The Forkhead-associated Domain of NBS1 Is Essential for Nuclear Foci Formation after Irradiation but Not Essential for hRAD50-hMRE11-NBS1 Complex DNA Repair Activity*

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NBS1 (p95), the protein responsible for Nijmegen breakage syndrome, shows a weak homology to the yeast Xrs2 protein at the N terminus region, known as the forkhead-associated (FHA) domain and the BRCA1 C terminus domain. The protein interacts with hMRE11 to form a complex with a nuclease activity for initiation of both nonhomologous end joining and homologous recombination. Here, we show in vivo direct evidence that NBS1 recruits the hMRE11 nuclease complex into the cell nucleus and leads to the formation of foci by utilizing different functions from several domains. The amino acid sequence at 665–693 on the C terminus of NBS1, where a novel identical sequence with yeast Xrs2 protein was found, is essential for hMRE11 binding. The hMRE11-binding region is necessary for both nuclear localization of the complex and for cellular radiation resistance. On the other hand, the FHA domain regulates nuclear foci formation of the multiprotein complex in response to DNA damage but is not essential for nuclear transportation of the complex and radiation resistance. Because the FHA/BRCA1 C terminus domain is widely conserved in eukaryotic nuclear proteins related to the cell cycle, gene regulation, and DNA repair, the foci formation could be associated with many phenotypes of Nijmegen breakage syndrome other than radiation sensitivity.

NBS1 is a responsible gene for Nijmegen breakage syndrome

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The abbreviations used are: NBS, Nijmegen breakage syndrome; FHA, forkhead-associated; BRCT, BRCA1 C terminus; PCR, polymerase chain reaction; DMEM, Dulbecco’s modified Eagle’s medium; Gy, gray; BD, binding domain; AD, activating domain; del, deletion.
of the pIREs-hyg vector (CLONTECH). The entire cDNA insert was verified by sequencing.

For construction of yeast two-hybrid vectors, full-length NBS1 cDNA or hMRE11 cDNA was ligated into pAS2-1 (CLONTECH) or into the GAL4-activating domain of pACT2 (CLONTECH). Partial deletion mutants of hMRE11 were constructed by PCR using full-length cDNAs as a template, Pyrobest DNA polymerase (TaKaRa), and oligonucleotides (22-24-mer) designed to make an in-frame deletion. The PCR products were then self-ligated, and the entire DNA sequence was verified.

**Cell Culture and Transfection—**GM166VA7 cells from a NBS patient were used as an NBS cell line. HeLa cells were used as a control cell line with normal radiation sensitivity. Cell cultures were maintained in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone). The vectors were transfected into GM166VA7 cells by electroporation using a GenePulser (Bio-Rad), and stable transformants were selected by incubation in medium containing 200 μg/ml hygromycin B (Wako).

**Cell Survival Assay—**Exponentially growing cells were trypsinized, re-suspended in mMEM, and sealed in a glass tube. The cells were then irradiated with 80 Gy at a dose rate of 1.0 Gy/min. Immediately after irradiation, an appropriate number of cells were plated in mMEM supplemented with 10% fetal bovine serum and 10% fetal calf serum. After 14 days of incubation, the cells were fixed with ethanol and stained with a 4% Giemsa solution (Katayama Chemical). Surviving fractions were calculated by comparing the number of colonies in the experimental cells with the number of colonies formed in nonirradiated control cells.

**Western Blotting and Immunofluorescent Staining—**Whole-cell extracts (2 × 10^6 cells) were prepared as described (8), and 40 μg of total protein was applied to an 8% SDS polyacrylamide gel. After electrophoresis, proteins were transferred to a blotting membrane using an electrophoret apparatus (ATTO), and immunoblots were performed as described previously (8).

For immunofluorescent staining, cells grown on a glass slide were fixed with cold methanol for 20 min, rinsed with cold acetone for several seconds, and then air-dried. The slides were stained as described previously (8). The primary antibodies used were as follows: anti-NBS1 (8, anti-hMRE11 (Novus Biologicals), and anti-hRAD50 (GeneTex). Alexa-488-conjugated anti-rabbit IgG (Molecular Probes) was used for visualization of NBS1 or hMRE11. Biotinylated anti-mouse IgG (Vector) and Alexa-488-conjugated streptavidin (Molecular Probes) were used for hRAD50. The 488-nm excited green fluorescence from the Alexa-488 dye was visualized with a laser scanning microscope (Olympus).

**Yeast Two-hybrid Analysis—**Full-length or mutated NBS1 cDNA was expressed as a fusion protein to a GAL4-DNA-binding domain (BD) from pAS2-1 (CLONTECH) or to a GAL4-activating domain (AD) from pACT2 (CLONTECH). The full-length or mutated NBS1-1BD (or -AD) plasmid was transfected into the yeast strain GC1945 (CLONTECH), along with a full-length hMRE11-AD (or -BD) plasmid. Interaction between the expressed proteins was detected by growth on a synthetic dropout (−Leu−Trp−His) plate and by β-galactosidase activity.

**RESULTS**

To locate the functionally important domain of the NBS1 gene involved in the human NBS phenotype, we tried to determine which sequences were conserved in NBS1 in higher eukaryotes. Cloning and sequencing of the chicken Nbs1 gene can be very useful for locating a conserved domain, because a low homology of the NBS1 amino acid sequence between the human sequence and the chicken sequence has been suggested (2). The amino acid sequence of chicken Nbs1 shows apparent homology with human and mouse NBS1 (62 and 63% identity, respectively) at the N terminus 200 amino acids, which contains both the FHA/BRCT domain and a phosphorylation site on a serine residue at 278 and 343 (Refs. 9 and 10 and data not shown). The N terminus 360 amino acids, which contains the FHA/BRCT domain and a phosphorylation site on a serine residue at 278 and 343 (Refs. 9 and 10 and data not shown) in the human sequence and the chicken sequence has been suggested (2).

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![Fig. 1. Comparison of amino acid sequence at the C-terminal region in human, mouse, and chicken NBS1.](image332x629 to 531x729)

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**analysis.** Significant interaction between NBS1 and hMRE11 was detected only when codons 665–693 were present in the construct even when N-terminal FHA/BRCT domains were deleted (Fig. 2). This C-terminal region contains a sequence highly conserved at codons 682–693 in the chicken, mouse, human NBS1, and yeast Xrs2 proteins, implying that the sequence might be a critical region for hMRE11 binding.

Because NBS1 is essential for the hMRE11-hRAD50-NBS1 complex to express nuclease activity or ATP-dependent DNA unwinding activity (11), the hMRE11-binding domain is probably necessary for the processing of damaged DNA. To assay the functions of the binding domain in vivo, we subcloned mutant NBS1 constructs into expression vectors (shown in Fig. 2) and transfected them into the NBS cell line GM7166VA7. All of the stable transformants expressed a significant amount of the mutant NBS1 protein (Fig. 3a). Although the expression of multiple smaller proteins was observed in the N-terminal-deleted mutants (Fig. 3a, FE3 and BRCT-d), the expected mutant NBS1 proteins in the FE3 and BRCT-d clones were still detected in their transformants (Fig. 3a). Because it is known that NBS1 directly binds to hMRE11 and indirectly to hRAD50 through Xrs2 (Fig. 1), we tested the ability of mutant NBS1 to form the complex. Full-length and S703 mutant protein, containing C-terminal conserved region, were able to form the triple-protein complex, because they coimmunoprecipitated with hRAD50. On the other hand, S590 and S670, in which the C-terminal conserves sequence was deleted, were not able to form the complex (Fig. 3b). The result is consistent with yeast two-hybrid experiment (Fig. 2), which demonstrated the essential C-terminal domain for hMRE11 binding at 665–693. Although the expected mutant protein from FE3 or BRCT-d was invisible in NBS1-blot for hRAD50 precipitates (Fig. 3b), a very weak signal was detected when the increased amount of the precipitate was used for analysis (data not shown). Because the expected FE3 or BRCT-d proteins were accompanied by the degraded small fragments (Fig. 3a), the N terminus mutant proteins could be unstable. The absence of the N terminus region of the protein in the FE3 and BRCT-d clones was confirmed by immunoblotting using antisera that recognizes the N-terminal end of NBS1 (data not shown). From these results, it appears that the multiple proteins expressed in the FE3 and BRCT-d mutants must contain function hMRE11-binding domains at the C-terminal NBS1 region.

Because restoration of radiation resistance was observed only when the mutant proteins contained the hMRE11-binding domain at C terminus (Fig. 4), this suggests that the hMRE11-binding domain is essential for radiation resistance. This was supported by finding that del 683–693 mutant lacking Xrs2-identical sequence could not restore radiation resistance (data not shown). Interestingly, cells transfected with mutant NBS1 lacking the FHA domain alone or lacking both the FHA and
BRCT domains (FE3 and BRCT-d) also became radiation resistant to a degree similar to that seen in C-terminal end-deleted mutant (S703 in Fig. 4). These results imply that the FHA/BRCT domain is not essential for restoration of radiation resistance, i.e. for DNA repair.

Subsequently, observations were made to see whether the various truncated NBS1 proteins could restore the focus formation activity of the hRAD50-hMRE11-NBS1 complex in NBS cells after irradiation, because it has been reported that this complex is required for repair of DNA double-strand breaks.

![Image of NBS1 constructs](image)

**FIG. 2.** NBS1 constructs used in the present study and their hMRE11 binding activity obtained from yeast two-hybrid analysis. Left, a brief protein structure and designed constructs of NBS1. FHA domain at 24–102, BRCT domain at 108–196, and possible phosphorylation site at Ser776 or Ser343 residues are indicated. Down arrows represent the mutation position in NBS patients (1–3). Right, the hMRE11 binding activity of various NBS1 constructs by means of yeast two-hybrid analysis. The columns NBS1-MRE11 or MRE11-NBS1 represent that NBS1 fused to GAL4-DNA-BD (or NBS1 fused to GAL4-AD) was coexpressed with a full-length hMRE11 fused to AD (or BD) in yeast strain GC-1945 (CLONTECH). β-Galactosidase activities of each transfectant are indicated by the following symbols: +++, color change within 1 h; +, color change within 3 h; −, no color change or weak color change over 12 h; U, undetermined. Note that a weak color change for S670-AD-hMRE11-BD (and for del 682–693) was detected after a 24-h incubation. The black box represents the putative domain that is essential for hMRE11 binding.

![Image of NBS1 expression](image)

**FIG. 3.** Expression of NBS1 protein in the NBS1-transfected cell lines. **a**, immunoblot analysis of NBS cells (GM7166VA7) and various NBS1 transfectants. **b**, formation of the triple-protein complex in NBS cells or in NBS1-transfected cells. Whole-cell extract was immunoprecipitated with anti-hRAD50 antibody, and the precipitants were analyzed by immunoblotting with anti-NBS1 (upper panel) or anti-hMRE11 antibody (lower panel). hMRE11 was always coimmunoprecipitated with hRAD50, because they directly interact each other. NBS1 was coimmunoprecipitated with hRAD50 when the mutant protein contained an hMRE11-binding domain (Full, S703, FE3, and BRCT-d).

![Image of radiation sensitivity](image)

**FIG. 4.** Radiation sensitivity of parental NBS (GM7166VA7) and NBS1-transfected cells. Exponentially growing cells were exposed to 4 Gy of γ rays, and survivals were determined by colony formation. Each point represents mean ± S.D. from at least five independent clones with duplicate experiments.

BRCT domains (FE3 and BRCT-d) also became radiation resistant to a degree similar to that seen in C-terminal end-deleted mutant (S703 in Fig. 4). These results imply that the FHA/BRCT domain is not essential for restoration of radiation resistance, i.e. for DNA repair.

Subsequently, observations were made to see whether the various truncated NBS1 proteins could restore the focus formation activity of the hRAD50-hMRE11-NBS1 complex in NBS cells after irradiation, because it has been reported that this
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triple-protein complex forms foci at DNA double-strand breaks in the nucleus after exposure to ionizing radiation (12). An absence of hMRE11 and hRAD50 in the nucleus was observed in NBS cells (GM7166VA7), and foci did not form when cells were irradiated with γ rays (Fig. 5, GM7166VA7; see Ref. 2). In contrast, nuclear localization of NBS1, hMRE11, and hRAD50 was detected in NBS cells transfected with full-length NBS1, and foci formation in the nucleus was clearly observed after irradiation (Fig. 5, +Full). A mutant of NBS1 containing the hMRE11-binding domain showed nuclear localization of hMRE11 and hRAD50 (Fig. 5, +S703, +FE3, and +BRCT-d), and the lack of the hMRE11-binding domain in the S670, S590, or the del 682–693 clone resulted in the cytoplasmic localization of the proteins. Because the cells in the absence of triple complex in nuclei (S590, S670, and del 682–693) remained radiation-sensitive (Fig. 4 and data not shown), these results suggest that the nuclear localization of hMRE11 and hRAD50 is necessary to restore the radiation resistance. Surprisingly, the FE3 and BRCT-d cells could not form foci in response to DNA damage (Fig. 5, +IR lanes, +FE3 and +BRCT-d) even though the triple-protein complex was able to localize in the nucleus. To confirm the inability of nuclear foci formation in FE3 mutant NBS1 protein, we transfected this mutant cDNA into HeLa cells. Significant reduction of foci formation after irradiation was observed in FE3-expressed HeLa cells, possibly by dominant negative effects (Fig. 6). This FE3-expressing HeLa cell clone showed no alteration of radiation sensitivity (data not shown), supporting the finding that FHA domain is not essential for restoration of radiation resistance.

**DISCUSSION**

NBS1 is reported to be essential for nuclear localization of hRAD50-hMRE11 complex (2) and to enhance the nuclear activity of the complex (7, 11). These observations suggest the function of NBS1 as a key regulator of both localization and activity of the triple-protein complex. We identified the essential region at codons 665–693 of NBS1 for hMRE11 binding. The present results also showed that the FHA/BRCT domain of NBS1 is essential for nuclear foci formation after DNA damage but not for cellular survival after irradiation. This is confirmed by evidence that foci formation was repressed in FE3-transfected HeLa cells. Zhao et al. (10) reported that the alteration of the phosphorylation site at both Ser273 and Ser343 residues markedly reduced the foci formation and radiation resistance, suggesting phosphorylation of NBS1 is necessary for both foci formation and DNA repair after irradiation. It is consistent with our result that the expression of a mutant NBS1 protein lacking both phosphorylation site and FHA/BRCT domain in GM7166VA cells was unable to complement not only nuclear foci formation of the complex but also radiation resistance of the cells (data not shown). Therefore, we conclude that FHA/BRCT domain, possibly sole FHA domain, is essential for the nuclear foci formation of the triple-protein complex together with the presence of both hMRE11-binding domain and the serine residues for phosphorylation.

A number of DNA repair-related proteins are known to form nuclear foci in response to DNA damage, such as RAD51, BRCA1, and BLM, as well as the hRAD50-hMRE11-NBS1 complex (1, 8, 12, 13), and these might be affected or regulated by phosphorylation signals. In view of this, the failure of the triple-protein complex in the FE3 and BRCT-d clones to form nuclear foci supports the putative functions of the FHA and BRCT domains, namely the FHA domain motif is for protein-protein interactions that recognize the phosphorylation state of the target protein (14), and the BRCT domain might provide a DNA-binding domain for repair-related proteins (15). The results shown here indicate that FHA/BRCT domain is essential for nuclear foci formation activity following DNA damage, even though they are not directly related to the DNA repair ability itself. This finding is consistent with the fact that most of the DNA double-strand breaks are rejoined within the first hour after irradiation (16), but foci formation persists even 5–8 h after irradiation (8, 12). Taken together, it is suggested that the nuclear foci formation is not a strict hallmark of DNA repair. Because the FHA/BRCT domain is conserved in eukaryotic NBS1 homologue, they might be involved in other crucial phenotypes of NBS, such as in insuring the fidelity of the rejoined DNA.

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