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NBS1 is regulated by two kind of mechanisms: ATM-dependent complex formation with MRE11 and RAD50, and cell cycle–dependent degradation of protein

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ABSTRACT

Nijmegen breakage syndrome (NBS), a condition similar to Ataxia-Telangiectasia (A-T), is a radiation-hypersensitive genetic disorder showing chromosomal instability, radio-resistant DNA synthesis, immunodeficiency, and predisposition to malignancies. The product of the responsible gene, NBS1, forms a complex with MRE11 and RAD50 (MRN complex). The MRN complex is necessary for the DNA damage–induced activation of ATM. However, the regulation of MRN complex formation is still unclear. Here, we investigated the regulatory mechanisms of MRN complex formation. We used an immunoprecipitation assay to determine whether levels of the MRN complex were increased by radiation-induced DNA damage and found that the levels of these proteins and their mRNAs did not increase. ATM-dependent phosphorylation of NBS1 contributed to the DNA damage–induced MRN complex formation. However, pre-treatment of cells with an ATM-specific inhibitor did not affect homologous recombination (HR) and non-homologous end-joining (NHEJ) repair. G0 phase cells, decreasing NBS1 and HR activity but not NHEJ, gained HR-related chromatin association of RAD51 by overexpression of NBS1, suggesting that the amount of NBS1 may be important for repressing accidental activation of HR. These evidences suggest that NBS1 is regulated by two kind of mechanisms: complex formation dependent on ATM, and protein degradation mediated by an unknown MG132-resistant pathway. Such regulation of NBS1 may contribute to cellular responses to double-strand breaks.

KEYWORDS: DNA damage, NBS1, ATM, cell cycle checkpoint, homologous recombination

INTRODUCTION

DNA double-strand breaks (DSBs) often occur in genomic DNA after exposure to ionizing radiation (IR). Unrepaired DSBs can have severe consequences, such as the induction of genomic instability and subsequent tumorigenesis, or the promotion of apoptosis. Therefore, cells are able to activate DNA damage responses (DDRs) immediately after the detection of DSBs in genomic DNA, such as cell cycle checkpoint and DNA repair mechanisms. DSBs are repaired mainly through non-homologous end-joining (NHEJ) and homologous recombination (HR). NHEJ directly rejoins two DSB ends, but sometimes requires end-resection, particularly for radiation-induced DSB ends. Hence NHEJ is known to be an error-prone DSB repair mechanism

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repair mechanism, leading to nucleotide loss. In contrast, during HR repair, the non-damaged homologous DNA region is used as a template for the repair of DSB regions, which is why HR is an error-free DSB repair mechanism.1

Radiation-hypersensitive genetic disorders, such as Ataxia-Telangiectasia (A-T) and Nijmegen breakage syndrome (NBS) have been studied in order to elucidate the underlying mechanisms of DSB-induced cellular responses.2, 3 Their cellular phenotypes are similar as well, showing radiation hypersensitivity, chromosome instability, and radiation-resistant DNA synthesis, and it was suggested that the genes responsible for these diseases (ATM in A-T and NBS1 in NBS) play physical or functional roles during the DSB-induced response. The product of ATM is a protein kinase, while the product of NBS1 is an adaptor protein that regulates DDRs through the formation of a protein complex.1, 2 ATM kinase is activated in response to DSBs and has a fundamental role in the regulation of cell cycle checkpoints through the phosphorylation of DDR proteins, including p53, Chk2 and NBS1.6 Bakkenist and Kastan5 showed that the generation of radiation-induced DSBs results in an intermolecular modification within ATM dimers that leads to their activation through autophosphorylation at serine 1981. This phosphorylation triggers dimer dissociation and the free monomers subsequently phosphorylate several nuclear targets that recruit DDR proteins.5

The product of human NBS1 is a 754-amino acid (a.a.) protein that contains several functional domains, mainly located in the N- and C-termini. The N-terminus includes a forhead-associated (FHA) domain and two BRCA1 C-terminus (BRCT) domains, which contribute to the binding of several phosphorylated proteins1. The C-terminus of NBS1 protein interacts with several functional proteins as well, including ATM and MRE11, and these interactions are vital for various DNA damage responses6, 7. MRE11 is a mutated gene responsible for another radiation-hypersensitive disorder (Ataxia-Telangiectasia-like disorder; AT-LD), which codes for a 708-a.a. protein that possesses DNA nuclease activity.8

NBS1 forms a complex with MRE11 and RAD50 through its C-terminal region (a.a. 682–693), and this interaction facilitates the localization of the complex to the nucleus.6 Since HR activity is considerably reduced in NBS1- or MRE11-defective chicken DT40 cells and in patient cells, this indicates that the formation of this complex and its nuclear localization are crucial for activation of the HR pathway.9 Another conserved motif (a.a. 734–754) in the C-terminus of NBS1 directly interacts with ATM7. The cells of NBS patients less efficiently recruit ATM to DSB sites following IR, and show an insufficient activation of ATM kinase, suggesting an important role for the interaction between NBS1 and ATM via this motif during ATM activation.7 Additionally, NBS1 interacts with RNF20 (histone modification enzyme) for HR repair, and with Rad18 for translesion DNA synthesis, through the C-terminal conserved motifs10, 11. Therefore, NBS1 represents a multifunctional protein involved in several DDR pathways.

Formation of the MRE11/NBS1/RAD50 (MRN) complex was discovered 15 years ago. It is believed that this complex is stable under most cellular conditions, and NBS1 may be regulated through the formation of the MRN complex. Therefore, we investigated the regulatory mechanisms of MRN complex formation, and its role in DNA damage responses. We showed that the rate of NBS1 interactions with MRE11 and RAD50 increased following the generation of DSBs by gamma irradiation. We discuss the relationship between this complex formation and DDRs.

MATERIALS AND METHODS

Cell culture

HeLa, U2OS, hTERT-immortalized human fibroblast (48BR), SV40-transformed normal fibroblast (MRCSV), SV40-transformed AT-patient-derived fibroblast (AT5BIVA), SV40-transformed AT-LD patient–derived fibroblast (ATLD2) and SV40-transformed NBS patient–derived fibroblast (GM7166SV) cells12, 13 were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and antibiotics. NBS cells, expressing wild-type (WT) or mutated NBS1 (serine substituted with alanine at a.a. 278 and 343) were prepared as reported previously14. Growth arrest occurred at G0 in 48BR or U2OS-DRGFP and U2OS-pEJ by exchanging serum-free media for the serum-free media. Generally, 3 days after the exchange, these cells were considered as G0 cells15.

Antibodies

The following antibodies were used for western blot analysis: phospho-ATM (S1981), γ-H2A histone family X (H2AX) mouse monoclonal and H2B rabbit polyclonal antibodies (Millipore Corp.); MDC1, and phospho-KAP1 rabbit polyclonal antibodies (Bethyl Laboratories Inc.); Chk2, phospho-Chk2 (T68), phospho-Chk1 (S317), and phospho-Rad17 rabbit polyclonal antibodies (Cell Signaling Technology); human meiotic recombination 11 (MRE11), RAD50, and NBS1 rabbit polyclonal antibodies (Novus Biologicals); RPA32, RPA70 mouse monoclonal and ATM rabbit polyclonal antibodies (Merk Millipore); NBS1, MRE11, and RAD50 mouse monoclonal antibodies (GeneTex); RAD51 rabbit polyclonal antibody (Bioacademia); beta-actin mouse monoclonal antibody (Sigma-Aldrich); c-myc mouse monoclonal antibody (Covance).

Irradiation with γ-ray

Irradiation of cells with gamm rays was carried out with Gammarcell 40Ex (MDS Nordion; Dose rate 0.9 Gy/min) at room temperature.

myc-His-NBS1 transfection

Plasmids expressing WT and two different types of mutated NBS1 gene (serine substitution with alanine at a.a. 278 and 343, or MRE11-binding domain deletion) were prepared as reported previously14. Subconfluent MRCSV or AT5BIVA cells, seeded in the culture dishes a day before the transfection, were transfected with these plasmids using FuGene HD (Promega). Two days later, these cells were re-seeded, and they were used for immunoprecipitation assays the following day.

Immunoprecipitation assays

Immunoprecipitation was performed as reported previously12. Immunoprecipitation was performed by incubating the samples with anti-MRE11 rabbit polyclonal antibody (Novus Biologicals) or anti-NBS1 mouse monoclonal antibody (GeneTex). Co-immunoprecipitates were detected by western blot analysis.
Western blot analysis

Western blot analyses were carried out as described previously [12]. Target proteins were detected with the primary antibodies listed above and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or anti-mouse IgG antibodies (GE Healthcare). The bands of target proteins were visualized using the ECL plus chemiluminescence system (GE Healthcare). Quantification of the visualized bands was carried out by ImageJ software, and the ratios to quantities in unirradiated samples were calculated.

Analysis of complex formation using cross-linking with formalin

The protein extracts were prepared with lysis buffer (10 mM Tris/HCl pH7.8, 1% NP-40, 150 mM NaCl, 1 mM EDTA). After centrifugation, formalin (final concentration 4%) was added to the supernatants and they were incubated at 37°C for 5 min. Then, cross-linking complexes were detected with western blot analysis using anti-NBS1 or MRE11 rabbit polyclonal antibodies.

Quantitative PCR analysis

Total RNA was isolated from HeLa, U20S, 48BR and GM7166SV (NBS) cells by the RNAqueous Total RNA Isolation Kit (Ambion). cDNA molecules were synthesized with the SuperScript III First-Strand Synthesis System (Invitrogen), and quantitative PCR analysis was performed with the 7500 Real time PCR system (Applied Biosystems) using the Power SYBR Green PCR Master Mix (Applied Biosystems), NBS1 primers (Qiagen #249900) and GAPDH primers (forward primer, 5′-TCTCCCCACACACATGCACCTT; reverse primer, 5′-CCTAGTCCAGGGCTTTGATT).

HR and NHEJ activity analyses

HR and NHEJ analyses were performed as previously reported [16, 17]. Briefly, in order to measure the HR or NHEJ repair of I-SceI-generated DSBs, 50 μg of the I-SceI expression vector (pCBASce) was introduced to 1 × 10⁶ U2OS-DRGFP (for HR) or U2OS-pEJ (for NHEJ) cells by electroporation (GenePulser; BIO-RAD). To determine the level of HR or NHEJ activity, the percentage of GFP-positive cells were quantified at Day 3 after electroporation using FACScalibur (Becton Dickinson).

RESULTS

The rate of MRN complex formation increased in response to DSB generation

First, we examined whether the formation of MRN complex increases after gamma-ray IR using immunoprecipitation with anti-MRE11 antibody (Fig. 1). Anti-MRE11 antibody co-precipitates NBS1 and RAD50, forming the MRN complex with MRE11, and the amount of co-precipitated NBS1 increased in HeLa cells following IR (Fig. 1A and B), but the levels of co-precipitated RAD50 were unchanged after IR. In U2OS cells, a similar increase in the

Fig. 1. Immuno precipitation with anti-MRE11 antibody detected increases of MRN complex in response to DSB damages.

(A) Extracts from HeLa cells without (−) or with 10 Gy of IR were immunoprecipitated with anti-MRE11 antibody, and then the immuno-complexes were detected by Western blot analysis using the indicated antibodies. WCE = whole cell extract. Quantification was carried out by ImageJ software, and the ratios to unirradiated samples were calculated as shown in (B).

(C) Detection of MRN complex with cross-linking by formalin. Whole cell extracts from irradiated (10 Gy) or unirradiated cells were treated with formalin, and then cross-linked MRN complex (arrows) was detected by Western blot analysis using the indicated antibodies. (D) The levels of NBS1 mRNA were quantified as shown in Materials and Methods using unirradiated and irradiated cells (10 Gy).
amount of MRN complex components was observed following the IR (Fig. S1A). However, the amount of NBS1 in whole cell extracts (WCEs) was unchanged (Fig. 1A and B). Anti-NBS1 antibody is also able to detect the increase in MRN complex formation (Fig. S1B). When we treated cell extract with formalin to stabilize complex formation through cross-linking, we observed the appearance of two bands at >250 kD after IR using the anti-NBS1 antibody (Fig. 1C), suggesting an increase in the MRN complex following IR. On the other hand, anti-MRE11 detected 150 kD bands with and without IR, suggesting that MRE11 might always form the stable homodimer. In the case of mRNA expression, we did not observe a remarkable increase in NBS1 at the same level to that of immunoprecipitated NBS1, as shown in Fig. 1A and B (Fig. 1D). These results suggest that the number of MRN complexes might increase upon IR-induced DSB formation, but this increase may not depend on increase in the amounts of protein or mRNA of the individual components of this complex.

DSB-induced increase in the MRN complex was independent of the stabilization of each component

Previously, it was reported that in some DNA damage–related complexes, such as the XRCC4/ligase IV complex, the formation of the complex can lead to the stabilization of their components [18]. In order to investigate this, we used proteasome inhibitor, MG132. When 48BR cells were pre-treated with MG132, we did not observe any increase in NBS1, MRE11 or RAD50 levels in WCEs, or any disturbance in MRN complex formation following the IR (Fig. 2A). The same treatment of cells with MG132 stabilized cyclin A (G0+MG132; Fig. 4B), which is known to be degraded by a proteasome-dependent mechanism [19]. This pre-treatment did not influence ATM-dependent phosphorylation of KAP1 and Chk2 (Fig. 2B). It has been reported that MRE11-deficient AT-LD cells have not only less MRE11 protein, but also less NBS1 and RAD50 protein due to a decrease MRN complex formation [20]. We also investigated whether such instability in ATLD2 was overcome by MG132 treatment; however, the treatment did not increase the levels of the proteins (Fig. 2C). Taken together, each component of the MRN complex and the complex formation might be regulated independently of the proteasome pathway.

ATM was necessary for DSB-dependent formation of the MRN complex

NBS1 is indispensable for the DSB-dependent activation of ATM [7], and since NBS1 is a substrate of ATM kinase, this phosphorylation could lead to intra-S phase checkpoint, namely inhibition of DNA synthesis [21, 22]. We investigated whether this phosphorylation might contribute to DSB-dependent formation of the MRN complex. Pre-treatment of U2OS cells with an ATM-specific inhibitor, KU55933, inhibited the phosphorylation of KAP1 and Chk2, known to be ATM-dependent (Fig. 3A), and also repressed an IR-dependent increase in the levels of co-immunoprecipitated NBS1 (IP:MRE11; Fig. 3A). ATM-defective A-T patient fibroblasts (ATS5BIVA) also showed a similar tendency to immunoprecipitate NBS1 (Fig. S2A). These results suggest that the kinase activity of ATM is necessary for DSB-dependent formation of the MRN complex.

Figure 2. Treatment of MG132 did not disturb DSB damage-dependent increase in MRN complex. (A) 48BR cells were treated with MG132 (10 μM) 1 h before 10 Gy of IR. The extracts were immunoprecipitated with anti-MRE11 antibody, and then the immuno-complexes were detected by Western blot analysis using the indicated antibodies. (B) U2OS cells were treated with MG132 (10 μM) 1 h before 10 Gy of IR (0.5 h of post-irradiation). Then, ATM-dependent phosphorylation was confirmed by Western blot analysis using the indicated antibodies. (C) MRC5SV and MRE11-defective ATLD2SV cells were treated with MG132 (10 μM) 1 h before 5 Gy of IR (0.5 h of post-irradiation), and analyzed using the indicated antibodies.
As serine residues 278 and 343 in NBS1 are phosphorylated by ATM, leading to regulation of the intra-S phase checkpoint [21, 22], we examined the relationships between the phosphorylation of these sites and DSB-dependent formation of MRN complex, using an NBS1 mutant in which both serine 278 and 343 were replaced by alanine. When we transfected MRCS5 cells with WT or mutant NBS1, anti-MRE11 antibody was able to co-precipitate WT-NBS1 following the IR, but one of the mutated NBS1 genes (del-MRE11bd), lacking the MRE11-binding domain [6], could not be co-immunoprecipitated using the anti-MRE11 antibody (Fig. 3B). The other NBS1 mutant, 278/343SA-NBS1, was co-immunoprecipitated with the antibody used, but the levels of this molecule did not increase after IR. In the case of AT cells (Fig. 3C) we were not able to observe any increase in the levels of either WT or mutated NBS1 between unirradiated and irradiated cells. MDC1 is another component of the MRN complex.

Fig. 3. ATM was indispensable for DSB damage-dependent complex formation of MRN. (A) U2OS cells were treated with KU55933 (10 μM; +inhibitor) 1 h before 10 Gy of IR (1 h of post-irradiation). The extracts were immunoprecipitated with anti-MRE11 antibody, and then the immuno-complexes were detected by Western blot analysis using the indicated antibodies. The intensity of the bands was quantified by ImageJ software and the ratio was calculated as shown for the lower positions of each band. (B)(C) NBS1-WT or mutated NBS1 (278/343SA or del-MRE11bd) were transfected to MRCS5 (B) or AT5BIVA (C). After 2 days, their cells were irradiated with 10 Gy of IR (1 h of post-irradiation), and then extracts were prepared from them. Their extracts were immunoprecipitated with anti-MRE11 antibody and then the immuno-complexes were detected by Western blot analysis using anti-c-myc (for mycHisNBS1) and anti-MRE11 mouse monoclonal antibodies.

Fig. 4. Regulation of MRN components in G0 phase. (A) Extracts from growing (log) and growth-arrested serum-starved (G0) cells (48BR cells) were analyzed by Western blot using the indicated antibodies. (B) MG132 treatment did not recover NBS1 expression. G0 cells were pre-treated with MG132 (10 μM), and then irradiated with 10 Gy of IR (1 h of post-irradiation). Western blot analysis was performed using extracts from the cells and the indicated antibodies.
complex, which directly binds NBS1 [23]. MDC1-depleted U2OS cells by siRNA showed reduced DSB-dependent increase in MRN complex formation, suggesting that MDC1 may be important for the formation of this complex (Fig. S2B).

Regulation of MRN components in the G0 phase
The expression of many molecules involved in HR, such as RAD51 and BRCA, is induced in S phase, because HR is activated by DSBs in late S and G2 phases [24]. As the MRN complex is important for HR as well [9], we investigated the cell cycle–dependent role of MRN using G0 (growth-arrest phase) cells. When we maintained hTERT-immortalized human fibroblasts (48BR cells) in serum-free media, cyclin A (S/G2 phase marker protein) levels decreased at 2 and 3 days after medium change (Fig. S3A) and were arrested at G0 phase [15]. Cell cycle analysis by flow cytometry showed that the cells after 3 days of serum starvation arrested at G0 phase (G0/G1: 87.5%; Fig. S3B). NBS1 levels also decreased at these time-points as well, but the levels of other components, MRE11 and RAD50, were unchanged. We also verified that NBS1 mRNA levels were unchanged after serum starvation (Fig. S3C). The phosphorylation of ATM substrates (KAP1, Chk2 and ATM itself) was induced in the cells in the growth (log) phase, but their phosphorylation levels were considerably reduced in G0 phase (Fig. 4A), while the growth arrest at G0 phase did not reduce the expression of ATM or that of the substrates, KAP1 and Chk2 (Fig. S3A; right panel). Figure 4A also showed that γH2AX levels increased in G0 phase after irradiation, suggesting that the reduction in NBS1 levels may lead to DSB repair defects. Additionally, we examined whether the decrease in NBS1 levels in G0 phase was due to the changes in stability, caused by the components of the proteasome pathway. Although the pre-treatment of MG132 was able to recover cyclin A levels in the G0 phase, NBS1 levels were unchanged by this treatment (Fig. 4B), suggesting that the decrease in NBS1 may be regulated by an MG132-resistant pathway at the protein level. We also measured MRN complex formation in the G0 phase. As expected, the co-immunoprecipitated NBS1 levels completely decreased with and without the induction of DNA damage [IR and camptothecin transfection. Then, I-Sce1 expression plasmids were transfected to these cells by electroporation and analyzed by flow cytometry. Each result represents an average value and standard deviation from three experiments. Lower panel: The cells were treated with KU55933 (10 μM; KU) 1 h before 5 Gy of IR (0.5 h of post-irradiation). Then, ATM-dependent phosphorylation was confirmed by Western blot analysis using the indicated antibodies. (B) Serum-starved (for 3 days) or growing U2OS-DRGF or U2OS-pEJ cells were electroporated with I-Sce1 expression plasmids and analyzed by flow cytometry. Each result represents an average value and standard deviation from three experiments. (C) Chromatin-associated proteins such as RAD51 and RPA were prepared from precipitates after RIPA buffer extraction and were then detected by Western blot analysis using the indicated antibodies.
Dissecting NBS1 roles in DNA damage responses, we found that ATM-dependent phosphorylation of NBS1 is indispensable for regulation of the ATM-dependent checkpoint, but not for HR repair (Fig. 5B and C). It is likely that the MG132-resistant system might degrade NBS1 protein in G0 phase, and the system may be important for avoiding accidental activation of HR.

We next investigated the relationship between DSB-dependent phosphorylation, which is related to stabilization of the MRN complex, and the ATM-related pathway. We investigated the cell cycle distribution after unirradiated and irradiated cells, using propidium iodide (PI) staining (Fig. S4A). At 6 h after irradiation, the percentage of WT-NBS1-expressing cells in G2 phase considerably increased, due to the activation of the G2 checkpoint, but an increased number of cells in S phase was not observed. After 12 h, some of the cells were released from G2 checkpoint arrest, and these cells were able to progress to G1 phase. Both NBS cells and 278/343A-NBS1-expressing cells showed S phase arrest at 6 h after IR, and the percentage of the cells in G1 phase was low at 12 h. We also confirmed the induction of apoptosis by PI staining (Fig. S4B). Camptothecin treatment was able to induce the death of >10% of NBS cells, but the rates of apoptosis were reduced in WT- and 278A/343A-mutated NBS1-expressing cells (Fig. S4B). Taken together, ATM-dependent phosphorylation of NBS1, and subsequent amplification of the MRN complex may be important for cell cycle checkpoints, but not for the apoptosis pathway.

**CONFLICT OF INTEREST**

The authors declare that there are no conflicts of interest.

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**SUPPLEMENTARY DATA**

Supplementary data are available at the Journal of Radiation Research online.
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