC pattern, respectively). The molecular masses of the isolated peptides were determined by MALDI-TOF MS [Figure 3B, HF(−), and Table 1]. To examine whether these peptides contained phosphate groups, peptides were treated with HF and then the mass shifts derived from the dissociation of phosphate group(s) were analyzed [Figure 3B, HF(+)]. Mass shifts of 80 Da were observed for all isolated peptides, indicating that these peptides were phosphopeptides and that one site in their sequences was phosphorylated (Figure 3B and Table 1). Isolated phosphopeptides contained several possible phosphorylation sites, except peak 3. MS/MS sequencing (Figure 3C) revealed three M-phase-specific phosphorylation sites, Thr-209, Ser-351, and Ser-402, and one cell cycle-independent phosphorylation site, Ser-463 (Table 1). Importantly, a phosphorylation site in the LEM domain was never identified, as is the case for emerin (*22*). Although Olsen et al. reported that Ser-141, Ser-144, Ser-259, and Ser-260 are phosphorylated in asynchronous HeLa cells (*29*), we did not detect these residues as cell cycle-dependent phosphorylation sites (see Discussion).

The significance of the LEM domain in the mitotic phosphorylation of MAN1-N was examined by deletion mutagenesis. The phosphopeptides derived from MC-treated MAN1-N (Figure 4, MAN1-N) and MAN1-NΔLEM (Figure 4, MAN1- NΔLEM) were isolated. The magnitude of peak

Table 1: Phosphopeptides Detected by MALDI-TOF MS/MS[a]

| peak | peptide (*m/z*) | calcd molecular mass (Da) | sequence | phosphorylation site |
|---|---|---|---|---|
| 1 | 2329.4 | 2329.6 (1P) | EEV***S***PTGSFSAHPLERPHRD (460−479) | 463 |
| | 1979.9 | 1980.0 (1P) | REEV***S***PTGSFSAHPLER (459−475) | 463 |
| | 1823.8 | 1823.9 (1P) | EEV***S***PTGSFSAHPLER (460−475) | 463 |
| 2 | 1979.9 | 1980.0 (1P) | REEV***S***PTGSFSAHPLER (459−475) | 463 |
| | 1823.7 | 1823.9 (1P) | EEV***S***PTGSFSAHPLER (460−475) | 463 |
| 3 | 1428.6 | 1428.5 (1P) | RPAGPELQ***T***PPGK (201−213) | 209 |
| 4 | 2475.3 | ND[b] | ND[b] | ND[b] |
| 5 | 2538.1 | 2538.5 (1P) | NLEEAAAAEQGGGCDQVD***S***SPVPR (333−356) | 351 |
| | 1709.6 | 1709.7 (1P) | TNNHIGGGAFSVD***S***PR (389−404) | 402 |
| 6 | 2329.2 | 2329.4 (1P) | EEV***S***PTGSFSAHPLERPHRD (460−479) | 463 |
| 7 | 1823.9 | 1823.9 (1P) | EEV***S***PTGSFSAHPLER (460−475) | 463 |
| | 1980.0 | 1980.0 (1P) | REEV***S***PTGSFSAHPLER (459−475) | 463 |

[a] Data obtained in the MALDI-TOF MS and MS/MS experiments shown in Figure 3. Phosphoamino acids are indicated by the bold italicized letters. 1P indicates the number of phosphate groups. [b] Not determined.

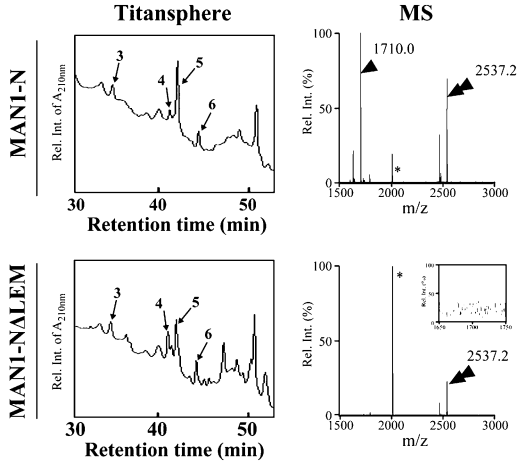


FIGURE 4: LEM domain of MAN1-N needed to phosphorylate Ser-402. Approximately 50 $\mu$g of MAN1-N and MAN1-NΔLEM were treated with MC at 23 °C for 1 h. After being washed, these proteins were subjected to SDS−PAGE and stained with CBB. Full-length MAN1-N and MAN1-NΔLEM were excised and digested with trypsin. The tryptic peptides were subjected to a phosphopeptide isolation system, and phosphopeptides were isolated as described in the legend of Figure 3. The peak numbers indicated by the arrows correspond to the peak numbers in Figure 3. The mass of the isolated peptides in peak 5 was determined. The asterisk indicates the same nonspecific peptide. The mass from 1650 to 1750 Da is enlarged in the inset.

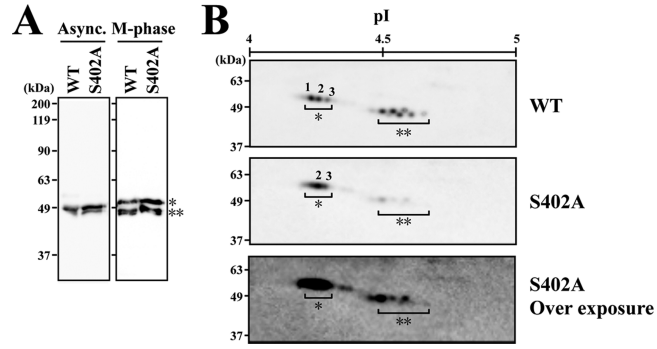


FIGURE 5: Ser-402 is phosphorylated in M-phase HeLa cells. (A) Flag-tagged MAN1 (corresponding to amino acids 1−554) without or with point mutation (WT and S402A) was transiently expressed in HeLa cells, and the extracts from asynchronous and mitotic-arrested cells were prepared as described in Materials and Methods. The extracts were subjected to SDS−PAGE, and Flag-tagged WT and S402A were detected by Western blotting. Asterisk and double asterisk bands correspond to the spots shown in panel B. Molecular mass markers are indicated at the left. (B) Mitotic extracts prepared as described in panel A were subjected to 2D PAGE, and Flag-tagged WT and S402A were detected by Western blotting. The pI values are indicated at the top, and molecular mass markers are indicated at the left.

5 (corresponding to peak 5 in Figure 3A) was decreased in MAN1-NΔLEM (in Figure 4, Titansphere, compare the top left panel with the bottom left panel). Since this peak had contained two phosphopeptides (*m/z* 1709.6 and 2538.1), we used mass spectrometry to determine if one or both phosphopeptides disappeared. We found that the phosphopeptide corresponding to amino acids 389−404 (*m/z* 1709.6) had completely disappeared (Figure 4, MS, arrowhead); thus, Ser-402 of MAN1-NΔLEM was not phosphorylated by the MC treatment. However, we detected the phosphopeptide corresponding to amino acids 333−356, which includes the mitotic phosphorylation on Ser-351 (Figure 4 MS, double arrowhead). Therefore, we concluded that the LEM domain of MAN1-N is required for the phosphorylation of Ser-402 by an unidentified kinase during mitosis.

To verify the mitotic phosphorylation of Ser-402 in vivo, we transiently expressed Flag-tagged N-terminal regions of MAN1 with or without (WT) a point mutation at Ser-402 (S402A) in HeLa cells. After nocodazole treatment, mitotic HeLa cells were harvested, lysed, and then subjected to SDS−PAGE and two-dimensional (2D) PAGE (panels A and B of Figure 5, respectively). In the SDS−PAGE gel, mitotic band shifts were observed in both WT and S402A (Figure 5A, band indicated by the asterisk in the M phase; compare with the band derived from the asynchronous cells) and in the in vitro phosphorylation assay (compare with Figure 1B). In the 2D PAGE gel, the band denoted with an asterisk in WT was separated into three spots (Figure 5B, spots 1−3 in WT). In contrast, the band denoted with an asterisk in the S402A mutant resolved into two spots. The spot corresponding to spot 1 in WT completely disappeared in the S402A mutant, and spot 2 became stronger than that of WT (Figure 5B, spot 2 in S402A). This tendency is a reasonable change due to the loss of a phosphorylation site. There were no differences between other spots, i.e., spot 3 and double asterisk spots. This result suggests that Ser-402 is phosphorylated in the M phase in vivo.

*Mitotic Phosphorylation at Ser-402 Inhibits the Binding of MAN1 and BAF.* Unphosphorylatable and phosphomimetic point mutants in MAN1-N were generated by replacing Ser-402 with alanine and glutamic acid (N-S402A and N-S402E, respectively). In N-S402A, the mitotic phosphorylation of Ser-402 completely disappeared without any effect on the other mitotic phosphorylations of MAN1-N (Figure 6A). An in vitro BAF binding assay involving N-S402A demonstrated

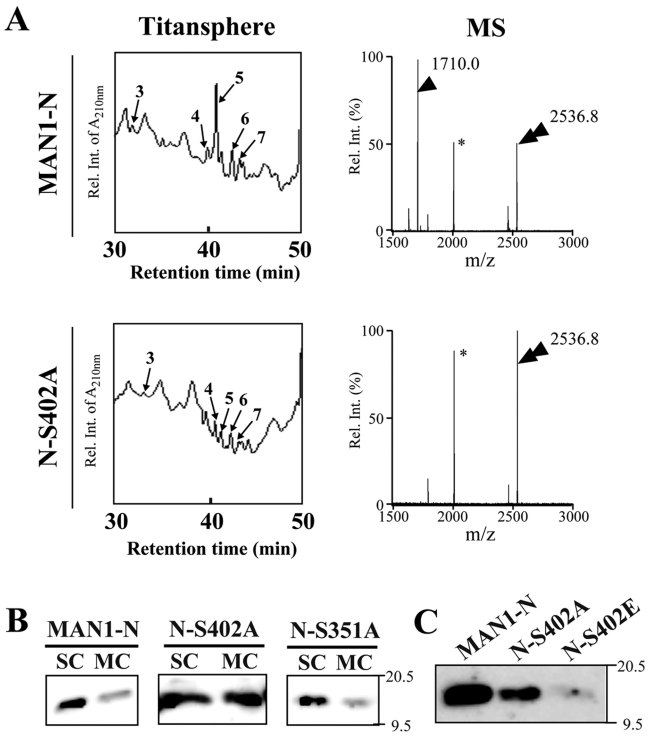


FIGURE 6: Phosphorylation at Ser-402 of MAN1-N regulates the binding of MAN1 to BAF. (A) Separation pattern of phosphopeptides derived from MAN1-N and N-S402A. Approximately 50 $\mu$g of MAN1-N and N-S402A treated with MC was trypsinized, and the obtained peptides were applied to a phosphopeptide isolation system as described in the legend of Figure 3. The peak numbers indicated by the arrows correspond to the peak numbers in Figure 3. Right panels are the MS analyses of peak 5. An asterisk indicates a nonspecific peak. (B) N-S402A prevented the mitotic dissociation of BAF. Beads bearing MAN1-N, N-S402A, and N-S351A ($\sim$3 $\mu$g) were treated with SC or MC at 23 °C for 1 h. The beads were incubated with an *E. coli* extract expressing His-tagged BAF, separated via 12% SDS−PAGE, and then transferred to a PVDF membrane. BAF that bound to the beads was detected with an anti-His antibody as described in the legend of Figure 2. (C) N-S402E did not bind to BAF. Beads bearing MAN1-N, N-S402A, and N-S402E ($\sim$3 $\mu$g) were directly treated with an *E. coli* extract expressing His-tagged BAF, and the BAF bound to beads was detected as described in the legend of Figure 2. The molecular masses of the marker proteins are indicated at the right.

that the BAF binding avidity of N-S402A did not change between the SC and MC treatments, although the binding avidity of wild-type MAN1 (MAN1-N) was suppressed by the MC treatment (Figure 6B). We generated the point mutant replacing Thr-209 and Ser-351 with alanine (N-T209A and N-S351A, respectively) to examine the effect of another mitotic phosphorylation site for MAN1-BAF binding. Unfortunately, N-T209A was completely degraded in *E. coli* (data not shown). MC-treated N-351A did not bind to BAF like wild-type MAN1, suggesting that the mitotic phosphorylation at Ser-402 may be a specific site for regulating the binding of MAN1 to BAF. Furthermore, N-S402E did not bind to BAF even with the buffer-only treatment (Figure 6C). Thus, Ser-402 of MAN1-N is the critical phosphorylation site for regulating the binding of MAN1-N to BAF in a cell cycle-dependent manner.

## DISCUSSION

*Cell Cycle-Dependent Phosphorylation of MAN1.* Our results demonstrate that the N-terminal region of MAN1 outside of the LEM domain is highly phosphorylated at both the S phase and the M phase. Among the phosphorylation sites that we identified, Thr-209, Ser-351, and Ser-402 were specifically phosphorylated during the M phase. The amino acid sequences around the phosphorylation sites are -S/T-P-. This sequence is similar, but not identical, to the consensus sequences for cdc2 kinase (-S/T-P-x-R/K-) and p38 MAP kinase (-L/P-x-S/T-P-) (*33*). It has been suggested that mitotic phosphorylation by cdc2 kinase leads to the breakdown of various nuclear structures, e.g., the dissociation of LBR and LAP2$\beta$ from chromatin (*14, 34*−*36*), the disassembly of nuclear lamina (*37*−*39*), and the disassembly of NPCs (*40*). Reasonably, cdc2 kinase is also expected to phosphorylate MAN1-N. A kinase prediction server, NetPhosK1.0 (*41*), predicted that Thr-209, Ser-351, and Ser-402 can be phosphorylated by cdc2 kinase with moderate scores (0.44, 0.49, and 0.47, respectively), although the highest scores at Thr-209 and Ser-402 were obtained for cdk5 and p38MAP kinase, respectively.

We did not identify the phosphorylation sites reported by Olsen et al. (*29*). One possible reason for this apparent discrepancy is that we used cell cycle-specific *Xenopus* egg cytosol to phosphorylate MAN1, whereas Olsen et al. used serum-starved interphase HeLa cells stimulated by epidermal growth factor. Phosphorylation status should be changed among cells in interphase, the S phase, and the M phase. Another possible explanation for the different results is that the phosphopeptide recovery rate of our system is higher than that of the tip-base method (Y. Iwase et al., unpublished observation). It is likely that we did not detect some of the minor phosphopeptides, because we fractionated the isolated phosphopeptides according to UV absorbance. Importantly, our results indicated that at least Ser-402 is phosphorylated in both the *Xenopus* egg system and the HeLa cell, and that Ser-402 is an M-phase-specific phosphorylation site (Figures 3−5).

*Mechanisms and Roles of Ser-402 Phosphorylation.* The LEM domain interacts with BAF. Interestingly, all of the MAN1 phosphorylation sites that we identified are outside of the LEM domain (Figure 3 and Table 1), and the mitotic phosphorylation at Ser-402 is required for the regulation of the binding of MAN1 to BAF (Figure 6B,C).

How does the mitotic phosphorylation at Ser-402 affect the binding of MAN1 and BAF? BAF mainly binds to the LEM domain of MAN1 (*21*). In the primary MAN1 sequence, Ser-402 is located very far from the LEM domain. The mitotic phosphorylation of Ser-402 may induce a conformational change around this portion and inhibit the BAF binding activity of the LEM domain, resulting in the dissociation of MAN1 from BAF. However, this phosphorylation does not seem to be sufficient to completely abolish the interaction between MAN1 to and BAF. Our in vitro pull-down assay showed that the phosphorylation at Ser-402 reduced the affinity between MAN1-N and BAF, but weak binding between MAN1-N and BAF was still observed. It has been reported that BAF in the M phase is also phosphorylated by vaccinia-related kinase 1 (VRK1) and that this phosphorylation reduces the affinity for the LEM domain (*42*). It is likely that the mitotic phosphorylation of both MAN1 and BAF guarantees the complete dissociation of MAN1 and BAF in the M phase.

How is the mitotic phosphorylation on Ser-402 regulated? This phosphorylation did not occur when the LEM domain was deleted (Figure 6). Thus, the LEM domain is essential for the mitotic phosphorylation of Ser-402. These results support the following two hypotheses. (1) The LEM domain directly binds to a LEM protein kinase that has not yet been identified, and (2) BAF bridges the binding between the LEM proteins and a LEM protein kinase. VRK1 is a candidate for the kinase that acts on both the LEM proteins and BAF at the same time in the M phase. Recently, Gorjanacz and co-workers reported that *C. elegans* VRK1 localizes to the NE at prophase and controls the reassembly of BAF and LEM proteins onto the chromatin (*43*). Furthermore, in the VRK1-depleted embryos of *C. elegans*, LEM2 (a homologue of MAN1) and emerin seem to remain associated with the chromatin throughout mitosis, and VRK1 is co-immunoprecipitated with BAF. Therefore, we speculate that VRK1 might accumulate on the LEM protein−BAF complexes at prophase and phosphorylate both BAF and the LEM proteins. Unfortunately, the consensus sequence for VRK1 phosphorylation sites remains unclear.

The BAF binding activity of the LEM domain of MAN1 is regulated by phosphorylation at a remote region. This type of regulation also occurs in the interaction between the LEM domain of emerin and BAF (*22*). Thus, a similar regulatory mechanism underlies both the emerin−BAF and MAN1−BAF interactions. Other LEM proteins such as LAPs are also phosphorylated during the M phase (*14, 44*). Although the mitotic phosphorylation sites of the LAPs, especially the membrane-embedded isoform LAP2$\beta$, have not been identified, we propose a general model in which the phosphorylation at remote regions induces a conformational change and promotes the dissociation of LEM proteins from BAF. This effective dissociation of LEM proteins from BAF perhaps stimulates the dissociation of the nuclear membrane from chromatin. On the other hand, an unidentified phosphatase may dephosphorylate the mitotic phosphorylation of LEM proteins to form a trimeric complex of LEM proteins, BAF, and chromatin, and this dephosphorylation may induce the nuclear membrane assembly.

*Roles of Phosphorylation Sites with the Exception of Ser-402.* Most NE structures, except some nucleoporin complexes (*45*), disassemble at prophase. For this disassembly, the mitosis promoting factor (MPF) is likely to phosphorylate nuclear proteins (*46*). Since MAN1 binds to various proteins such as BAF, emerin, both A- and B-type lamin, and Smads (*8, 21, 47*−*49*), the phosphorylation sites that we identified may regulate the binding between MAN1 and its binding partners. The N-terminal region of MAN1 is responsible for binding to emerin and lamin. Since MAN1, emerin, and lamins dissociate with each other and disperse into the endoplasmic reticulum and cytoplasm at the onset of mitosis (*46*), the binding among them may be regulated by mitotic phosphorylation. Thr-209 and/or Ser-351 of MAN1 could be the target sites of such phosphorylation. However, the MAN1 binding region of emerin is also phosphorylated at M phase (*22*). The mitotic phosphorylations of MAN1 and emerin may cooperatively dissociate the proteins from each other.

With regard to BAF binding, MAN1-C seems to be able to interact with BAF (*21, 26*), although we did not detect the binding of MAN1-C to BAF under the conditions used in our study. We mention two points as causes of this apparent contradiction. The first is a preparation procedure of BAF. We purified His-tagged BAF from *E. coli* by sonication because we should use nonmodified BAF for the binding assay to verify the effect of cell cycle-dependent MAN1 modification only. However, previous reports used an in vitro translation system to purify BAF (*21, 50*). Then, the contradiction might come from the modification state of BAF. The second is the DNA binding properties of MAN1-C, because this region binds to both DNA and BAF (*51*). The DNA binding of MAN1-C may interfere with the BAF−MAN1-C interaction. However, our in vitro pull-down assay performed in the presence of DNase I did not detect the binding of MAN1-C to BAF (our unpublished observation). It is interesting that the C-terminal region of MAN1 is also specifically phosphorylated in the M phase. Although the phosphorylation level is very low, this phosphorylation might regulate the binding of its interactants such as DNA and Smad (*51, 52*).

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