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Biochemical and Biophysical Research Communications 331 (2005) 247–252
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Protein kinase CK2 interacts with Chk2 and phosphorylates Mre11 on serine 649 ☆

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Received 21 March 2005
Available online 2 April 2005

Abstract

The Mre11–Rad50–Nbs1 protein complex has been known to be involved in a variety of DNA metabolic events that involve DNA double-strand breaks (DSBs). The phosphorylation of Mre11 is increased in response to ionizing radiation, which suggests that phosphorylation of Mre11 may be an important regulatory mechanism of this complex. Mre11-phosphorylating kinase activities were observed in Chk2 immunoprecipitates and HeLa nuclear extracts. Through the tandem affinity tagging system and conventional chromatography, this kinase was purified and identified as protein kinase CK2. CK2 phosphorylates Mre11 in vitro. In vitro kinase assay with a series of truncated Mre11 proteins as substrates for CK2 and site-directed mutagenesis showed that serine 649 of Mre11 is mainly phosphorylated by CK2 in vitro. In vivo labeling and phosphopeptide mapping analysis revealed that this phosphorylation occurs in vivo. These data implicate CK2 as a potential upstream regulator of Mre11 function.

Keywords: Mre11; Protein kinase CK2; Chk2; Phosphorylation; DNA damage response pathway

DNA double-strand breaks (DSBs) induce a complex cellular response designed to activate cell cycle checkpoints, DNA repair, and the onset of apoptosis. DSBs are not only generated by exogenous DNA-damaging agents such as ionizing radiation, but also arise during normal DNA replication. They can be also generated in meiosis, mating-type switching, and V(D)J recombination [1–4]. DSBs are repaired by two repair pathways, nonhomologous end joining and homologous recombination. Ataxia telangiectasia-mutated (ATM) protein kinase and the Mre11–Rad50–Nbs1 (MRN) complex are required for rapid activation of these signaling cascades. Mutations in the ATM, MRE11, or NBS1 cause Ataxia telangiectasia (A-T), Ataxia telangiectasia-like disorder (A-TLD), and Nijmegen breakage syndrome (NBS), respectively [5–7]. These inherited cancer-prone diseases have similar, overlapping phenotypes. The cells derived from these patients show chromosome fragility, radiosensitivity, and cell cycle checkpoint defects. ATM kinase plays a key role in the DNA damage response pathway and is rapidly activated by the presence of DSBs. In undamaged cells, the ATM protein remains inactive as a dimer. In response to the formation of DNA DSBs, the autophosphorylation of serine 1981 results in dimer dissociation and activation of the kinase activity of ATM [8]. The subsequent phosphorylations of target proteins, including p53, Chk2, Mdm2, Smc1, Nbs1, FanCD2, Brca1, and H2AX, result in the activation of cell cycle checkpoints and DNA repair [9–16].

☆ *Abbreviations:* CK2, protein kinase CK2; DSB, double-strand break; IR, ionizing radiation; ATM, Ataxia telangiectasia-mutated; MRN, Mre11–Rad50–Nbs1; IPTG, isopropyl β-D-thiogalactoside; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TAP, tandem affinity tagging; DRB, 5, 6-dichloro-1-(β-D-ribofuranosyl)benzimidazole.

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doi:10.1016/j.bbrc.2005.03.162

Mre11 forms a complex with Rad50 and Nbs1 [17], a complex which is involved in a variety of DNA metabolism such as homologous recombination, nonhomologous end joining, telomere maintenance, DNA damage sensing, and cell cycle checkpoint signaling. In vitro, the Mre11 complex acts as an Mn^{2+}-dependent double-stranded DNA 3′–5′ exonuclease and a single-stranded endonuclease [18,19]. These activities are enhanced by Nbs1 [20]. In vivo, Mre11 facilitates the generation of 3′ tails for strand invasion in homologous recombination [21]. The Mre11 complex forms foci at the sites of DNA damage and disperse after the completion of repairs. Upon IR, Mre11 is phosphorylated in an ATM- and Nbs1-dependent manner [22,23], but questions regarding the nature and functional consequences of this phosphorylation remain unanswered. Like several other ATM downstream target proteins, the recruitment of the Mre11 complex to DNA damage sites is ATM-independent. Another member of this complex, Nbs1, is also phosphorylated by ATM in response to DNA DSBs and this phosphorylation is required for a proper S-phase checkpoint [13]. Nbs1 seems to act as a downstream adaptor for optimal ATM-dependent phosphorylation of downstream target proteins involved in cell cycle checkpoints.

Protein kinase CK2 is a ubiquitous protein kinase that promotes cell growth and cell survival through the regulation of a variety of different pathways. CK2 holoenzyme is composed of two catalytic subunits (αα, αα′ or α′α′) and a dimer of two β subunits. The catalytic subunits (αα, αα′, or αα′) are essential for cell viability, while the function of the dimer of β subunits remains unknown. CK2 is required for the transition of G0–G1 transition and G1–S transition in the cell cycle [24], and has been reported to be oncogenic in culture cells and in transgenic animals [25,26]. CK2 is implicated in cellular responses to a variety of stresses, including UV, heat shock, inflammation, and spindle damage [27]. CK2 has been reported to be involved in transcriptional responses to DNA damage [28]. CK2 is a component of FACT, a chromatin transcriptional elongation factor. In this complex, CK2 phosphorylates p53 on serine 392 to promote the transcriptional activity of p53 [29].

Although Mre11 is involved in several aspects of the cellular response to DNA damage, its regulation is still poorly understood. Here, I have revealed some unexpected roles of CK2 in the DNA damage response pathway. I identified CK2 as a binding partner of cell cycle checkpoint kinase, Chk2, and demonstrated that CK2 constitutively phosphorylates Mre11 protein.

Materials and methods

Expression and purification of Nus-Mre11. For *Escherichia coli* expression vector, two complementary oligonucleotides (5′-GACCGAGCTCATGAGTACTGCAGATGCACTTGAT-3′ and 5′-TTTGGTACCTCTTCTATTTCTTCTTAAAGA-3′) were used to amplify full length MRE11 and the *Sac*I–*Kpn*I fragment of PCR products was subcloned into pET43.1b (Novagen, WI). The construct was confirmed by restriction enzyme digestion and DNA sequencing. The pET43.1b-MRE11 construct was freshly transformed into BL21-Codonplus (DE3)-RIL (Novagen). One liter of Luria broth containing ampicillin was inoculated with 10 ml overnight growth of *E. coli* transformants bearing pET43.1b-MRE11 and incubated at 37 °C with vigorous shaking for 3 h until an OD_{600} of 0.7–0.8 was obtained. Then 0.4 mM IPTG was added and incubation was continued for 3 h. Cells were harvested by centrifugation at 7000*g* for 5 min and the cell pellets were resuspended in 100 ml of 20 mM Tris–HCl (pH 8.0) containing 300 mM NaCl, 100 mM imidazole, and 20% sucrose. The suspension was then lysed by sonication at 4 °C. The crude extract was clarified by centrifugation at 15,000*g* for 30 min. The supernatant containing Nus-Mre11 protein was loaded onto a pre-equilibrated Ni–NTA affinity column and washed with 100 ml of the same buffer, absent sucrose. Then Nus-Mre11 proteins were then eluted with a linear gradient of 50–200 mM imidazole in the same buffer at a flow rate of 2 ml/min, and 10 ml of fractions were collected. The Nus-Mre11 fractions were confirmed by SDS–PAGE and were pooled. Vectors that express GST-conjugated Mre11 peptides were made by cloning complementary oligonucleotides that encoded the desired peptides into the *Bam*HI–*Sma*I sites of pGEX-2T (Amersham Pharmacia Biotech). GST-conjugated Mre11 peptides were expressed and purified as described previously [30].

In vitro kinase assays. In vitro kinase assays for Chk2 and CK2 were performed as described previously [31]. Cell extracts were prepared from 293T cells transfected with Flag-Chk2 or Srt-CK2α by resuspension in modified TGN buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Tween 20, 0.3% Nonidet P-40, 1 mM sodium fluoride, 1 mM Na_3VO_4, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 μg/ml pepstatin). Cleared supernatants were immunoprecipitated with anti-flag antibody (M2) or anti-SRT antibody and protein A/G–agarose beads. The beads were washed twice with TGN buffer and twice with kinase buffer (20 mM Hepes, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT). Finally, the immunoprecipitate was resuspended in 50 μl kinase buffer containing 10 μCi [γ-^{32}P]ATP, and 1 μg Nus-Mre11 as a substrate. The kinase reaction was performed at 30 °C for 30 min and was stopped by the addition of SDS–polyacrylamide gel electrophoresis loading buffer. Proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Immunoprecipitated myc-Chk2 was confirmed by Western blotting with α-myc monoclonal antibody (9E10). Radiolabeled proteins were visualized and quantified on BAS (Fuji).

Metabolic labeling and immunoprecipitation of Mre11. 293T cells transfected with Flag-Mre11 wild-type or S649A mutant were grown in 60-mm dishes in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum at 37 °C and 5% CO_2. Transfected cells were washed with phosphate-free DMEM supplemented with 10% dialyzed fetal bovine serum and incubated for 2 h with [γ-^{32}P]orthophosphate (0.2 mCi/ml) at 37 °C. The labeled cells were then harvested by washing once in ice-cold PBS. Cells were lysed in ice-cold lysis buffer (250 mM NaCl, 1% NP-40, 20 mM Tris–HCl at pH 7.4, 1 mM EDTA, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM Na_3VO_4, 10 mM NaF, and 0.5 mM PMSF) at 4 °C. Lysates were clarified at 12,000*g* for 15 min at 4 °C. Flag-Mre11 was immunoprecipitated from 1.5 ml of cleared lysate using an α-Flag affinity gel. The beads were boiled in SDS sample buffer and immunoprecipitated Flag-Mre11 was electrophoresed on a 10% SDS–polyacrylamide gel. Protein was electrophoretically transferred to immobilon-P membrane [polyvinylidene difluoride (PVDF) membrane, Millipore]. Radiolabeled Mre11 protein was visualized by autoradiography. The appropriate band was cut out and prepared for tryptic digestions as described below.

Two-dimensional phosphopeptide mapping and phosphoamino acid analysis. After transfer to a PVDF membrane, the radiolabeled

Flag-Mre11 band was cut from the membrane and digested with TPCK-trypsin. Phosphopeptides were resolved in the first dimension by electrophoresis (1030 V for 35 min at 15 °C) using an HTLE 7000 apparatus (C.B.S. Scientific). They were resolved in the second dimension by chromatography in *n*-butanol, pyridine, glacial acetic acid, and water (5:3.3:1:4). ^{32}P-labeled tryptic peptides were visualized on BAS (Fuji). For phosphoamino acid analysis, the radiolabeled Flag-Mre11 was separated by SDS–polyacrylamide gel electrophoresis. After Coomassie blue staining, the Flag-Mre11 band was excised and the protein was extracted in 50 mM NH_4HCO_3, pH 7.4, containing 0.5% β-mercaptoethanol and 0.1% SDS. The proteins were precipitated in 15% trichloroacetic acid, washed in 100% ethanol, dried, and hydrolyzed in 6 N HCl for 1 h at 110 °C and the phosphoamino acid content of the hydrolyzed peptides was determined. Phosphoamino acid standards were visualized by spraying the plate with 1%(wt/vol) ninhydrin in acetone.

Results and discussion

Previous studies have reported that Mre11 goes through phosphorylation upon DNA damage treatment in ATM- and NBS-dependent manners [22,23]. To investigate whether ATM or its downstream kinase, Chk2, is responsible for this, I initially tested whether ATM or Chk2 kinase phosphorylates Mre11 in vitro. As full-length Mre11 protein or large amount of truncated Mre11 proteins were insoluble in the bacterial system, I subcloned full length Mre11 into the NusA system (Novagen) which aids many insoluble proteins in soluble expression. Nus-Mre11 was expressed in a soluble form and was purified to homogeneity. Although GST-conjugated Mre11 peptides containing serine 264 were phosphorylated well by ATM in previous study [30], Nus-conjugated full-length Mre11 protein remained unphosphorylated (data not shown). Chk2 phosphorylated Nus-Mre11 but not Nus protein. However, the kinase-inactive mutant Chk2 also unexpectedly phosphorylated Nus-Mre11, suggesting the existence of other contaminating or co-purified kinases, not Chk2 kinase itself, in Flag-Chk2 immunoprecipitates (Fig. 1A). Recently, I identified several Chk2-interacting proteins by using tandem affinity tagging system (TAP) and found that CK2αα and α′ interact with Chk2 (data not shown). I also investigated the existence of CK2α in Flag-Chk2 immunoprecipitates. CK2α was co-immunoprecipitated with Flag-Chk2 wild-type or the kinase-inactive form. IR did not affect the interaction between Chk2 and CK2αα (Fig. 1B). Endogenous CK2α also interacts with Chk2 (data not shown). The functional consequence of the interaction between Chk2 and CK2 remains unknown. To test whether CK2 in Flag-Chk2 immunoprecipitate phosphorylates Mre11 in vitro, a potent CK2 inhibitor, 5,6-dichloro-1-(β-D-ribofuranosyl)benzimidazole (DRB), was added to the in vitro kinase reaction. The addition of DRB leads to a decrease in the phosphorylation of Mre11 by Flag-Chk2 immunoprecipitates. This suggests that CK2 is a responsible kinase of

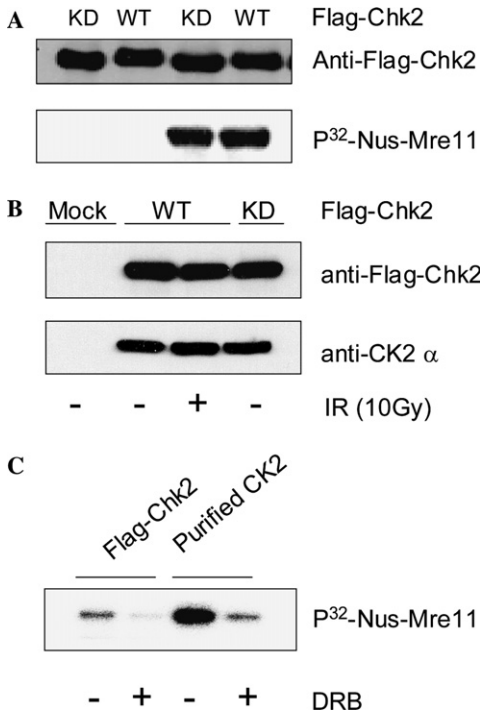

Fig. 1. Phosphorylation of Mre11 by CK II. (A) Nus-Mre11 was phosphorylated in vitro by an unknown kinase that was co-immunoprecipitated with Flag-Chk2. No phosphorylation of Nus was observed. (B) CK2α interacts with Chk2 in vivo. (C) Purified CK2 phosphorylates Nus-Mre11 in vitro. A potent CK2 inhibitor, DRB, was added to in vitro kinase reaction samples before the addition of [γ-^{32}P]ATP.

Mre11 (Fig. 1C). The commercially available CK2 complex (New England Biolabs) also phosphorylates Mre11 in vitro and the treatment of DRB inhibited the phosphorylation of Mre11 to a similar extent.

As Mre11-phosphorylating kinase activity was observed in HeLa and 293T nuclear extract, I attempted to purify the kinase that phosphorylates Mre11 through conventional chromatographic methods. To identify the major protein kinase(s) that might be responsible for the phosphorylation of Mre11, I proceeded with the fractionation of 293T cell lysates through a Q-Sepharose column, Heparin Sepharose, and Superdex 200 column. At each step, a single major peak of kinase activity phosphorylating Mre11, determined using Nus-Mre11 (full-length) protein as a substrate, was identified. After gel filtration chromatography on the Superdex 200, I examined the presence of CK2 in this partially purified sample. Western blot analysis with anti-CK2α antibody showed that CK2 was coeluted with the peak of Mre11 phosphorylation activity. Phosphorylation of Mre11 was nearly abrogated by a specific CK2 inhibitor, DRB, which suggests that CK2 is a major Mre11 kinase in the partially purified sample. CK2 is also inhibited by low concentrations of heparin, and treatment of heparin inhibited Mre11-phosphorylating activity (data not

shown). As a whole, these data strongly suggested that CK2 could be the major cellular kinase that phosphorylates Mre11.

CK2 preferentially phosphorylates serine or threonine followed by a stretch of acidic residues on the immediate carboxy-terminal side. Among these acidic residues, the +3 position after the phosphoacceptor is the most important determinant. The acidic domain is found at the +3 position in about 90% of identified phosphorylation sites and at +1 in 70% of the sites [32]. Mre11 possesses seventeen phosphorylation consensus sites for CK2 (S/T-X-X-D/E). To map the phosphorylation sites of Mre11 by CK2, a series of truncated Nus-Mre11 was generated (Fig. 2A). One truncated Nus-Mre11, containing amino acids 583–708, was phosphorylated well by CK2 (Fig. 2B) and the first half of this region was significantly phosphorylated (Fig. 2C). To examine which residues within this region might be phosphorylated, I compared the phosphorylation of five GST-conjugated peptides which spanned this region.

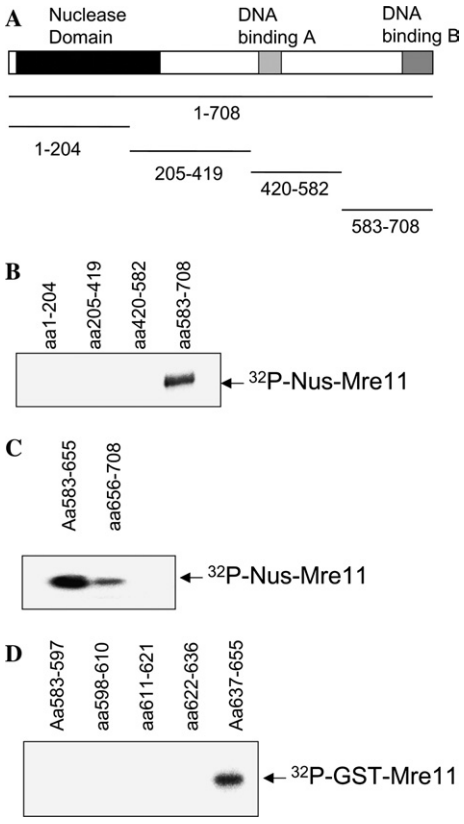

Fig. 2. Determination of the sites on Mre11 that are phosphorylated in vitro by protein kinase CK2. (A) Schematic diagram of Mre11 showing the location of nuclease domain and two DNA-binding domains. The size and location of Nus-fusion proteins used in the kinase reactions are shown below Mre11. (B,C) Nus-fusion proteins containing Mre11 amino acids 1–204, 205–419, 420–582, 583–708, 583–655, and 656–708 were used as substrates in an in vitro kinase assay with purified CK2 complex. (D) Phosphorylation of GST-fusion peptide containing Mre11 amino acids 583–597, 598–610, 611–621, 622–636, and 637–655 was tested.

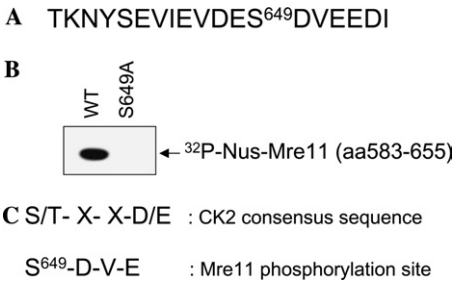

Fig. 3. Serine 649 is a major phosphorylation site of Mre11 by CK2. (A) Amino acid sequence of Mre11 surrounding serine 649. (B) Phosphorylation of Mre11 wild-type and mutant proteins (S649A). (C) Comparison of the CK2 consensus phosphorylation sequence and Mre11 phosphorylation sequence.

GST-peptide containing amino acids 637–655 was a good substrate of CK2 (Fig. 2D). In this peptide, there is one putative CK2 phosphorylation consensus sequence, serine 649 (S^{649}DVEED). The amino acid sequence surrounding serine 649 is well fit to the known CK2-phosphorylation consensus sequence (Fig. 3C). Mutations of serine 649 of Nus-Mre11 (aa 583–655) to alanine completely abrogated phosphorylation by CK2, suggesting that serine 649 is a major phosphorylation site of Mre11 by CK2 (Fig. 3B).

To test whether phosphorylation of serine 649 on Mre11 is observed in vivo, cells transfected with Flag-Mre11 wild-type or S649A mutant were labeled with [^{32}P]orthophosphate. Flag-Mre11 is phosphorylated in vivo and the mutation of serine 649 to alanine showed slightly less incorporation of [^{32}P]phosphate (Fig. 4A). To determine the nature of the phosphorylated residues, I performed phosphoamino acid analysis of radiolabeled Flag-Mre11. Phosphoamino acid analysis revealed that the phosphorylation of Mre11 occurred exclusively on serine and did not occur on threonine or on tyrosine residue (Fig. 4B). To test whether serine 649 is phosphorylated in vivo, radiolabeled Flag-Mre11 protein was digested with trypsin, and the digestion products were evaluated by phosphopeptide mapping. Three major phosphopeptides were observed in tryptic digests of wild-type Flag-Mre11, but one spot disappeared in those of S649A mutant Flag-Mre11, suggesting that serine 649 is phosphorylated in vivo. The addition of tryptic peptides from Mre11 (aa 583–655) phosphorylated by CK2 in vitro to peptides of Mre11 (S649A) leads to reappearance of this spot, confirming that this spot is phospho-serine 649 peptide.

The original purpose of the present study was to identify the kinase responsible for the IR-induced Mre11 phosphorylation. In the course of these studies, I found that CK2 interacts with Chk2 and phosphorylates serine 649 of Mre11 in vitro. In vivo labeling and phosphopeptide mapping analysis revealed that serine 649 on Mre11 is phosphorylated in vivo. At present, the effects of serine 649 phosphorylation on Mre11 function remain

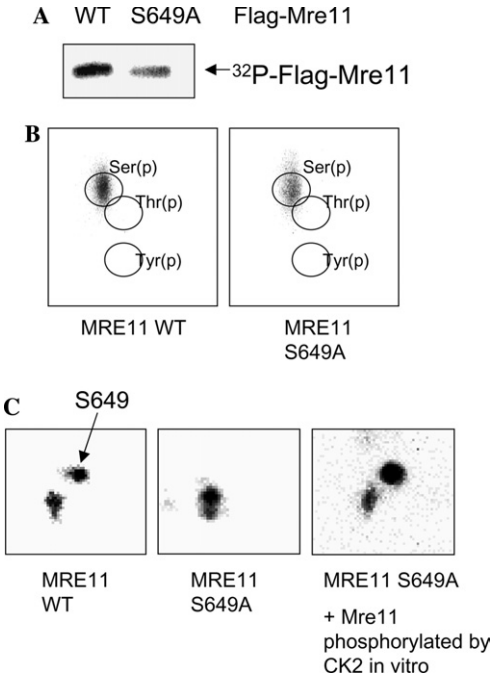

Fig. 4. (A) Flag-Mre11 (wild-type or S649A mutant) was transfected into 293T cells, labeled with [^{32}P]orthophosphate, and immunoprecipitated with anti-Flag antibody. (B) Phosphoamino acid analysis following in vivo labeling. ^{32}P-labeled Flag-Mre11 was analyzed on SDS–PAGE and stained with Coomassie blue, subsequently excised, and subjected to hydrolysis and two-dimensional phosphoamino acid analysis as described under Materials and methods. The phosphoamino acid standards, phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY), were marked after exposure to ninhydrin staining solution. (C) Phosphorylation of serine 649 of Mre11 in vivo. 293T cells transiently transfected with Flag-tagged Mre11 (wild-type) or Mre11(S649A) expression vectors were metabolically labeled with ^{32}P. Proteins were resolved by 10% SDS–PAGE and electrophoretically transferred to PVDF membrane. Radiolabeled Mre11 was cut from the membrane and digested with trypsin. Radiolabeled peptides were resolved by electrophoresis at pH 1.5 in the first dimension followed by ascending chromatography in the second dimension.

unknown. Additional experiments are necessary to address the consequence of phosphorylation on the function of Mre11.

Acknowledgments

This work was supported by a grant from Research for Pure Basic Science, Korea Research Foundation (2002-KRF-C00070, S.-T.K.). I thank Gil-Joo Seo and Jeong-weon Lee for technical assistances and Dr. Jaekyoon Shin for providing pCMV-SRT-CK2α.

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