Dissociation of emerin from barrier-to-autointegration factor is regulated through mitotic phosphorylation of emerin in a *Xenopus* egg cell-free system*

Yasuhiro Hirano[a, c], Masashi Segawa[a], Fumiko S Ouchi[b], Yoshio Yamakawa[b], Kazuhiro Furukawa[a], Kunio Takeyasu[c] and Tsuneyoshi Horigome[a, d, e]

[a]*Course of Functional Biology, Graduate School of Science and Technology, Niigata University, Igarashi-2, Niigata 950-2181, Japan;* [b]*Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan;* [c]*Graduate School of Biostudies, Kyoto University, Kitashirakawa-ohiwakecho, Sakyo-ku, Kyoto 606-8205, Japan; and* [d] *Center for Transdiciplinary Research, Niigata University, Igarashi-2, Niigata 950-2181, Japan*

Running title: Dissociation of emerin from BAF on mitotic phosphorylation

[e]To whom correspondence should be addressed. Tsuneyoshi Horigome, Department of Chemistry, Faculty of Science, Niigata University, Igarashi-2, Niigata 950-2181, Japan. Tel.: +81-25-262-6160; Fax: +81-25-262-6160; E-mail: thori@chem.sc.niigata-u.ac.jp.

Emerin is the gene product of *STA*, of which mutations cause Emery-Dreifuss muscular dystrophy. The protein 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 emerin, and this phosphorylation 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, ^{49}Ser, ^{66}Ser, ^{67}Thr, ^{120}Ser and ^{175}Ser, 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 MS/MS sequencing. An *in vitro* binding assay involving an emerin S175A point mutant protein suggested that phosphorylation at ^{175}Ser regulates the dissociation of emerin from BAF.

The nuclear envelope (NE) [1] is a highly dynamic structure that disassembles at the onset of mitosis and reassembles on the surface of chromatin during the telophase in vertebrates. These changes of NE are crucial for cell cycle progression. The NE consists of an outer nuclear membrane (ONM), inner nuclear membrane (INM), nuclear pore complex (NPC), and nuclear lamina. The INM contains integral membrane proteins, *i.e.*, lamin B receptor (LBR), lamina-associated polypeptide-2β (LAP2β), emerin, MAN1 and others, that interact with DNA and/or chromatin, and these proteins are proposed to participate in nuclear membrane targeting to chromatin at an early step of nuclear assembly (1). The interactions between some of the INM proteins and chromatin are regulated through phosphorylation of these INM proteins. The phosphorylation mechanisms for LBR, and LAP2 α and β 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). LAP 2β binds to lamin B1 and chromatin, and cell cycle-dependent phosphorylation of LAP 2β abolishes the binding to them (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 (LAP 2β, Emerin, MAN1) protein family, the members of which have an

about 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 disperse 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 the telophase (15,16). A 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-Dreifuss muscular dystrophy (EDMD) and cannot bind to lamin A, exhibits 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 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 ^{175}Ser phosphorylation is responsible for the dissociation of emerin from BAF.

MATERIALS AND METHODS

Construction of GST-fused emerin fragment proteins and His_6-Tagged BAF- Cloning of the nucleoplasmic region of human emerin (ΔTM, amino acid residues 1-213) was performed by PCR. PCR was carried out with a human testis cDNA library using the following primers: 5'-CGGGATCCCCATGGACAACTAGCAGAT-3' and 5'-CGGGATCCAGAGCACGGTTTTCAGG-3'. The PCR product was digested with BamHI, and then inserted into the pBluescript II SK(-) or pGEX 3X vector (Novagen) at the BamHI site at the 3' end of GST. To generate a point mutant with the serine at position 175 replaced with alanine (S175A-ΔTM), a GeneTailor mutagenesis kit (Invitrogen) was used according to the manufacturer's procedure. PCR was carried out with pBluescript II SK(-)-Emerin ΔTM using the following primers: 5'-CTGTTTCGCCTCCAGGGCCTCCCTGGACC-3' and 5'-CCTGGAGGCTGAAACAGGGCGGTAGTCGT-3', followed by verification by DNA sequence analysis. The pBluescript II SK(-)-S175A-ΔTM was digested with BamHI and the resulting fragment was inserted into the pGEX 5X-3 vector (Novagen) at the BamHI site. To construct the pGEX 3X-Emerin ΔLT plasmid, pBluescript II SK(-)-Emerin ΔTM was digested with BglII and BamHI, and the resulting fragment was inserted into the pGEX 3X vector using the BamHI site in the vector. The cDNA clone of human BAF (Accession No. BC005942) was purchased from Invitrogen. To obtain His-tagged BAF, the coding region of BAF was PCR-amplified using primers 5'-CGGGATCCCGATGACAACCTCCCAAAAGCA-3' and 5'-CGGAATTCATGCAAGAGCGAGAATCC-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 ΔTM and pET28c-BAF were confirmed using an ALF DNA sequencer (Phamacia).

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 ΔTM or GST-emerin ΔLT, 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 *E. coli* extract containing BAF was necessary, it was carried out as follows.

E. coli cells expressing His-tagged BAF were sonicated vigorously and then centrifuged at 12,000 g for 10 min. A 50 µl aliquot of the supernatant was reacted with beads bearing GST-emerin ΔTM in binding buffer (20 mM Tris-HCl (pH 7.6), 134 mM NaCl and 0.1 % Tween-20 either containing 125 µg/ml DNase or not) at 4 ℃ for 3h. The thus treated beads were washed three times with binding buffer and then used for the chromatin binding assay.

Typically, *Xenopus* sperm chromatin, which was demembranated with lysolecithin and subsequently decondensed with heated *Xenopus* egg cytosol, in 20 µl of extraction buffer (10,000 per µl) was added to the thus treated beads suspended in 10 µl of extraction buffer. After incubation at 4 °C for 10 min, the binding reaction was stopped by pipetting 15 µl samples onto glass slides spotted with 12 µl of a fixing solution (extraction buffer containing 3% formaldehyde and 6 µg/ml Hoechst dye 33342). The fixed samples were observed by fluorescence microscopy. One hundred to two hundred beads were observed for every sample and "the percentage of beads with bound chromatin" was determined. This value was used as an index of the affinity of beads bearing emerin fragments and chromatin. The values in the figures are indicated after subtraction of a blank value. The blank value (less than 10 %) was determined in each experiment using Sepharose beads with GST bound instead of GST-emerin fragments. The significance of the pretreatment of beads bearing emerin fragments with various reagents in the chromatin binding assay was evaluated by means of Student's t-test (n=3). In the preceding study, we compared this method and an established *in vitro* binding method involving soluble proteins and chromatin, showing that this bead method gives the same results as the established method (28). Therefore, we used this method to determine the affinity of protein fragments to chromatin in this study.

In vitro binding assay of emerin and BAF - A supernatant containing His-tagged BAF was prepared as described above. Beads bearing approximately 10 µg of ΔTM or S175A-ΔTM preincubated with *Xenopus* cytosol or an extraction buffer were washed twice with binding buffer (20 mM Tris-HCl (pH7.6), 134 mM NaCl and 0.1 % Tween 20), and then incubated with BAF-expressed *E. coli* extract at 4 ℃ for 3 h. The beads were washed three times with binding buffer. The thus obtained sample was subjected to SDS-PAGE and then transferred to a nitrocellulose membrane. BAF bound to beads was detected with anti –His-tag antibody and chemical luminescence. Emerin phosphorylated with a *Xenopus* egg cytosol fraction was separated by 10 % gel SDS-PAGE and the emerin band was excised. Phosphorylation of the protein was detected with ProQ diamond stain (Molecular Probes) according to the manufacturer's instructions and a reference (29).

In vitro dissociation assay of emerin and BAF- Beads bearing approximately 10 µg of ΔTM were pretreated with a supernatant containing His-tagged BAF as described above. After three times wash, thus treated beads were treated with buffer or cell cycle dependent *Xenopus* egg cytosol fraction at 23℃ for 1h. The beads were washed, subjected to SDS-PAGE and transferred to a nitrocellulose membrane. BAF bound to beads was detected as described above.

Phosphorylation assay of emerin fragments with a Xenopus egg cytosol fraction- Approximately 3 µg of GST-fused emerin bound to glutathione-Sepharose beads was incubated with S-phase and M-phase egg cytosol fractions containing 1 µCi [γ-^{32}P]ATP at 23 ℃ for 1 h. After washing twice with phosphate buffer saline containing 0.05 % Tween 20 (PBS-T), the beads were supplemented with 2 mM ATP and then incubated at 4 ℃ for 10 min. The thus treated proteins 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 ΔTM bands were excised from the gel and the phosphorylated emerin ΔTM was quantified by scintillation counting.

Phosphopeptide mapping- Approximately 30 µg of emerin ΔTM or ΔLT was phosphorylated as described above, separated by SDS-PAGE and then transferred to a nitrocellulose sheet. The full length emerin band was excised, soaked in 0.5% poly(vinyl pyrrolidone) K-30 in 100 mM acetic acid for 30 min at 37 ℃ and then washed extensively with water. The protein was digested with trypsin or chymotrypsin in 50 mM NH_4HCO_3 for 16 h at 37 ℃. The released peptides were dried, dissolved in water and then loaded onto a cellulose TLC plate (Funacell;

Funakoshi Co., Tokyo). Electrophoresis, in the first dimension, was performed at pH 8.9 (1 % ammonium carbonate) for 20 min at 1,000 V, and ascending chromatography, in the second dimension, was performed with a solvent system of 37.5 % 1-butanol, 25 % pyridine and 7.5 % acetic acid in water (v/v). The dried plate was exposed to Fuji X-ray film.

Separation of phosphopeptides derived from emerin ΔTM treated with mitotic cytosol using a Titansphere column – This experiment was carried out according to the method of *Kuroda et al.* with some modification (30). Beads bearing approximately 100 μg of GST-fused emerin ΔTM were treated with a *Xenopus* egg M-phase cytosol fraction as described above except that [γ-^{32}P]ATP was omitted. The thus treated beads were separated by 10 % gel SDS-PAGE and visualized by CBB R-250 staining. Emerin ΔTM bands were excised from the gel and then in-gel digested with chymotrypsin or trypsin in 50 mM NH_4HCO_3 at 37 ℃ for 16 h. The thus 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 I.D. ×10 mm column; GL Science Co., Japan) equilibrated with solvent A at the 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 (pH8.0)) at the flow rate of 0.5 ml/min for 15 min. The thus eluted phosphopeptides were directly applied to a reversed-phase silica-base CAPCELL PACK C8 column (4.6 mm I.D. ×150 mm column; Shiseido, Japan), briefly washed with solvent A and then eluted with a 90 min linear gradient, 0 to 45 %, of solvent B (acetonitrile containing 0.0886 % (v/v) TFA) at the flow rate of 1 ml/min. The thus isolated phosphopeptides were analyzed with an AXIMA-CFR MALDI-TOF MS (Shimadzu Co., Japan) using CHCA as a matrix.

Dephosphorylation of phosphopeptides- Dephosphorylation of phosphopeptides derived from M-phase cytosol-treated emerin ΔTM was carried out according to the method of Kuyama *et al.* (31). The phosphopeptides were dried, dissolved in 40 μl of 46 % hydrofluoric acid (HF; Wako, Japan), and then incubated at room temperature for 1.5 h. The thus treated peptides were dried, dissolved in 1 μl of 40 % acetonitrile containing 0.1 % (v/v) TFA and then analyzed with an AXIMA-CFR MALDI-TOF MS.

MS/MS sequencing – Phosphopeptides, which were separated with the Titansphere and C8 columns, were applied to an Inertsil ODS column (0.2 mm I.D. ×50 mm; GL Science Co., Japan) equilibrated with solvent D (2 % (v/v) acetonitril containing 0.1 % (v/v) formic acid) and then eluted with a 20 min linear gradient, from 5 to 55 % a solvent E (98 % (v/v) acetonitrile containing 0.1 % (v/v) formic acid) at the flow rate of 1.5 μl/min using a MAGIC 2002 system (Michrome BioResources, Inc). The thus eluted phosphopeptides were directly introduced to ESI-IT MS, LCQ Deca XP (Thermo Electron, San Jose, CA, USA), equipped with a nanospray interface (AMR Inc, Japan) and a metal nanosprayer (GL Science Co., Japan). To obtain sequence information on the eluting phosphopeptides, the mass spectrometer was operated in the ion select mode, where MS scan was followed by the MS/MS scans of the calculated mass of the phosphopeptide as parent mass.

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 almost all LEM and transmembrane domains. GST-fused emerin ΔTM (ΔTM) and GST-fused emerin ΔLT (ΔLT) were purified form *E.Coli* extract using a GSH-Sepharose bead (Fig.1B). Western blotting with anti-GST antibody of ΔTM and ΔLT preparations that purified by GSH-Sepharose showed that smaller protein 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 bead 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 a little (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 columns 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 thus expressed emerin fragment 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 and ii) the stimulation was not observed for ΔLT, which lacks the BAF binding domain (mentioned below in more detail). On the other hand, suppression of the binding of SC-treated ΔTM to chromatin on subsequent treatment with MC should be caused by the phosphorylation of emerin because the suppression was prevented by apyrase or a kinase inhibitor, *i.e.,* staurosporine (columns 8-11 in Fig. 2).

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-tagged BAF. Thus treated beads were used for the chromatin binding assay (Fig. 3). ΔTM treated with the *E coli* extract containing His-tagged BAF (Fig.3A, lane 2) bound to chromatin, although beads treated with buffer or the blank *E. coli* extract (Fig.3A, lane 1 or 3, respectively) could not bind to chromatin. The binding of His-tagged BAF to the beads bearing ΔTM in this assay system was confirmed by Western blotting with an anti His-tag antibody (Fig. 3B). These results clearly show that the binding of beads bearing ΔTM to chromatin is mediated by BAF and also support our idea that the stimulation of the binding of beads bearing ΔTM to chromatin on pretreatment with SC is mediated by the binding of BAF to emerin.

M-phase specific phosphorylation of emerin ΔTM suppressed the binding to BAF – We examined 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-tagged BAF to bind His-tagged BAF to ΔTM. Thus treated beads were further treated with buffer or cell cycle dependent *Xenopus* egg extract (Fig. 4A). In the cases of the buffer- and an SC-treatment, the His-tagged BAF bound to ΔTM was remained on beads (Fig. 4A, buffer and SC). However, His-tagged BAF bound to ΔTM was 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-tagged BAF, and 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 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 above 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 (Fig. 4B, ProQ stain. Compare 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 band- shift on SDS-PAGE of emerin on treatment with MC (Fig. 4C, MC and SC-MC) was consistent with 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. A part of phosphorylation of ΔTM may be caused by staurosporine insensitive kinase(s) because 5μM staurosporine could not completely inhibit (Fig. 4C, ProQ stain, MC+Sta.). 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 abolished this suppression activity (Fig. 4B and C, MC+Apy. and MC+Sta.). Therefore, we concluded that the binding of emerin to BAF is suppressed by M-phase specific phosphorylation. Thereafter, we focused on analysis of the cell cycle-dependent phosphorylation of emerin, especially mitotic phophorylation 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). Beads bearing ΔTM was treated with egg cytosol fractions containing [γ-^{32}P] ATP and the thus treated proteins were analyzed by SDS-PAGE. The gel was stained with CBB and then subjected to autoradiography. The ΔTM was strongly phosphorylated on treatment with MC, although the phosphorylation by SC was very low (Fig. 5A, *Autoradiography*). (9)A mitotic phase-specific band shift was also observed for ΔLT treated with MC (Fig. 5A, ΔLT). These results show that a phosphorylation site(s) that is outside of the LEM domain caused the main band shift because ΔLT lacking the almost all LEM domain showed a clear band shift on treatment with MC (Fig. 5A, ΔLT lanes). To compare the amounts of incorporated phosphate groups, ΔTM treated with SC or MC in the presence of [γ-^{32}P]ATP was electrophoresed, and ΔTM bands were excised from the gel and counted (Fig. 5B). The ΔTM 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 what differences are there in the phosphorylation site(s) between ΔTM and ΔLT, we performed phosphopeptide mapping (Fig. 6). Beads bearing ΔTM and ΔLT treated with SC or MC in the presence of [γ-^{32}P]ATP. After wash, thus treated beads bearing ΔTM and ΔLT were subjected to SDS-PAGE and transferred to nitrocellulose membrane. Then, the full-length emerin bands were excised from membrane and digested with trypsin (Fig. 6A) or chymotrypsin (Fig. 6B). The generated peptides were subjected to two-dimensional separation on a cellulose plate. Although many spots of phosphorylated peptides were observed for the preparations derived from both ΔTM and ΔLT treated with MC, the major spots indicated with arrows in Fig. 6 were not observed for ΔLT. When ^{32}P-labeled ΔTM and ΔLT were digested with chymotrypsin, well-focused patterns were obtained (Fig. 6B). In these patterns, major spots 1 and 2 and three weak spots,8, 10 and 11, were absent in ΔLT map (compare Fig. 6B ΔTM and ΔLT). 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, 3) BAF maybe bind an emerin kinase and recruit it to ΔTM, and then its kinase phosphorylates ΔTM. However, BAF cannot recruit the kinase to ΔLT because ΔLT lacks the BAF binding domain. These three possibilities discuss in ”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, 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 tryptic phosphopeptides derived from phosphorylated ΔTM were bound to the Titansphere column and then eluted with 0.5 M phosphate buffer (pH 8.0). The thus obtained phosphopeptide fraction was directly introduced onto the reverse phase C8 column, eluted with a linear gradient and then fractionated (Fig. 7A). The molecular masses of the thus obtained peptides were determined by MALDI-TOF MS. Some of the determined masses were consistent with the calculated phosphopeptide masses (Fig. 7B, HF(-), and Table 1). 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, respectively (Fig. 7B and Table 1). As can be seen in Table 1, 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 ($(M+1H)^+$ and $(M+2H)^{2+}$ of the 1367.7 Da peptide, respectively) allowed localization of the phosphorylation site to residue ^{175}Ser by b and y ion series. Because we could detect the b_7 ion (RPVSASR) without a phosphate group on product ion m/z 1367.7 sequencing and the b_8 (RPVSASRS) ion, which includes a phosphate group, and the y_4 ion (SLDL) 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, ^{175}Ser or ^{176}Ser, was phosphorylated in 2080.0 Da peptide. However, we focused on only ^{175}Ser, because ^{175}Ser but not ^{176}Ser 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 (^{49}Ser, ^{66}Ser, ^{67}Thr and ^{120}Ser) were also determined (Table 1). However, we could not completely exclude the possibility that another site in the 2396.8 Da peptide was phosphorylated, that is, ^{52}Ser, ^{53}Ser or ^{54}Ser was possibly phosphorylated. In the case of the 4109.6 Da peptide, we could not determine the phosphorylated residue, although candidates include either ^{123}Ser, ^{141}Ser, ^{142}Ser or ^{143}Ser. In the cases of the 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 phospho-tyrosine by phospho-amino acid analysis (data not shown). Finally, we identified five phosphorylation sites, ^{49}Ser, ^{66}Ser, ^{67}Thr, ^{120}Ser and ^{175}Ser. These phosphorylation sites matched the consensus sequences of well-known kinases *i.e.*, ^{49}Ser: protein kinase A (PKA), calmodulin-dependent kinase II (CaMK II), and glycogen synthesis kinase 3β (GSK 3β); ^{66}Ser: GSK3β; and ^{175}Ser: GSK3β.

^{175}Ser phosphorylation of emerin by MC responsible for the emerin-BAF dissociation — Although five mitotic phosphorylation sites of emerin were identified 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 miner 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 ^{175}Ser (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 ^{175}Ser, 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 ^{175}Ser 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 ^{175}Ser-containing peptide (Fig. 8A, S175A). Furthermore, we performed phosphopeptide mapping to confirm that the ^{175}Ser 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). These results suggested that the LEM domain is necessary for the ^{175}Ser phosphorylation by MC. Then, we carried out an *in vitro* binding assay using S175A-ΔTM and BAF to determine whether the ^{175}Ser 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 ^{175}Ser 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.* indicated that the interaction of emerin and actin is increased by dephosphorylation of emerin (20), 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* at least five specific residues, four serine and one threonine residues; ^{49}Ser, ^{66}Ser, ^{67}Thr, ^{120}Ser and ^{175}Ser (Table 1). These phosphorylation sites are interestingly located at the binding region for many emerin-binding proteins: ^{49}Ser, ^{66}Ser and ^{67}Thr for GCL, YT521-B and Btf (23-25); ^{120}Ser for lamin A and actin (12,21); and ^{175}Ser 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 ^{120}Ser and/or ^{175}Ser 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 indicated that the dissociation of emerin from BAF takes place through mitotic phosphorylation of emerin in a *Xenopus* cell-free system. Unfortunately, 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 ^{175}Ser, 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 residues 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 ^{175}Ser on MC treatment may induce a conformational change of the BAF binding surface because the region around ^{175}Ser has been predicted to be a flexible region that consists of a poly-Ser cluster (32). This conformational change might cause the BAF dissociation.

The lack of ^{175}Ser phosphorylation of ΔLT by MC may be explained as following three hypotheses: First, removal of the LEM domain from ΔTM may cause a conformational change around ^{175}Ser and thus phosphorylation may be blocked. Second, a kinase may bind to the LEM domain and then phosphorylate ^{175}Ser of ΔTM in which case ΔLT lacking the LEM domain may not be phosphorylated at ^{175}Ser because the kinase cannot be recruited. Last, BAF maybe recruits kinase(s) that phosphorylate ^{175}Ser of emerin. To exclude this possibility, we performed the emerin phosphorylation assay using BAF-binding proteins depleted MC. BAF-binding proteins were depleted from MC by the incubation of the Ni-NTA agarose beads bearing His-BAF, and then beads bearing ΔTM was phosphorylated by thus treated MC in present of [γ-^{32}P]ATP. However, we could not detect the difference of phosphorylation level

between non-depleted MC treatment and BAF-binding proteins depleted MC treatment (data not shown). Therefore, we should further study ^{175}Ser phosphorylation mechanism from viewpoint of emerin.

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 the 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 a kinase(s) 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 participate in 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 the prophase accumulates in the 'core' region of the chromosome in the 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 participate in the telophase recruitment of emerin to BAF around the "core" region of chromatin. Our unpublished observation[2] also support this idea: the binding of emerin to chromatin, once suppressed on treatment with MC, is recovered by subsequent treatment with 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, since Haraguchi *et al.* have indicated that emerin cannot be re-localized to the chromatin surface in the late telophase without BAF re-localization (16).

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FOOTNOTES

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[1]The abbreviations used are: GST, glutathione-S-transferase; ΔTM, GST-fused fragment comprising amino acid residues 1-213 of human emerin; ΔLT, GST-fused fragment comprising amino acid

residues 37-213 of human emerin; 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, ER; endoplasmic reticulum, ESI-IT MS; electrospray ionization-ion trap mass spectrometry, CHCA; α-cyano-4-hydroxycinnamic acid, TFA; trifluoroacetic acid, HF; hydrofluoric acid

[2]Hirano Y., Segawa M., Furukawa K. and Horigome T. Unpublished observation

LEGENDS FOR FIGURES

Fig. 1. N-Terminal fragments of emerin expressed as GST-fusion proteins – (A) Schematic diagram of N-terminal fragments of emerin expressed as GST fusion proteins. The binding sites for emerin-binding proteins are shown as reported previously (upper, 11-13, 17-25). Slashed boxes indicate the hydrophobic amino acid rich region, which was demonstrated by Wolff *et al.* (31). Two GST-fused emerin fragments were constructed and used in this study (middle and lower). (B) SDS-PAGE of GST fusion proteins. GST-emerin ΔTM (ΔTM) and GST-emerin ΔLT (ΔLT) were expressed in *E. coli* and then purified with glutathione-Sepharose bead. Beads bearing ΔTM (1), ΔLT (2), or marker proteins (M) were analyzed by SDS-PAGE on a 10 % gel and then stained with CBB. The bands indicated asterisk at the right are GST- containing degradation products of ΔTM and ΔLT (data not shown). The values at the left are the relative molecular masses of the marker proteins.

Fig. 2. The suppression of the binding of emerin to chromatin is caused by mitotic phosphorylation of emerin –Beads bearing ΔTM (about 6 μg) were used, and the beads for columns 4, 9 and 10 were pretreated with a synthetic phase cytosol (columns 4, 10, 11), a mitotic phase cytosol (column 5) or buffer (columns 1-3, 6-9) at 23 ℃ 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 mU apyrase or 5 μM staurosporine (columns 6 and 7; SC+Apy. and SC+Sta., respectively) or MC pretreated with 8 mU apyrase or 5 μM staurosporine (columns 8-11; MC+Apy., MC+Sta., SC-MC+Apy., and SC-MC+Sta, respectively) at 23 ℃ for 20 min. Thus treated beads were incubated with 20,000 decondensed sperm chromatin at 4 ℃ 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 (about 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. * indicates a significant difference from the respective control ($P<0.05$).

Fig. 3. The binding of emerin to chromatin is caused by BAF – (A) *In vitro* chromatin binding assay involving ΔTM treated with BAF. Beads bearing ΔTM (about 10 μg) were treated with buffer (buffer), an *E. coli* soluble fraction containing His-tagged BAF (BAF (+) *E.coli* extract), or a blank *E coli* soluble fraction expressing a His-tagged insoluble protein (BAF (-) *E.coli* extract) for 3h. 25,000 decondensed sperm chromatin was added to thus treated beads, 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. * indicates 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 and then incubated with anti-His antibody. Bound antibodies were detected as enhanced chemiluminescence.

Fig. 4. Phosphorylation of emerin by M-phase cytosol fraction caused dissociation of emerin from BAF – (A) BAF dissociate from emerin by MC treatment. Beads bearing ΔTM (about 10 μg) were pretreated with *E. coli* extract containing His-tagged BAF at 4 ℃ for 3h. 10% of loading amount of His-BAF in the reaction was detected with anti His-tag antibody (Load). After wash, thus treated

beads were treated with buffer (Buffer) or cell cycle dependent *Xenopus* egg cytosol fraction (SC, MC) at 23 ℃ for 1h. Then, thus treated beads were subjected to 12 % gel SDS-PAGE, transferred to a nitrocellulose membrane and then incubated with anti His-tag antibody. Bound antibodies were detected as enhanced chemiluminescence. The values at the right are the relative molecular mass of the marker protein. (B) The binding of BAF to emerin was suppressed by mitotic phosphorylation of emerin. Beads bearing ΔTM (about 10μg) 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+Apy.), or MC pretreated with staurosporine (MC+Sta.), respectively. BAF that bound to beads were detected anti His-tag antibody as in (A). The values at the right are the relative molecular mass of the marker protein. (C) The phosphorylation states of emerin. Beads bearing ΔTM treated as in (B) were separated by 10 % gel SDS-PAGE and then stained with ProQ diamond to confirm the phosphorylation states of emerin (ProQ stain). Total emerin protein was stained by CBB (CBB stain). The values at the right are the relative molecular mass of the marker protein.

Fig. 5. Emerin is strongly phosphorylated in the M-phase - (A) Detection of phosphorylation. Beads bearing 3 μg of emerin ΔTM or ΔLT pretreated with SC (SC-MC) ornot That beads were incubated with 20 μl of a synthetic (SC) or mitotic (MC and SC-MC) phase egg cytosol fraction containing 1μCi [γ-^{32}P]ATP at 23 ℃ for 1 h. Thus treated beads were subjected to SDS-PAGE, followed by CBB staining and autoradiography. Arrows, arrowheads and double arrowheads indicate ΔTM, the mitotic-specific band shift of ΔTM and emerin ΔLT bands, respectively. The values at the right are the relative molecular masses of the marker proteins. (B) The ΔTM bands in A were excised from the gel, and then the levels of phosphate groups incorporated into ΔTM were compared by scintillation counting. The results are shown as means ± S.D. for three independent experiments.

Fig. 6. Tryptic and chymotryptic phosphopeptide maps of ΔTM and ΔLT - Beads bearing about 30 μg of ΔTM or ΔLT were incubated with 20 μl of SC or MC containing 20 μCi [γ-^{32}P]ATP at 23 ℃ for 1 h. The thus treated proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The ΔTM or ΔLT bands were excised and digested with trypsin (A) or chymotrypsin (B). The thus obtained phosphopeptides were separated by electrophoresis at pH 8.9 (horizontal direction; cathode to the left) and by ascending chromatography. Excised bands contained 1 kcpm, 6 kcpm, 0.6 kcpm, 4 kcpm, 6 kcpm and 4 kcpm for 6A-SC-ΔTM, 6A-MC-ΔTM, 6A-SC-ΔLT, 6A-MC-ΔLT, 6B-MC-ΔTM, and 6B-MC-ΔLT, respectively. The points of sample application can be seen as dots near the bottom-left corners. The arrows indicate major spots lacking for ΔLT treated with MC.

Fig. 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-^{32}P]ATP was chymotrypsinized or trypsinized. The thus obtained peptides were applied to a Titansphere column and eluted with 0.5 M phosphate buffer (pH 8.0). The retained chymotryptic (upper panel) or tryptic (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 base line 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 RT for 1.5 h. The thus treated peptides were dried again and then analyzed by MALDI-TOF MS. A values shown in the figures indicate the monoisotopic peptide masses. (C) Identification of phosphorylation sites. A mass spectrum of the 1367.7 (((M+1H)$^{1+}$, left) and 685.1 (M+2H)$^{2+}$, 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 the b and y ion ones. The peptide sequence is RPVSASRSSLDL. In the spectrum, the b_8 ion still has the phosphate moiety, but the b_7 and y_4 ones do not. This indicates that the phosphate moiety must be located at ^{175}Ser, indicated by an asterisk.

Fig. 8. ^{175}Ser phosphorylation by MC participates in emerin-BAF dissociation (A) The

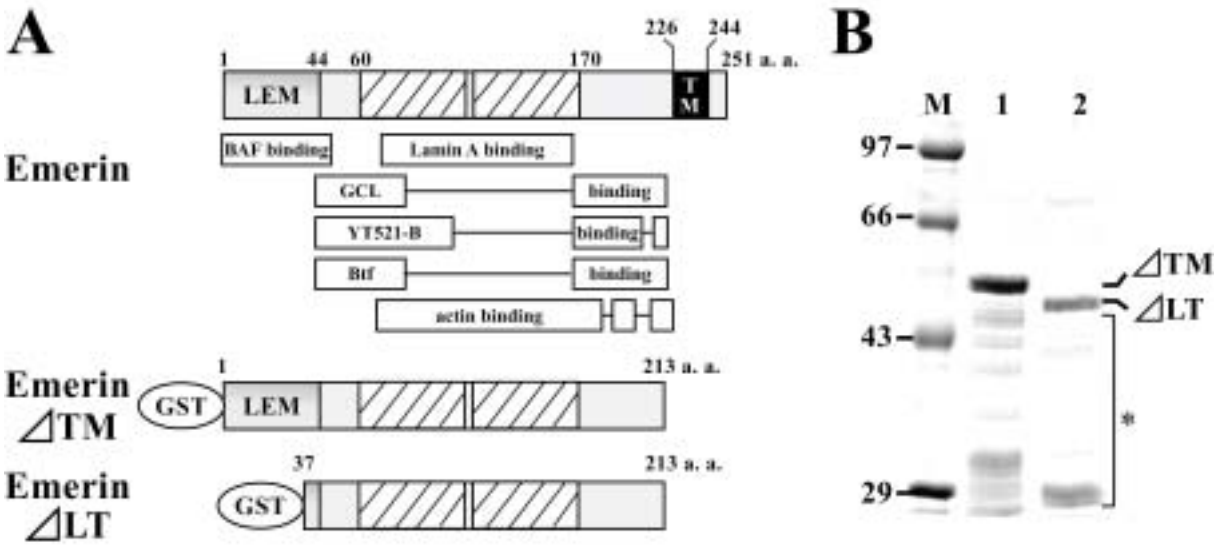
separation pattern of phosphopeptides derived from ΔLT and S175A-ΔTM. Approximately 100 μg of ΔTM, ΔLT or S175A-ΔTM treated with MC as in Fig. 7 was chymotrypsinized. The thus obtained peptides were applied to a phosphopeptide separation system as in Fig. 7. The peak indicated by the arrow coincides with peak 1 in Fig. 7. (B) Beads bearing about 30 μg of ΔTM, ΔLT or S175A-ΔTM were incubated with 20 μl of SC or MC containing 10 μCi [γ-^{32}P]ATP at 23 ℃ for 1 h. The thus treated proteins were separated by SDS-PAGE, transferred to a PVDF membrane and then chymotrypsinized. The thus obtained phosphopeptides were separated by electrophoresis at pH 8.9 (horizontal direction; cathode to the left) and by ascending chromatography, as in Fig. 6. The points of sample application can be seen as dots near the bottom-left corners. (C) S175A-ΔTM prevented the mitotic dissociation of BAF. Beads bearing ΔTM or S175A-ΔTM (about 10μg) were treated with MC at 23 ℃ for 1 h. The thus treated beads were incubated with an *E. coli* extract expressing His-tagged BAF, separated by 12% gel SDS-PAGE and then transferred to a PVDF membrane. BAF that bound to the beads was detected with Anti-His antibody as in Fig. 4.

Table 1. Phosphopeptides detected by ESI-IT MS. This table comprises data obtained in the MALDI-TOF MS and ESI-IT MS experiments shown in Fig. 7. Phosphoamino acids are given in bold and italicized letters. pyE: pyroglutamic acid (considered as an artificial modification probably arising during sample preparation). 1P and 2P indicate the number of phosphate group.

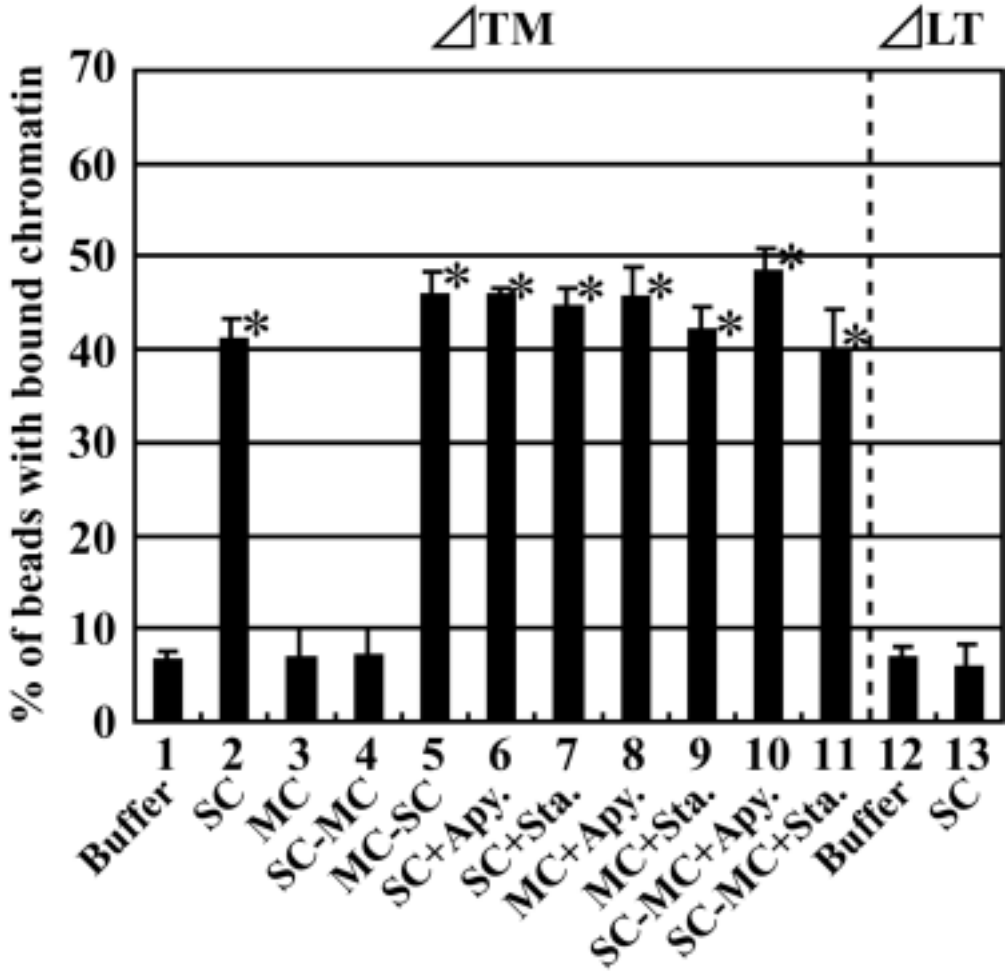
Peptide (m/z)	Phosphopeptide mass (Da)	Peak number	Sequence (residue)	Phosphorylated residue
2080.2	2080.0 (1P)	1	pyESITHYRPVSASR***SS***LDL (162-179)	^{175}Ser or ^{176}Ser
1367.7	1367.7 (1P)	2	RPVSASR***S***SLDL (168-179)	^{175}Ser
1758.5	1758.7 (1P)	3	SFSDLN***ST***RGDADMY (60-74)	^{66}Ser, ^{67}Thr (mixture)
1847.0	1846.9(1P)	4	RAVRQ***S***VTSFPDADAF (115-130)	^{120}Ser
2739.9	2738.2(2P)	5	GEPESAGPSRAVRQSVTSFPDADAF (106-130)	ND*
2658.2	2658.2 (1P)	6	GEPESAGPSRAVRQSVTSFPDADAF (106-130)	ND*
2396.8	2397.0 (1P)	7	RL***S***PPSSSAASSYSFSDLNSTR (47-68)	^{49}Ser (possibly also^{52}Ser, ^{53}Ser or ^{54}Ser)
4109.6	4108.8 (1P)	8	AVRQSVTSFPDADAFHHQVHDDD LLSSSEEECKDR (116-150)	ND*

* ND, not determined.

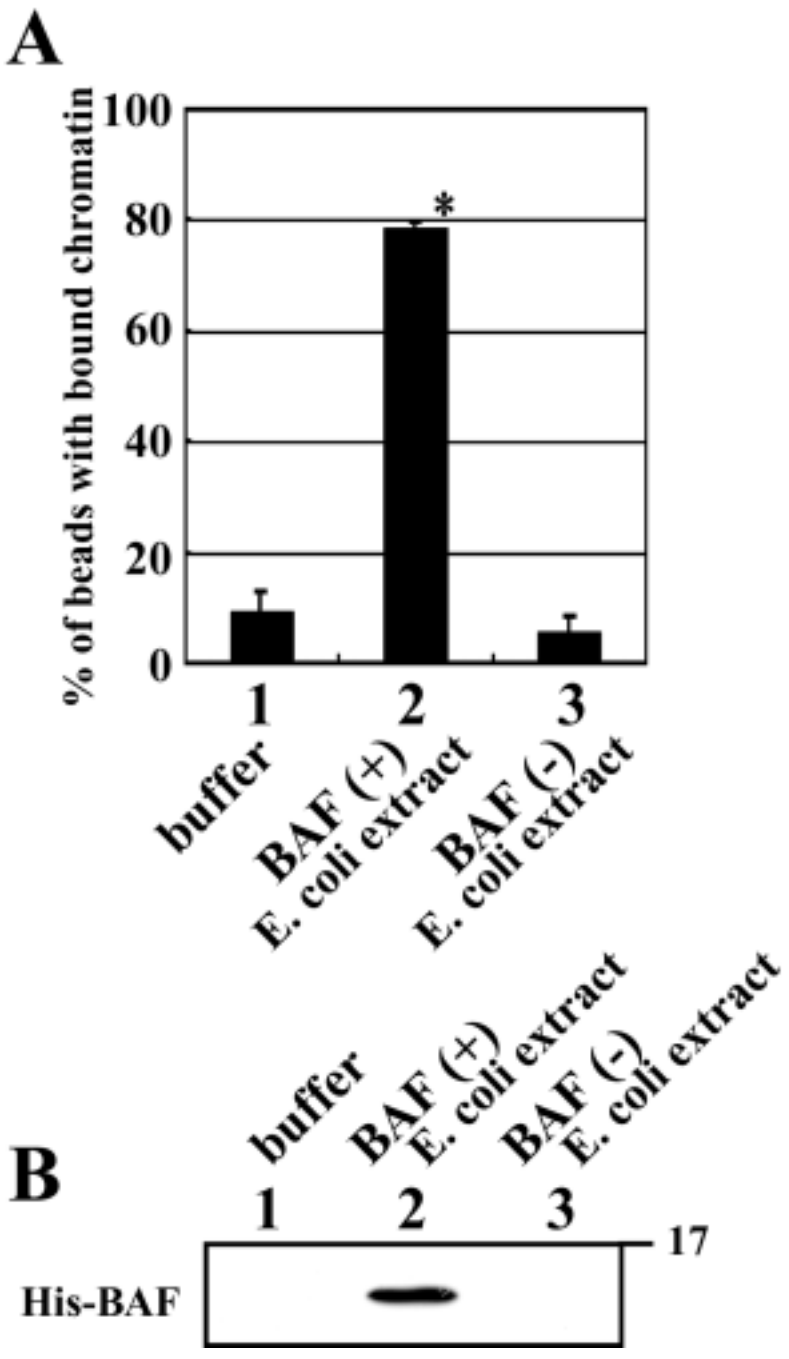
(Fig. 1.)


A
1
44
60
170
226
244
251 a. a.
LEM
T
M
Emerin
BAF binding
Lamin A binding
GCL
binding
YT521-B
binding
Btf
binding
actin binding
Emerin
⊿TM
1
213 a. a.
GST
LEM
Emerin
⊿LT
37
213 a. a.
GST
B
M
1
2
97
66
43
29
⊿TM
⊿LT
*

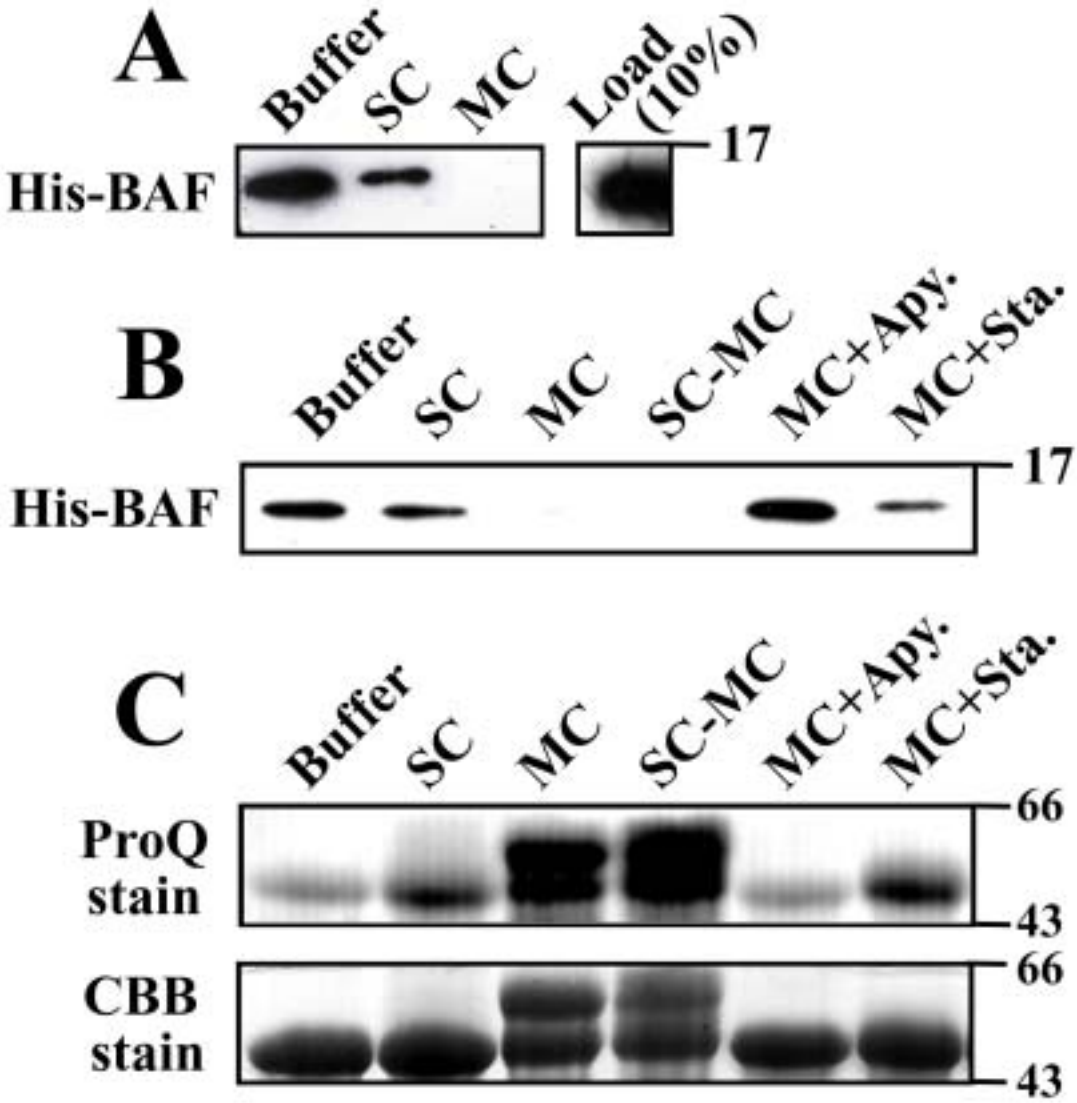
(Fig. 2.)


⊿TM
⊿LT
% of beads with bound chromatin
70
60
50
40
30
20
10
0
1 Buffer
2 SC
3 MC
4 SC-MC
5 MC-SC
6 SC+Apy.
7 SC+Sta.
8 MC+Apy.
9 MC+Sta.
10 SC-MC+Apy.
11 SC-MC+Sta.
12 Buffer
13 SC

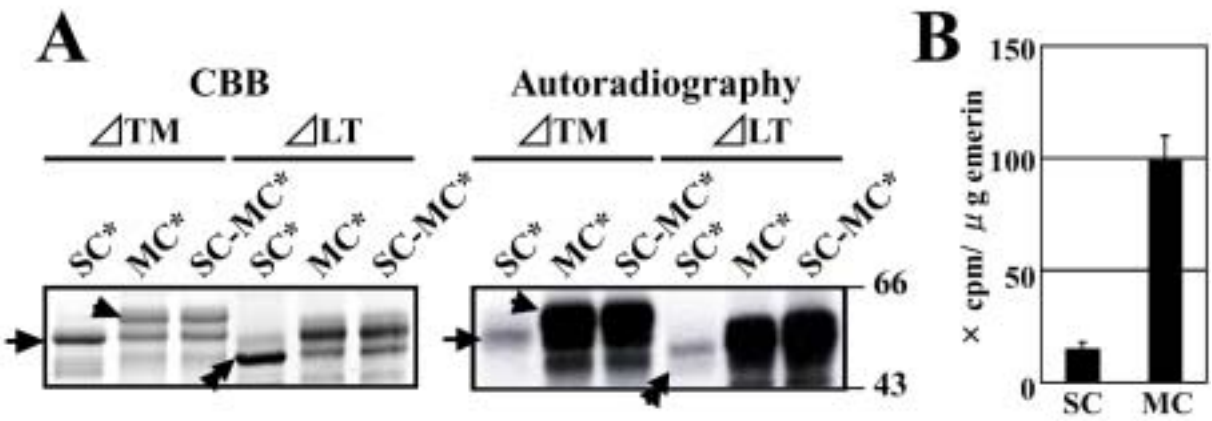
(Fig. 3.)


A
% of beads with bound chromatin
100
80
60
40
20
0
*
1
2
3
buffer
BAF (+) E. coli extract
BAF (-) E. coli extract
B
buffer
BAF (+) E. coli extract
BAF (-) E. coli extract
1
2
3
17
His-BAF

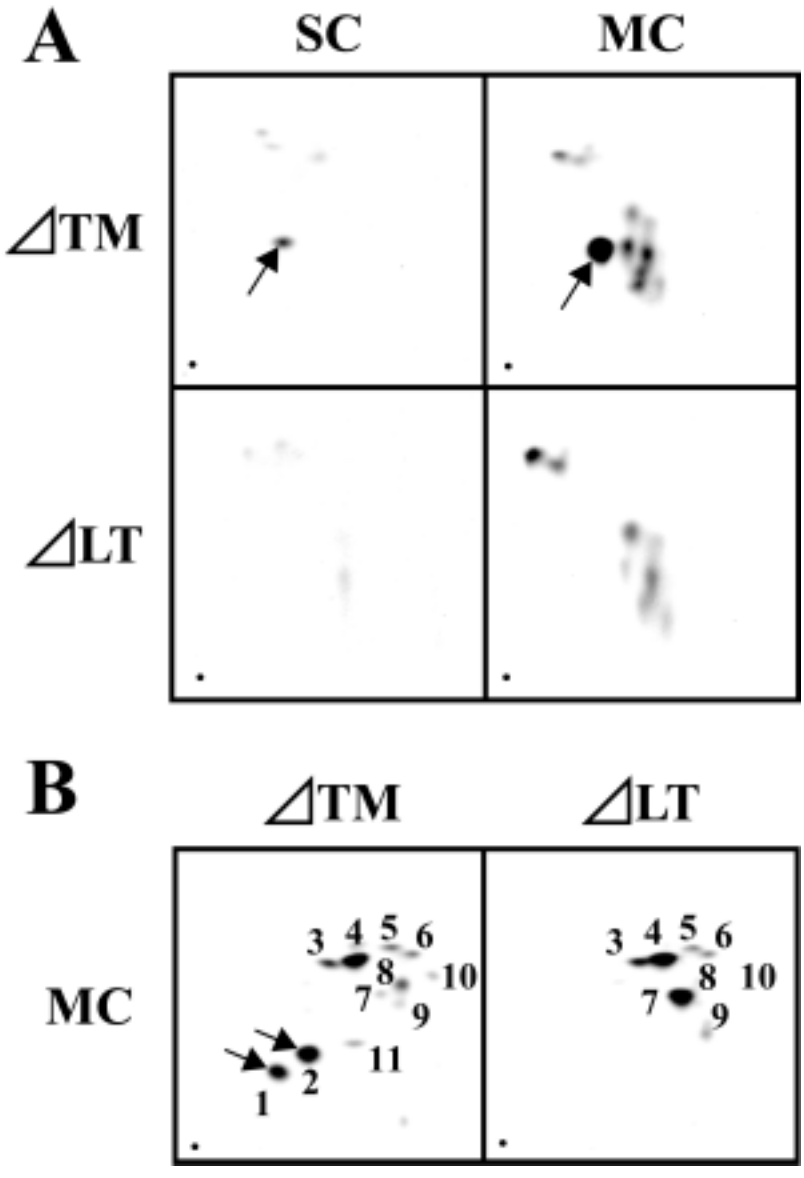
(Fig. 4.)


A
Buffer
SC
MC
Load (10%)
17
His-BAF
B
Buffer
SC
MC
SC-MC
MC+Apy.
MC+Sta.
17
His-BAF
C
Buffer
SC
MC
SC-MC
MC+Apy.
MC+Sta.
66
43
ProQ stain
66
43
CBB stain

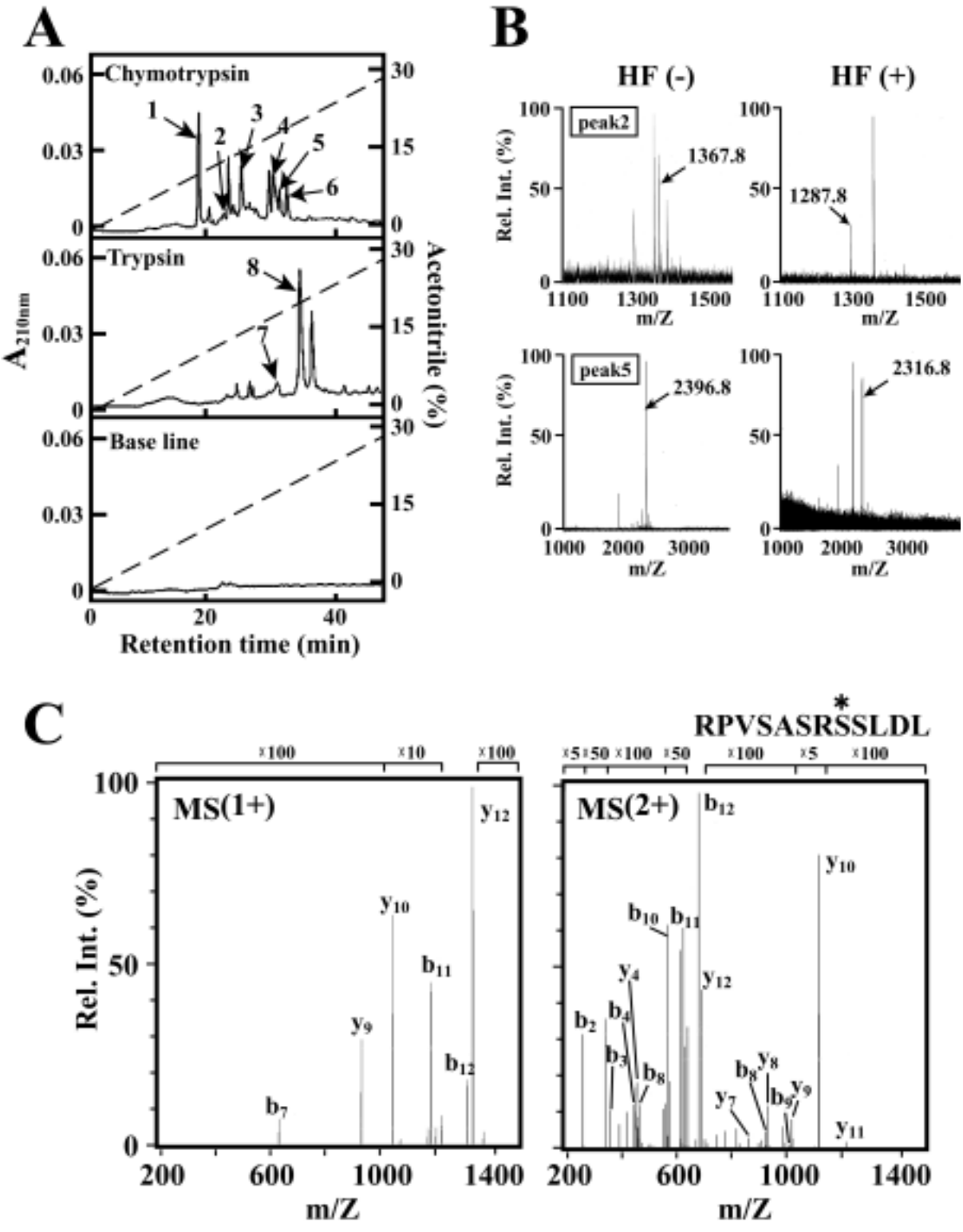
(Fig. 5.)


A
CBB
Autoradiography
⊿TM
⊿LT
⊿TM
⊿LT
SC*
MC*
SC-MC*
SC*
MC*
SC-MC*
SC*
MC*
SC-MC*
SC*
MC*
SC-MC*
66
43
B
150
100
50
0
× cpm/ μg emerin
SC
MC

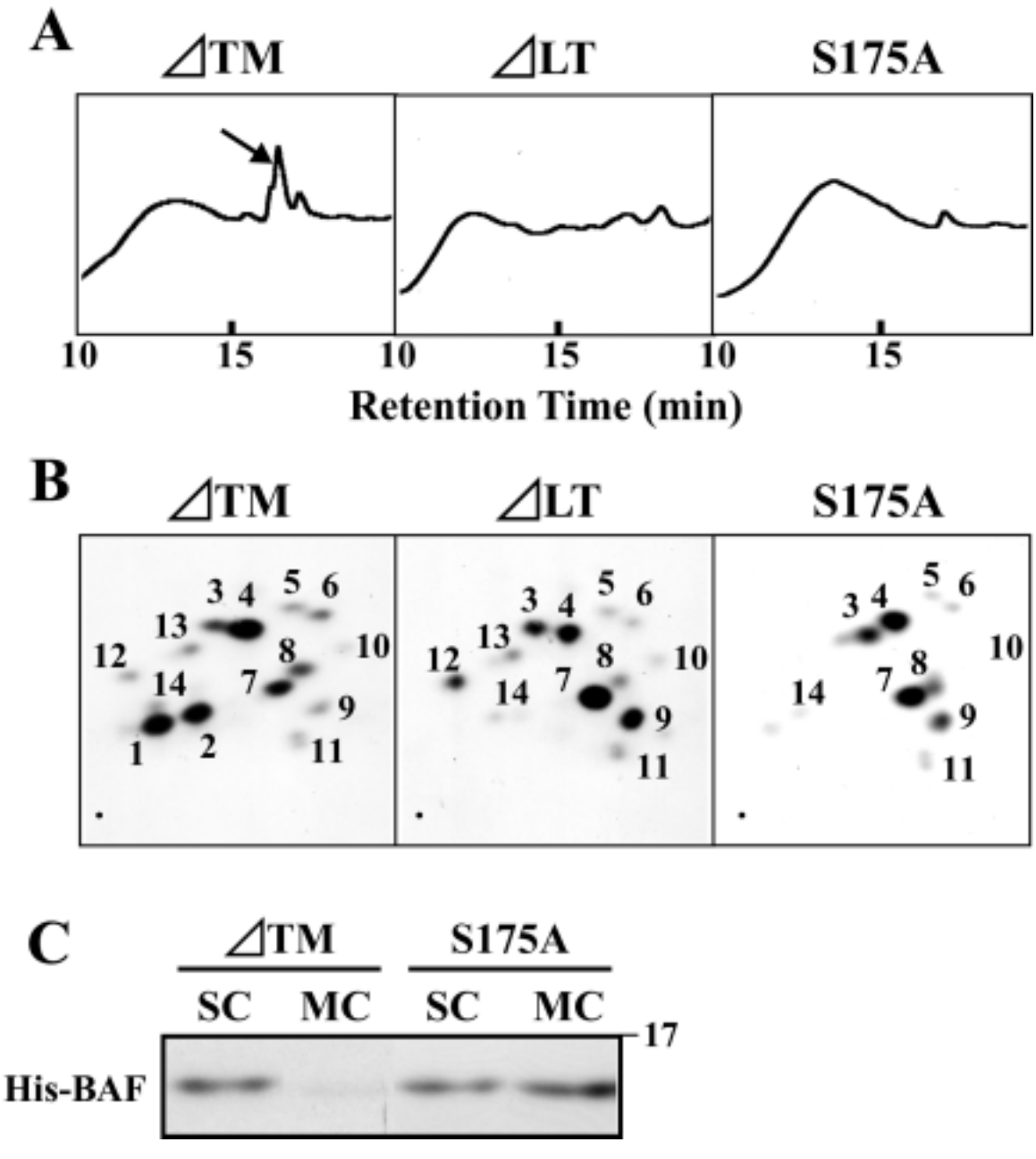
(Fig. 6.)



(Fig. 7.)


A
Chymotrypsin
Trypsin
Base line
1
2
3
4
5
6
7
8
A210nm
Acetonitrile (%)
Retention time (min)
B
HF (-)
HF (+)
peak2
1367.8
1287.8
peak5
2396.8
2316.8
Rel. Int. (%)
m/Z
C
RPVSASRSSLDL
MS(1+)
MS(2+)
Rel. Int. (%)
m/Z

(Fig. 8.)


A
⊿TM
⊿LT
S175A
10
15
10
15
10
15
Retention Time (min)
B
⊿TM
⊿LT
S175A
1
2
3
4
5
6
7
8
9
10
11
12
13
14
C
⊿TM
S175A
SC
MC
SC
MC
17
His-BAF