Dissociation of Emerin from Barrier-to-autointegration Factor Is Regulated through Mitotic Phosphorylation of Emerin in a Xenopus Egg Cell-free System*

Received for publication, March 23, 2005, and in revised form, September 6, 2005. Published, JBC Papers in Press, October 3, 2005, DOI 10.1074/jbc.M503214200

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Emerin is the gene product of STA whose mutations cause Emery-Dreiffus muscular dystrophy. It is an inner nuclear membrane protein and phosphorylated in a cell cycle-dependent manner. However, the means of phosphorylation of emerin are poorly understood. We investigated the regulation mechanism for the binding of emerin to chromatin, focusing on its cell cycle-dependent phosphorylation in a Xenopus egg cell-free system. It was shown that emerin dissociates from chromatin depending on mitotic phosphorylation of the former, and this plays a critical role in the dissociation of emerin from barrier-to-autointegration factor (BAF).

Then, we analyzed the mitotic phosphorylation sites of emerin. Emerin was strongly phosphorylated in an M-phase Xenopus egg cell-free system, and five phosphorylated sites, Ser49, Ser66, Thr67, Ser120, and Ser175, were identified on analysis of chymotryptic and tryptic emerin peptides using a phosphopeptide-concentrating system coupled with a Titansphere column, which specifically binds phosphopeptides, and tandem mass spectrometry sequencing. An in vitro binding assay involving an emerin S175A point mutant protein suggested that phosphorylation at Ser175 regulates the dissociation of emerin from BAF.

The nuclear envelope (NE) is a highly dynamic structure that disassembles at the onset of mitosis and reassembles on the surface of chromatin during telophase in vertebrates. These changes of NE are crucial for cell cycle progression. The NE consists of an outer nuclear membrane, inner nuclear membrane, nuclear pore complex, and nuclear lamina. The inner nuclear membrane contains integral membrane proteins, i.e. lamin B receptor (LBR), lamina-associated polypeptide-2β (LAP2β), emerin, MAN1, and others, which interact with DNA and/or chromatin, and these proteins are proposed to participate in nuclear membrane targeting to chromatin at an early stage of nuclear assembly (1). The interactions between some of the inner nuclear membrane proteins and chromatin are regulated through phosphorylation of these inner nuclear membrane proteins. The phosphorylation mechanisms for LBR and LAP2α and 2β are well understood (2–7). LBR directly binds to DNA in vitro and dissociates on phosphorylation by cdc2 kinase and other kinase(s) in a mitotic egg extract (3). LAP2β binds to lamin B1 and chromatin, and cell cycle-dependent phosphorylation of LAP2β cancels this binding (4). Phosphorylation of these inner nuclear proteins, therefore, is likely to be one of the key mechanisms that control the interactions between the inner nuclear proteins and components of the nuclear lamina as well as chromatin. In this study, we focused on the mitotic phosphorylation of emerin, one of the inner nuclear membrane proteins.

Human emerin is a serine-rich protein exhibiting an apparent mass of 34 kDa on SDS-PAGE (8) and is phosphorylated in a cell cycle-dependent manner (9). Emerin belongs to the LEM (LAP2B, emerin, MAN1) protein family, whose members have approximately a 40-residue domain named the LEM (10). These proteins directly bind to barrier-to-autointegration factor (BAF) (11–13). BAF is a DNA-bridging protein with a dimer mass of 20 kDa and is highly conserved in metazoans, and the BAF interactions with both DNA and LEM proteins are critical for nuclear membrane targeting to chromatin and chromatin decondensation during nuclear assembly (14). At the onset of mitosis, emerin disperses from the NE to the endoplasmic reticular (ER) network, and is re-localized to the surface of the central region of chromatin, called the “core” region, during telophase (15, 16). An LEM domain deletion mutant of emerin cannot be re-localized to this region, suggesting that the binding of emerin to BAF through the LEM domain is essential for this recruitment (16). It is also known that emerin has many binding partners, including transcriptional repressors and intermediate filament proteins (17–25). In particular, binding to A-type lamin is essential for the retention of emerin in the NE in the interphase. Furthermore, a deletion mutant of emerin residues 95–99 (Δ95–99), which causes Emery–Dreiffus muscular dystrophy and cannot bind to lamin A, exhibits an aberrant cell cycle-dependent phosphorylation forms (9). The study also suggested that the phosphorylation of emerin regulates the binding of emerin to lamin A (9). Thus, we were interested in the cell cycle-dependent regulation of the binding of emerin to chromatin and BAF.

We first examined the binding of emerin to chromatin by means of a binding assay involving a GST-fused N-terminal fragment of emerin and chromatin in a Xenopus egg cell-free system. We also analyzed the cell cycle-dependent phosphorylation states and sites of emerin. Phosphopeptides derived from emerin treated with a Xenopus egg mitotic
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cytosol were separated by means of a Titansphere column, and five phosphorylation sites were identified on mass spectrometry. Furthermore, an in vitro binding assay involving an emerin point mutant revealed that Ser\textsuperscript{175} phosphorylation is responsible for the dissociation of emerin from BAF.

MATERIALS AND METHODS

Construction of GST-fused Emerin Fragment Proteins and His\textsubscript{6} Tag BAF—Cloning of the nucleoplasmic region of human emerin (\Delta TM), amino acid residues 1–213 was performed by PCR. PCR was carried out with a human testis cDNA library using the following primers: 5'-CGGGATCCCTGGAAACTGAGAT-3' and 5'-CGGGATCCAGGACGCAGGGTTCAGG-3'. The PCR product was digested with BglII and BamHI, and the resulting fragment was inserted into the pBluescript II SK\textsuperscript{(+)}. PCR was carried out with the pBluescript II SK\textsuperscript{(−)}-emerin \Delta TM using the following primers: 5'-CTGTAAGAAGATATATAGCTGAAACAGGCGGTAGTCGT-3' and 5'-CCTGGAGGACGTCGAAAAACAGGGCGGTATGCT-3', followed by verification by DNA sequence analysis. The pBluescript II SK\textsuperscript{(−)}-S175A-\Delta TM was digested with BamHI and EcoRI, and then inserted into the pGEX 5X-3 vector (Novagen) at the BamHI site. To construct the pGEX 3X-emerin \Delta TM plasmid, the pBluescript II SK\textsuperscript{(−)}-emerin \Delta TM was digested with BglIII and BamHI, and the resulting fragment was inserted into the pGEX 3X vector using the BamHI site in the vector. The cDNA sequence of human BAF (accession no. BC005942) was purchased from Invitrogen. To obtain His tag BAF, the coding region of BAF was PCR-amplified using primers 5'-GGGGATCCCTGGAAACTGAGAT-3' and 5'-CCTGGAGGACGTCGAAAAACAGGGCGGTATGCT-3'. The PCR product was digested with BamHI and EcoRI, and then inserted into the pET28c vector at the BamHI and EcoRI sites. The DNA sequences of the inserts in plasmid pGEX 3X-emerin \Delta TM and pET28c-\textsuperscript{c}-BAF were confirmed using an ALF DNA sequencer (Amersham Biosciences).

Preparation of Xenopus Egg Cytosol Fractions—Xenopus eggs were collected, dejelled, and then lysed to prepare S-phase and M-phase cytosol fractions as described previously (26, 27).

Chromatin Binding Assay—Using beads bearing GST-emerin \Delta TM or GST-emerin \Delta LT, the chromatin binding assay was carried out as previously described (3, 28) except for the use of 20,000 Xenopus sperm chromatin per assay. When pretreatment of the beads bearing GST-emerin with an Escherichia coli extract containing BAF was necessary, it was carried out as follows. E. coli cells expressing His tag BAF were sonicated vigorously and then centrifuged at 12,000 \times g for 10 min. A 50-\mu l aliquot of the supernatant was reacted with beads bearing GST-emerin \Delta TM in binding buffer (20 mM Tris-HCl (pH 7.6), 134 mM NaCl, and 0.1% Tween 20) either containing 125 \mu Ci of [\gamma\textsuperscript{32P}]ATP at 23 °C for 1 h. After washing twice with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), the beads were washed with 2 mM ATP and then incubated at 4 °C for 10 min. The proteins thus treated were separated by SDS-PAGE and visualized by CBB R-250 staining. After drying the gel, phosphorylation was detected with Fuji X-ray film. The emerin \Delta TM bands were excised from the gel and the phosphorylated emerin \Delta TM was quantitated by scintillation counting.

Phosphopeptide Mapping—Approximately 3 \mu g of GST-fused emerin bound to glutathione-Sepharose beads was incubated with S-phase and M-phase egg cytosol fractions containing 1 \mu Ci of [\gamma\textsuperscript{32P}]ATP at 23 °C for 1 h. After washing twice with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), the beads were washed with 2 mM ATP and then incubated at 4 °C for 10 min. The proteins thus treated were separated by SDS-PAGE and visualized by CBB R-250 staining. After drying the gel, phosphorylation was detected with Fuji x-ray film. The emerin \Delta TM bands were excised from the gel and the phosphorylated emerin \Delta TM was quantitated by scintillation counting.
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RESULTS

The Binding of Emerin ΔTM to Chromatin—Two kinds of emerin fragments, i.e. emerin ΔTM (residues 1–213) and emerin ΔLT (residues 37–213), fused to GST were expressed in *E. coli* and used in this study (Fig. 1A). Emerin ΔTM lacks the transmembrane domain, and emerin ΔLT lacks both the transmembrane domain and most of LEM domain. GST-fused emerin ΔTM (ΔTM) and GST-fused emerin ΔLT (ΔLT) were purified from *E. coli* extract using a GSH-Sepharose bead (Fig. 1B). Western blotting with anti-GST antibody of ΔTM and ΔLT preparations, which were purified by GSH-Sepharose showed that smaller phosphopeptide bands observed in Fig. 1B (lanes 1 and 2) indicated by asterisk were GST-containing degradation products of ΔTM and ΔLT (data not shown). All experiments in this study were done using beads bearing GST-emerin ΔTM or ΔLT. To determine whether ΔTM interacts with chromatin and its interaction is regulated in a cell cycle-dependent manner, like for some other inner nuclear membrane proteins, i.e. LBR and LAP2β, or not, we performed an *in vitro* chromatin binding assay. We previously developed this assay method to analyze the binding of inner nuclear membrane proteins to chromatin (28). When beads bearing ΔTM were preincubated with buffer in the absence of the Xenopus egg cytosol fraction, they bound to chromatin slightly (column 1 in Fig. 2). However, when they were preincubated with a synthetic phase cytosol fraction (SC), the binding of chromatin to beads was stimulated (compare column 1 and 2 in Fig. 2). Preincubation with a mitotic phase cytosol fraction (MC) did not stimulate the binding (column 3 in Fig. 2). Moreover, the once-stimulated chromatin binding activity of SC-treated beads was suppressed on subsequent incubation with MC (compare columns 2 and 4 in Fig. 2). On the other hand, the once-suppressed chromatin binding activity of MC-treated beads was activated on subsequent incubation with SC (compare columns 3 and 5 in Fig. 2). These results demonstrated that the emerin fragment thus expressed can bind to chromatin, and that the chromatin binding assay method can be used to analyze the cell cycle-dependent binding of emerin to chromatin *in vitro*. The stimulation of the binding of ΔTM to chromatin on SC treatment seemed to be independent of phosphorylation of ΔTM, because the stimulation was not suppressed on pretreatment of SC with apyrase for ATP depletion or a wide-spectrum protein kinase inhibitor, i.e. staurosporine (compare columns 2, 6, and 7 in Fig. 2). Furthermore, the stimulation of the binding did not occur on SC treatment of the beads bearing GST-emerin ΔLT (compare columns 12 and 13 in Fig. 2). These results indicated that the stimulation might be caused by the binding of the BAF in SC to ΔTM, which is known to mediate the binding of emerin to chromatin, because (i) the stimulation of the binding was not suppressed by kinase inhibitors, (ii) the stimula-
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FIGURE 2. The suppression of the binding of emerin to chromatin is caused by mitotic phosphorylation of emerin. Beads bearing ΔTM (−6 μg) were used, and the beads for columns 4, 10, and 11 were pretreated with a synthetic-phase cytosol (columns 4, 10, and 11), a mitotic phase cytosol (column 5) or buffer (columns 1–3 and 6–9) at 23 °C for 20 min. The beads were subsequently treated with buffer (column 1; Buffer), SC (columns 2 and 5; SC and MC-SC, respectively), MC (columns 3 and 4; MC and SC-MC, respectively), SC pretreated with 8 milliunits of apyrase or 5 μM staurosporine (columns 6 and 7; SC + Apy and SC + Sta, respectively) or MC pretreated with 8 milliunits of apyrase or 5 μM staurosporine (columns 8–11; MC + Apy, MC + Sta, SC-MC + Apy, and SC-MC + Sta, respectively) at 23 °C for 20 min. The beads thus treated were incubated with 20,000 decondensed sperm chromatin at 4 °C for 10 min, and then observed by fluorescence microscopy after staining of DNA with Hoechst 33342. The “percentage of beads with bound chromatin” values were determined as described under “Materials and Methods” after subtraction of the value for blank GST beads. Beads bearing ΔLT (−4 μg) treated with buffer (column 12) or SC (column 13) were reacted with chromatin in the same way as for the beads bearing ΔTM. The results are the means ± S.D. for three independent experiments. *, a significant difference from the respective control (p < 0.05).

Participation of BAF in the Binding of Emerin ΔTM to Chromatin—To clarify the stimulation mechanism for the binding of ΔTM to chromatin on SC treatment, we examined whether the binding of emerin to chromatin is mediated or not by BAF in our assay system. Beads bearing ΔTM were treated with an E. coli extract containing expressed His tag BAF. The beads thus treated were used for the chromatin-binding assay involving ΔTM treated with BAF. Beads bearing ΔTM (−10 μg) were treated with buffer (buffer), an E. coli-soluble fraction containing His tag BAF (BAF (+) E. coli extract), or a blank E. coli-soluble fraction expressing a His tag insoluble protein (BAF (−) E. coli extract) for 3 h. 25,000 decondensed sperm chromatin were added to the beads thus treated, followed by incubation for 10 min. Then, the “percentage of beads with bound chromatin” values were determined as described in Fig. 2. The results are the means ± S.D. for three independent experiments. *, a significant difference from the respective control (p < 0.05). B, confirmation of the binding of emerin to BAF. Proteins bound to beads treated as in A were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and then incubated with an anti-His antibody. Bound antibodies were detected as enhanced chemiluminescence. A bar at the right indicates the position of 17-kDa marker protein electrophoresed.

M-phase-specific Phosphorylation of Emerin ΔTM Suppressed the Binding to BAF—We examined whether the binding of emerin ΔTM beads to BAF is cell cycle-dependent or not. Beads bearing ΔTM were pretreated with E. coli extract containing His tag BAF to bind His tag BAF to ΔTM. The beads thus treated were further treated with a buffer or cell cycle-dependent Xenopus egg extract (Fig. 4A). In the cases of treatments with the buffer and SC, His tag BAF bound to ΔTM remained on beads (Fig. 4A, buffer and SC). However, His tag BAF bound to ΔTM disappeared by MC treatment (Fig. 4A, MC). These results showed that MC, but not SC, treatment of ΔTM-BAF complex causes dissociation of BAF from ΔTM. On the other hand, beads bearing ΔTM pretreated with buffer, SC, or MC were treated with the E. coli extract containing His tag BAF, then proteins bound to the beads were separated by SDS-PAGE and transferred to a nitrocellulose membrane, and BAF was detected with an anti-His tag antibody (Fig. 4B). The binding of BAF was suppressed by the pretreatment of ΔTM beads with MC but not that with buffer or SC (Fig. 4B, buffer, SC, and MC). Furthermore, SC treatment followed by MC treatment (SC-MC) suppressed the binding of BAF to beads bearing ΔTM (Fig. 4B, SC-MC). These results were consistent with the earlier results. The phosphorylation levels of emerin shown in Fig. 4C were demonstrated by ProQ diamond staining, which is known as a means of phosphoprotein staining (compare Fig. 4C, ProQ stain with CBB stain) (29). The weak “Pro Q staining” observed for the band of ΔTM treated with buffer (Fig. 4C, buffer) is nonspecific staining. Therefore, staining over the background can be considered to be phosphoprotein-specific staining. The bandshift on SDS-PAGE of emerin on treatment with MC (Fig. 4C, MC and SC-MC) was consistent with in vivo phosphorylated emerin in mitotic-phase lymphoblastoid cells (9). However, the four bands depending on the phosphorylation states reported for lymphoblastoid cell were not clear in this system. ΔTM preparations treated with MC (MC and SC-MC) were strongly phosphorylated and the binding of His-BAF was strongly suppressed. On the other hand, pretreatment of MC with apyrase or staurosporine to prevent the mitotic phosphorylation of emerin canceled this suppression activity (Fig. 4, B and C, MC + Apy. and MC + Sta). A part of phosphorylation of ΔTM by MC seems to be
caused by staurosporine-insensitive kinase, because 5 μM staurosporine could not cause complete inhibition (Fig. 4, ProQ stain and MC + Sta.). Therefore, we concluded that the binding of emerin to BAF is suppressed by M-phase-specific direct or indirect phosphorylation. Thereafter, we focused on analysis of the cell cycle-dependent phosphorylation of emerin, especially mitotic phosphorylation sites of emerin.

Cell Cycle-dependent Phosphorylation States of Emerin—We next examined the cell cycle-dependent phosphorylation of emerin in a Xenopus egg cell-free system (Fig. 5). ∆TM on beads was treated with egg cytosol fractions containing [γ-32P]ATP, washed with binding buffer, and then analyzed by SDS-PAGE. The gel was stained with CBB and subjected to autoradiography. The ∆TM was strongly phosphorylated upon treatment with MC, although the phosphorylation by SC was very low (Fig. 5A, Autoradiography). A mitotic phase-specific band-shift was also observed for ∆LT treated with MC (Fig. 5A, ∆LT). These results show that one or more phosphorylation sites that are outside of the LEM domain caused the main band-shift, because ∆LT lacking almost all of the LEM domain showed a clear band-shift on treatment with MC (Fig. 5A, ∆LT lanes). To compare the amounts of incorporated phosphate groups, ∆TM treated with SC or MC in the presence of [γ-32P]ATP was electrophoresed, and ∆TM bands were excised from the gel and counted (Fig. 5B). The ∆TM phosphorylated in the M-phase was 6.6 times as strong as that in the S-phase. These results demonstrated that the phosphorylation of emerin in the S-phase is very low.

To determine the mitotic phosphorylation state of emerin, and the differences in the phosphorylation site(s) between ∆TM and ∆LT, we performed phosphopeptide mapping (Fig. 6). Beads bearing ∆TM and ∆LT were treated with SC or MC in the presence of [γ-32P]ATP. After washing, the beads thus treated bearing ∆TM and ∆LT were subjected to SDS-PAGE, and proteins were transferred to a nitrocellulose membrane. Then, the full-length emerin bands were excised from the membrane and digested with trypsin (Fig. 6A) or chymotrypsin (Fig. 6B). The peptides generated were subjected to two-dimensional separation on a cellulose plate. Although many spots of phosphorylated peptides were observed for the preparations derived from both ∆TM and ∆LT treated with MC, the major spots indicated with arrows in Fig. 6 were not observed for ∆LT. When 32P-labeled ∆TM and ∆LT were digested with chymotrypsin, well focused patterns were obtained (Fig. 6B). In these patterns, major spots I and 2 and three weak spots, 8, 10, and 11, were absent in ∆LT map (compare Fig. 6B, ∆TM and ∆LT). These results suggested the following three possibilities: 1) at least one major phosphorylation site exists in the LEM domain, 2) deletion of the LEM domain causes obstruction of a major phosphorylation site present in other than the LEM domain, and 3) BAF binds an emerin kinase and

FIGURE 4. Phosphorylation of emerin by M-phase cytosol fraction caused dissociation of emerin from BAF. A, BAF was dissociated from emerin by MC treatment. B, Beads bearing ∆TM (~10 μg) were pretreated with E. coli extract containing His tag BAF at 4 °C for 3 h. After washing, these beads were treated with extraction buffer (buffer) or a cell cycle-dependent Xenopus egg cytosol fraction (SC or MC) at 23 °C for 1 h. Then, proteins on beads thus treated were subjected to 12% gel SDS-PAGE, transferred to a nitrocellulose membrane, and then incubated with an anti-His tag antibody. Ten percent of loading amount of His-BAF in the reaction mixture was also reacted with an anti-His tag antibody (Load). Bound antibodies were detected by enhanced chemiluminescence. C, the binding of BAF to emerin was suppressed by mitotic phosphorylation of emerin. Beads bearing ∆TM (~10 μg) were pretreated with SC in the case of (SC-MC), and then subsequently treated with buffer (Buffer), SC (SC), MC (MC and SC-MC), MC pretreated with apyrase (MC+Ap), or MC pretreated with staurosporine (MC+Sta), respectively. BAF bound to beads were detected by anti-His tag antibody as in A. C, the phosphorylation states of emerin. Beads bearing ∆TM treated as in B were subjected to 10% gel SDS-PAGE, and then gel was stained with ProQ diamond to confirm the phosphorylation states of emerin (ProQ stain). Total emerin protein was stained by CBB (CBB stain). Bars at the right indicate positions of 17-, 43-, and 66-kDa marker proteins electrophoresed.

FIGURE 5. Emerin was strongly phosphorylated in the M-phase. A, detection of phosphorylation. Beads bearing 3 μg of emerin ∆TM or ∆LT pretreated with SC (SC-MC) or not were incubated with 20 μl of a synthetic (SC) or mitotic (MC and SC-MC) phase egg cytosol fraction containing 1 μCi of [γ-32P]ATP at 23 °C for 1 h. The beads thus treated were washed with binding buffer and then subjected to SDS-PAGE. The gel was stained by CBB and subjected to autoradiography. Arrows, arrowheads, and double arrowheads indicate ∆TM, the mitotic-specific band-shift of ∆TM, and emerin ∆LT bands, respectively. Bars at the right indicate positions of 43- and 66-kDa marker proteins electrophoresed. B, the ∆TM bands in A were excised from the gel, and then phosphate groups incorporated into ∆TM were compared by their radioactivities. Results are shown as means ± S.D. for three independent experiments.

FIGURE 6. Tryptic and chymotryptic phosphopeptide maps of ∆TM and ∆LT. Beads bearing ~30 μg of ∆TM or ∆LT were incubated with 20 μl of SC or MC containing 20 μCi of [γ-32P]ATP at 23 °C for 1 h. The proteins thus treated were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The ∆TM or ∆LT bands were excised and digested with trypsin (A) or chymotrypsin (B). The phosphopeptides thus obtained were separated by electrophoresis at pH 8.9 (horizontal direction; cathode to the left) and by ascending chromatography. Excised bands contained 1, 6, 0.6, 4, 6, and 4 kcpm for 6A-SC-∆TM, 6A-MC-∆TM, 6A-SC-∆LT, 6A-MC-∆LT, 6B-SC-∆TM, and 6B-MC-∆LT, respectively. The points of sample application can be seen as dots near the bottom-left corners. The arrows indicate major spots lacking for ∆LT treated with MC.
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FIGURE 7. Identification of the phosphorylated peptides derived from ΔTM treated with MC. A, separation of phosphopeptides derived from ΔTM. Approximately 100 µg of ΔTM treated with MC as in Fig. 6 without [γ-32P]ATP was chymotrypticized or trypsinized. The peptides thus obtained were applied to a TitanSphere column and eluted with 0.5 M phosphate buffer (pH 8.0). The retained chymotryptic (upper panel) or trypsic (middle panel) phosphopeptides were separated by subsequent C8 chromatography (see “Materials and Methods” for details). The collected fractions were analyzed by MALDI-TOF MS. The baseline is indicated in the lower panel. The numbered peak fractions contained phosphopeptides. B, detection of phosphopeptides of emerin on MALDI-TOF MS. One-eighth of the obtained peptides in A were analyzed by MALDI-TOF MS using 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 peptides thus treated were dried again and then analyzed by MALDI-TOF MS. Values shown in the figures indicate the monoisotopic peptide masses. C, identification of phosphorylation sites. A mass spectrum of the 1367.7 ((M+H)+, left) and 685.1 (M+2H)+, right) Da phosphopeptide (corresponding to 1367.7 Da, residues 168–179, monophosphorylated) was obtained by ESI-IT MS/MS. The prominent fragment ion series are those having b and y ions. The peptide sequence is RPVSASRSSLDL. In the spectrum, the b1 ion still has the phosphate moiety, but the b7 and y4 ones do not. This indicates that the phosphate moiety must be located at Ser175, indicated by an asterisk.

recruits it to ΔTM. These three possibilities are explained under “Discussion.” The phosphorylation levels of emerin fragments treated with SC were much lower than for those treated with MC (Fig. 6A), although the phosphorylation patterns of SC-treated samples were found to be very similar to those of MC-treated ones when the autoradiography films were superposed (data not shown). Therefore, we only examined the mitotic phosphorylation sites in the following experiments.

Identification of the Mitotic Phosphorylation Sites of Emerin—To identify the mitotic phosphorylation sites of emerin by means of mass spectrometry sequencing, we used a phosphopeptide separation system comprising a combination of a TitanSphere column, which specifically binds phosphopeptides, and a reverse phase C8 column (see “Materials and Methods”). For this purpose, ΔTM was purified by glutathione-Sepharose and phosphorylated by MC and then digested with proteinases. We mainly used a chymotryptic digest of emerin, because a chymotryptic digest gave a clearer phosphopeptide map than a tryptic digest, as can be seen in Fig. 6. The chymotryptic or trypsic phosphopeptides derived from phosphorylated ΔTM were bound to the TitanSphere column and then eluted with 0.5 M phosphate buffer (pH 8.0). The phosphopeptide fraction thus obtained was directly introduced onto the reverse phase C8 column, eluted with a linear gradient, and then fractionated (Fig. 7A). The molecular masses of the peptides thus obtained were determined by MALDI-TOF MS. Some of the determined masses were consistent with the calculated phosphopeptide masses (Fig. 7B, HF(−), and TABLE ONE). To confirm that these peptides contained phosphate groups, samples were pretreated with HF to hydrolyze phosphate groups and then the mass shift was analyzed by MALDI-TOF MS (Fig. 7B, HF(+)). The mass shifts of 80 or 160 Da indicate that the obtained peptides were phosphopeptides and that one or two sites in their sequences were phosphorylated (Fig. 7B and TABLE ONE). As can be seen in TABLE ONE, every phosphopeptide had more than two possible phosphorylation sites. Therefore, we performed MS/MS sequencing by means of ESI-IT MS to determine which residues are phosphorylated. The mass spectrum of the product ions of m/z 1367.7 and 685.1 ((M+H)+ and (M+2H)+ of the 1367.7-Da peptide, respectively) allowed localization of the phosphorylation site to residue Ser175 by b and y ion series. This was so because we could detect the b1 ion (RPVSASR) without a phosphate group on product ion m/z 1367.7 sequencing and the b5 ion (RPVSASRSSLDL) without a phosphate group on product ion m/z 685.1 sequencing (Fig. 7C). In contrast to the case of 1367.7-Da peptide, we could not determine which site, Ser175 or Ser176, was phosphorylated in 2080.0-Da peptide. However, we focused on only Ser175 because Ser175, but not Ser176, was identified as a phosphorylation site by the analysis indicated above. Using the same approach for determination of the phosphorylated residue, the phosphorylation sites of other phosphopeptides (Ser69, Ser66, Thr70, and Ser70) were also determined (TABLE ONE). However, we could not completely exclude the possi-
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**TABLE ONE**

| Peptide | Phosphopeptide mass | Peak number | Sequence (residue) | Phosphorylated residue |
|---------|---------------------|-------------|-------------------|-----------------------|
| 2808.0 | 2808.0 (1P)         | 1           | PyEITHYPVSASRSSDL (162–179) | Ser^{175} or Ser^{176} |
| 1367.7  | 1367.7 (1P)         | 2           | RPVSAASRSSDL (168–179) | Ser^{175} |
| 1758.5  | 1758.7 (1P)         | 3           | SFSDLNSTRGDAMY (60–74) | Ser^{245}, Thr^{306} (mixture) |
| 1847.0  | 1846.9 (1P)         | 4           | RAVRQSVTSPFDADAF (115–130) | Ser^{320} |
| 2739.9  | 2738.2 (2P)         | 5           | GEPSAGPSAVRQSVTSPFDADAF (106–130) | ND^{+} |
| 2658.2  | 2658.2 (1P)         | 6           | GEPSAGPSAVRQSVTSPFDADAF (106–130) | ND^{+} |
| 2396.8  | 2397.0 (1P)         | 7           | RLSPPSASSASSYFSDFLNNSTR (47–68) | Ser^{49} (possibly also Ser^{49}, Ser^{55}, or Ser^{57}) |
| 4109.6  | 4108.8 (1P)         | 8           | AVRQSVTSPFDADAFHHQVHDDDLSSSEECKDR (116–150) | ND^{+} |

*1P and 2P indicate the number of phosphate group.

*pyE, pyroglutamic acid (considered as an artificial modification probably arising during sample preparation).

*ND, not determined.

bility that another site in the 2396.8-Da peptide was phosphorylated, that is, Ser^{52}, Ser^{53}, or Ser^{54} was possibly phosphorylated. In the case of the 4109.6-Da peptide, we could not determine the phosphorylated residue, although candidates include either Ser^{123}, Ser^{141}, Ser^{142}, or Ser^{143}. In the cases of 2739.9- and 2658.2-Da peptides, moreover, sufficient information to determine the phosphorylation site could not be obtained. On the other hand, we could not detect phosphotyrosine by phosphoamino acid analysis of ΔTM phosphorylated by MC in the presence of [γ-^{32}P]ATP (data not shown). Finally, we identified five phosphorylation sites, Ser^{49}, Ser^{66}, Thr^{27}, Ser^{120}, and Ser^{175}. These phosphorylation sites matched the consensus sequences of well known kinases; i.e. Ser^{12}: protein kinase A, calmodulin-dependent kinase II, and glycogen synthetase kinase 3β; Ser^{66}: glycogen synthetase kinase 3β; and Ser^{175}: glycogen synthetase kinase 3β.

Ser^{175} Phosphorylation of Emerin by MC Responsible for the Emerin-BAF Dissociation—Although five mitotic phosphorylation sites of emerin were identified and one possible phosphorylation site, i.e. Ser^{175}, was found on MS/MS sequencing, we could not detect any phosphorylation site in the LEM domain. When these identified phosphorylation sites and phosphopeptide maps of ΔTM and ΔLT were compared, it was suggested that the phosphopeptide spots absent for the ΔLT digest (major spots 1 and 2 and minor spots 10 and 11 in Fig. 6B) might not be due to the LEM domain and that phosphorylation at these sites is regulated through an unknown mechanism by the LEM domain. Then, we applied the phosphopeptide separation system to ΔTM and ΔLT phosphorylated with a mitotic extract to determine the phosphorylation site(s) corresponding to the absent spots in the phosphopeptide maps in Fig. 6B. Surprisingly, a peak corresponding to a peptide containing phosphorylated Ser^{175} (arrow in Fig. 8A, ΔTM, corresponding to peak 1 in Fig. 7A) had completely disappeared for the ΔLT digest (Fig. 8A, ΔLT), although there was no clear change in other peaks. We could not examine whether the peak corresponding to peak 2 in Fig. 7A, a peak corresponding to a fragment of the peak 1 material, disappeared or not, because the peak in Fig. 8A was too small. These results indicated that phosphorylation at Ser^{175}, which is located outside of the LEM domain, might be affected through some unknown mechanism by the LEM domain and suggested that the phosphorylation participates in emerin-BAF dissociation. Then, we generated a point mutant at Ser^{175} of ΔTM (S175A-ΔTM) replaced with an alanine. Using this mutant, we could confirm that the missing peak from the elution pattern of phosphopeptides derived from ΔLT is that of the Ser^{175}-containing peptide (Fig. 8A, S175A). Furthermore, we performed phosphopeptide mapping to confirm that the Ser^{175} phosphorylation was affected by deletion of the LEM domain. As can be seen in Fig. 8B, spots 1 and 2, which disappeared from the ΔLT pattern, also completely disappeared from the S175A-ΔTM pattern. Spots 12–14 appeared irregularly (compare ΔTM patterns in Figs. 6 and 8). Spot 4 in Fig. 8B S175A shifted up when compared with ΔTM and ΔLT in Fig. 8B. However, the shift seems to be within a fluctuation of the spot, because the spot shifts slightly from one gel to another (compare Fig. 6B, ΔTM to Fig. 8B, ΔTM and Fig. 6B, ΔLT to Fig. 8B, ΔLT). These results suggested that the LEM domain is necessary for the Ser^{175} phosphorylation by MC. Then, we carried out an in vitro...
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binding assay using S175A-ΔTM and BAF to determine whether the Ser\textsuperscript{175} phosphorylation regulates the dissociation of emerin and BAF or not (Fig. 8C). The S175A point mutant treated with MC retained binding activity toward BAF, although ΔTM treated with MC dissociated from BAF. This result showed that the Ser\textsuperscript{175} phosphorylation in the M-phase participates in the dissociation of emerin and BAF.

DISCUSSION

Mitotic Phosphorylation of Emerin—Although it has been known that emerin is highly phosphorylated at the M-phase in human lymphoblastoid cells (9), the role of phosphorylation of emerin has been poorly understood. Recently, Lattanzi et al. (20) indicated that the interaction of emerin and actin is increased by dephosphorylation of emerin, suggesting that phosphorylation of emerin regulates its binding to actin. By means of a phosphopeptide separation system involving a Titansphere column, we demonstrated that emerin is phosphorylated under mitotic conditions in vitro with at least five specific residues, four serine and one threonine residues: Ser\textsuperscript{49}, Ser\textsuperscript{66}, Thr\textsuperscript{87}, Ser\textsuperscript{120}, and Ser\textsuperscript{175} (TABLE ONE). These phosphorylation sites are interestingly located at the binding region for many emerin-binding proteins: Ser\textsuperscript{49}, Ser\textsuperscript{66}, and Thr\textsuperscript{87} for GCL, YT521-B, and Btf (23–25) Ser\textsuperscript{120} for lamin A and actin (12, 21); and Ser\textsuperscript{175} for GCL, YT521-B, Btf, and actin (21, 23–25), respectively. Therefore, phosphorylation at some of these sites possibly regulates their interactions. In particular, dephosphorylation of Ser\textsuperscript{120} and/or Ser\textsuperscript{175} may increase the binding of emerin to actin, because the phosphorylation sites are located in the actin binding domain.

The Regulation Mechanism for the Interaction of Emerin and BAF—In this study, it was suggested that the dissociation of emerin from BAF takes place through mitotic phosphorylation of emerin in a Xenopus cell-free system. However, these results do not rule out mitotic modification of BAF, which could independently regulate its binding to emerin. Surprisingly, our point mutant study suggested that the phosphorylation at Ser\textsuperscript{175}, which is located outside of the LEM domain, participates in the dissociation of emerin from BAF in the M-phase (Fig. 8C).

Bengtsson and Wilson (17) and the previous study by Lee et al. (12) indicated that when at least two emerin mutants with replacement of residues 76–83 and 207–208, which lie outside of the LEM domain, were incubated with BAF, the amount of bound BAF was decreased by these mutations. Their study suggested that not only the LEM domain but also other regions of emerin seem to participate in the binding of emerin to BAF. Our data coincide with this finding. Then, we expected that the LEM domain and another BAF binding region in emerin may comprise a “BAF binding surface,” and that the phosphorylation at Ser\textsuperscript{175} on MC treatment may induce a conformational change of the BAF binding surface, because the region around Ser\textsuperscript{175} has been predicted to be a flexible region that consists of a poly-Ser cluster (32). This conformational change might cause the BAF dissociation.

Although we could not clearly show why Ser\textsuperscript{175} of ΔLT was not phosphorylated by MC, the following three possibilities can be pointed out. First, deletion of the LEM domain from ΔTM caused a conformational change around Ser\textsuperscript{175} and phosphorylation was blocked. Second, a kinase bound to the LEM domain participated in the Ser\textsuperscript{175} phosphorylation of ΔTM; in this case, ΔLT lacking the LEM domain could not be phosphorylated at Ser\textsuperscript{175}, because the kinase could not be recruited. Third, BAF recruits one or more kinases that phosphorylate Ser\textsuperscript{175} of ΔTM; in this case, ΔLT lacking BAF-binding activity could not be phosphorylated. To exclude the third possibility, we performed the emerin phosphorylation assay using MC whose BAF-binding proteins were depleted. BAF-binding proteins were depleted from MC by incubation with nickel-agarose beads bearing His-BAF, and then beads bearing ΔTM were phosphorylated in the presence of [γ\textsuperscript{32}P]ATP by thus treated MC. No difference was observed between MC- and BAF-binding protein-depleted MC in phosphorylation of ΔTM (data not shown). Therefore, the first and the second possibilities should be ascertained further to clarify the Ser\textsuperscript{175} phosphorylation mechanism.

Cell Cycle-dependent Phosphorylation and Dephosphorylation of Inner Nuclear Membrane Proteins—Major inner nuclear membrane proteins, i.e. LBR, LAP2β, emerin, and MAN1, are known to bind directly or indirectly to chromatin and to participate in stabilization of the heterochromatin structure, regulation of transcription, and other processes (17, 33, 34). On nuclear envelope breakdown in prophase, these proteins should become dissociated from chromatin and the nuclear lamina to disperse to the ER membrane network (16, 35). In the case of LBR, we have demonstrated that phosphorylation of LBR in the RS-region by cdc2 kinase and an unknown kinase in an M-phase egg extract causes dissociation from chromatin (3). In this study, we analyzed the LEM domain protein-chromatin interaction mechanism, focusing on emerin. Our findings suggested that the binding of emerin to chromatin mediated by BAF in a synthetic egg extract was suppressed by phosphorylation of emerin by one or more kinases in a mitotic egg extract. Our suggested dissociation/association mechanism for the binding of emerin to chromatin may be applicable to other LEM proteins, including MAN1 and LAP2β, because these proteins are known to bind to BAF through a common LEM domain (11, 13). Indeed, in the case of LAP2β, dissociation from chromatin on treatment with a mitotic HeLa cell extract has been reported (4). Dissociation of LBR and LEM proteins from chromatin through these phosphorylation mechanisms may cause the release of the nuclear membrane from chromatin at the onset of mitosis.

It has been shown that emerin dispersed to the ER membrane in prophase accumulates in the “core” region of the chromosome in telophase in HeLa cells (16, 35). In this process, BAF is recruited to the core region faster than emerin, and this recruitment is required for the assembly of emerin (16). This observation and our results suggest that the dephosphorylation of emerin may lead to telophase recruitment of emerin to BAF around the core region of chromatin. Our observations also support this idea: the binding of emerin to chromatin, once suppressed on treatment with MC, is recovered by subsequent treatment with SC (Fig. 2, MC-SC), and the recovery of the binding activity with SC is inhibited by pretreatment of SC with okadaic acid, which is known as a wide spectrum serine/threonine protein phosphatase inhibitor. On the other hand, it is also important to determine what kind of mechanism regulates the BAF-chromatin interaction to understand BAF-mediated interaction of emerin and chromatin, because Haraguchi et al. (16) have indicated that emerin cannot be re-localized to the chromatin surface in late telophase without BAF re-localization.

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