Distinct Functions of Nijmegen Breakage Syndrome in Ataxia Telangiectasia Mutated-Dependent Responses to DNA Damage

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Abstract
Phosphorylation of NBS1, the product of the gene mutated in Nijmegen breakage syndrome (NBS), by ataxia telangiectasia mutated (ATM), the product of the gene mutated in ataxia telangiectasia, is required for activation of the S phase checkpoint in response to ionizing radiation (IR). However, NBS1 is also thought to play additional roles in the cellular response to DNA damage. To clarify these additional functions of NBS1, we generated NBS cell lines stably expressing various NBS1 mutants from retroviral vectors. The ATM-dependent activation of CHK2 by IR was defective in NBS cells but was restored by ectopic expression of wild-type NBS1. The defects in ATM-dependent activation of CHK2, S phase checkpoint control, IR-induced nuclear focus formation, and radiation sensitivity apparent in NBS cells were not corrected by expression of NBS1 mutants that lack an intact MRE11 binding domain, suggesting that formation of the NBS1-MRE11-RAD50 complex is required for the corresponding normal phenotypes. Expression of NBS1 proteins with mutated ATM-targeted phosphorylation sites (serines 278 or 343) did not restore S phase checkpoint control but did restore the ability of IR to activate CHK2 and to induce nuclear focus formation and normalized the radiation sensitivity of NBS cells. Expression of NBS1 containing mutations in the forkhead-associated or BRCA1 COOH terminus domains did not correct the defects in radiation sensitivity or nuclear focus formation but did restore S phase checkpoint control in NBS cells. Together, these data demonstrate that multiple functional domains of NBS1 are required for ATM-dependent activation of CHK2, nuclear focus formation, S phase checkpoint control, and cell survival after exposure to IR.

Introduction
Damage to DNA triggers specific cellular responses that ensure the maintenance of genomic integrity. The induction of DNA strand breakage by ionizing radiation (IR) results in the activation of signaling pathways that lead either to elimination of the damaged cell by programmed cell death or to arrest of cell cycle progression and repair of the DNA breaks (1, 2). Among the various proteins that contribute to the DNA damage response, ataxia telangiectasia mutated (ATM) plays a prominent role. Cells from individuals with ataxia telangiectasia (AT) exhibit defects in cell cycle checkpoints operative in G1, S, and G2 phases as well as radiation hypersensitivity and an increased frequency of chromosome breakage (3).

ATM is a serine-threonine kinase that is both related to phosphoinositide 3-kinase and activated by exposure of cells to IR. Activated ATM, in turn, triggers the activation of cell cycle checkpoints and DNA repair through the phosphorylation of various proteins, including p53, MDM2, FAnCD, CHK2, BRCA1, SMC1, and NBS1 (3–17). In G1 checkpoint control, ATM phosphorylates p53 on Ser15 and MDM2 on Ser395, and these phosphorylation events appear to be required for the optimal induction and activation of p53 (4, 5, 14). In addition, the phosphorylation of CHK2 by ATM results in the phosphorylation of p53 on Ser20 by CHK2, contributing to inhibition of the binding of p53 to MDM2 and consequent p53 accumulation (13, 14, 18, 19). CHK2 also phosphorylates Ser123 of CDC25A in response to IR and thereby targets this protein for rapid degradation, resulting in inhibition of DNA replication (20).

NBS1 also plays an important role in the cellular response to DNA damage (21, 22). Mutations in the NBS1 gene are responsible for Nijmegen breakage syndrome (NBS) (23, 24), which, similar to AT, is characterized by hypersensitivity to IR, the failure of cells to arrest DNA synthesis in response to IR (radioresistant DNA synthesis, or RDS), chromosomal instability, and predisposition to cancer (25). NBS1 shares a low level of sequence homology with Saccharomyces cerevisiae Xrs2p and...
contains a forkhead-associated (FHA) domain and a BRCA1 COOH terminus (BRCT) domain in its NH2-terminal region (21). IR induces the formation of nuclear foci that contain a complex of NBS1 with MRE11 and RAD50 proteins, and these foci may represent sites of ongoing repair of DNA double-strand breaks (23, 26). Germ-line mutation of the MRE11 gene also results in an AT-like disorder (27). The phenotypic similarities among this latter disorder, AT, and NBS suggest that the NBS1-MRE11-RAD50 complex functions in ATM-dependent signaling in response to DNA damage.

Although a contribution of NBS1 to IR-induced activation of the S phase checkpoint has been demonstrated (9–12), the role of NBS1 in the G1 checkpoint has remained unclear. For example, NBS cells have been shown to be defective in the induction of p53 and in G1 checkpoint control in some studies but not in others (9, 28–31). There are also conflicting reports on the importance of NBS1 in the G2 checkpoint (29, 32, 33). Finally, studies on the role of NBS1 phosphorylation by ATM in radiation sensitivity, in the IR-induced formation of NBS1-MRE11-RAD50 foci, and in G2 checkpoint control have drawn inconsistent conclusions (9–12). The accumulated evidence indicates, however, that the phenotypic abnormalities shared by individuals with AT and those with NBS are not explained simply by the enzyme-substrate relation between ATM and the NBS1-MRE11-RAD50 complex. Consistent with this conclusion, NBS1 has recently been shown to be required for the regulation of additional targets of ATM, including CHK2 and SMC1 (15, 16, 31, 32).

To clarify the role of NBS1 in the cellular response to DNA damage and to provide insight into the abnormal phenotypes associated with NBS, we characterized the effects of mutation of the various structural and functional domains of NBS1 on ATM-dependent activation of CHK2, S phase checkpoint control, the IR-induced formation of nuclear foci, and radiation sensitivity. We performed these experiments by examining the ability of such NBS1 mutant proteins to complement the various abnormal phenotypes of NBS cells.

Results

Functional Domains of NBS1

To define the functional domains of NBS1 in DNA damage responses, we first established NBS cell lines expressing various hemagglutinin epitope (HA)-tagged NBS1 mutants from retroviral expression vectors based on pMSCV-puro. We focused our attention on the FHA domain (amino acids 24–100), the BRCT domain (residues 105–190), the ATM phosphorylation sites (Ser278, Ser343), and the MRE11 binding domain (residues 601–700) (Fig. 1A). We thus introduced point mutations at two important conserved residues (R28A, H45A) within the FHA domain, a small deletion in the BRCT domain, and point mutations at the ATM phosphorylation sites (S278A, S343A). We also generated NBS1 deletion mutants that are not able to associate with MRE11 and various other serial deletion mutants. Immunoprecipitation and immunoblot analysis of Low-dose IR.

The expression of the various mutants in NBS-ILB1 cells was confirmed by immunoblot analysis with antibodies either to HA (Fig. 1C) or to NBS1 (data not shown). Given that the level of expression differed among the mutant proteins, we also generated cell lines expressing some of the mutant proteins at a lower level from retroviral vectors based on pBABE-puro. No qualitative differences were apparent in the various assays between cell lines expressing the same mutant proteins from pMSCV-puro or from pBABE-puro, thus excluding the possibility that the different expression levels of the different mutants were responsible for observed functional differences.

Role of NBS1 in ATM-Dependent CHK2 Phosphorylation Induced by IR

Given that checkpoint activation requires the Xrs2p-Mre11p-Rad50p complex in yeast and that NBS1 is required for ATM-dependent activation of CHK2, we examined the ability of IR to induce CHK2 activation in our various NBS-ILB1 cell lines. Consistent with previous observations (32), we showed that IR (5 Gy) did not induce a shift in the electrophoretic mobility of CHK2 (reflecting CHK2 phosphorylation and its consequent activation) in NBS cells or AT cells examined 30 min after irradiation (Fig. 2A). Exposure of AT or NBS cells to various doses of IR up to 20 Gy also did not induce the activation of CHK2 examined after 30 min (Fig. 2B). Whereas the activation of CHK2 by IR (10 Gy) was apparent within 30 min of irradiation in normal cells, the electrophoretic mobility of CHK2 in AT and NBS cells remained unaffected until 2–4 h after irradiation (Fig. 2C). These results thus suggested that NBS is required for the rapid phosphorylation of CHK2 in response to low-dose IR.

Stable expression of wild-type NBS1 in NBS-ILB1 cells restored the ability of IR to induce the phosphorylation of CHK2 (Fig. 2A), demonstrating that the defect in CHK2 activation in NBS cells is attributable entirely to NBS1 deficiency. Whereas Buscemi et al. (32) also showed that CHK2 activation by IR requires NBS1, our results differ from those of this previous study in that expression of the S343A mutant of NBS1 in NBS-ILB1 cells restored the ability of IR to induce the shift in the electrophoretic mobility of CHK2. This observation thus suggests that the ATM-dependent phosphorylation of NBS1 on Ser343 is not required for the IR-induced activation of CHK2 and is similar to our previous observation that NBS1 phosphorylation is not required for ATM-dependent phosphorylation of SMC1 (15).

Role of NBS1-MRE11-RAD50 Complex Formation in ATM-Dependent CHK2 Phosphorylation Induced by IR

To identify the functional domains of NBS1 required for ATM-dependent CHK2 activation, we examined the effect of IR on the phosphorylation of CHK2 in NBS-ILB1 cells stably expressing other mutants of NBS1. Cells expressing NBS1 mutants lacking ATM phosphorylation sites (S343A, S278A, S343A, d1, d2) exhibited an apparently normal, dose-dependent activation of CHK2 in response to IR (Fig. 3A), again indicating that phosphorylation of NBS1 by ATM is not required for ATM-dependent CHK2 activation. In addition, the
FHA and BRCT domains of NBS1 did not appear to contribute to ATM-dependent CHK2 phosphorylation in response to IR. However, expression of the NBS1 deletion mutants d5 or d7, which lack the MRE11 binding domain, did not restore the ability of IR to induce phosphorylation of CHK2, suggesting that formation of the NBS1-MRE11-RAD50 complex is required for the ATM-dependent activation of CHK2 in response to IR. The mutant d6, which contains a deletion located near the MRE11 binding domain, also failed to restore IR-induced phosphorylation of CHK2, suggesting that the role of NBS1 in ATM-dependent phosphorylation of CHK2 is also likely mediated by a mechanism other than that dependent on MRE11 binding.

**Role of NBS1 in p53 Phosphorylation on Ser20**

Our observation that activation of CHK2 in response to IR appears to require both ATM and formation of the NBS1-MRE11-RAD50 complex prompted us to examine the CHK2-dependent phosphorylation of p53 on Ser20 in our NBS cell lines. Consistent with the results obtained for the analysis of CHK2 activation, the ability of IR to induce phosphorylation of p53 on Ser20 was defective in NBS-IBL1 cells and this defect was corrected by stable expression of wild-type NBS1 or of the various NBS1 mutants with the exception of d5, d6, and d7 (Fig. 3B). The defect in IR-induced phosphorylation of p53 on Ser20 in NBS cells thus reflects the failure of IR to activate CHK2 in these cells.

**NBS1 Domains Required for Radiation Resistance, Formation of NBS1-MRE11-RAD50 Foci, and S Phase Checkpoint Control**

The precise molecular mechanism (or mechanisms) responsible for the increased radiation sensitivity of AT and NBS cells is not clear. To determine the structural basis of the radioresistance afforded by NBS1, we examined the effect of expression of NBS1 mutants on the sensitivity of NBS-IBL1 cells to IR. Colony survival assays revealed that expression of wild-type NBS1 constructs (WT, S343A, S278A/S343A) or the deletion mutants d1, d3, or d4 also largely restored radiation resistance in NBS-IBL1 cells. However, we cannot rule out the possibility of
partial complementation of radiation sensitivity by these mutants because of the relatively small difference in radiation sensitivity of NBS cells expressing them. In contrast, the FHA domain mutants (R28A, H45A), the BRCT domain mutant, d5, or d7 failed to restore radiation resistance in NBS-ILB1 cells to the level observed in cells expressing wild-type NBS1. These results thus indicated that the FHA domain, the BRCT domain, and the MRE11 binding domain of NBS1 play important functional roles in cell survival after exposure to IR.

We next investigated the relations among radiation sensitivity, the IR-induced formation of NBS1-MRE11-RAD50 foci, which is implicated in the recognition and repair of DNA double-strand breaks (23, 26), and S phase checkpoint control (RDS phenotype). The formation of NBS1-MRE11-RAD50 foci was examined 30 min or 8 h after irradiation by immunofluorescence analysis with antibodies to NBS1 and to MRE11. Whereas NBS-ILB1 cells infected with the empty vector exhibited no detectable nuclear foci, such foci containing NBS1 and MRE11 were abundant at both time points after irradiation in cells stably expressing wild-type NBS1 (Fig. 4B, data not shown). IR also induced the formation of nuclear foci in cells expressing the S343A or S278A/S343A mutants, indicating that the phosphorylation of NBS1 by ATM is not required for this effect. Consistent with previous observations (35, 36), cells expressing NBS1 mutants that lack the MRE11 binding domain (d5, d7) exhibited a diffuse cytoplasmic localization of both NBS1 and MRE11, suggesting that the nuclear localization of MRE11 and RAD50 is required for the restoration of radiation resistance in NBS cells. Cells expressing the FHA or BRCT domain mutants, which did not restore radiation resistance, also did not form nuclear foci in response to IR, even though the NBS1-MRE11-RAD50 complex was localized to the nucleus.

RDS assays revealed that NBS-ILB1 cells transfected with empty vector or expressing the NBS1 mutants S343A, S278A/S343A, d2, d5, or d7 did not exhibit optimal inhibition of DNA synthesis on exposure to IR, whereas cells expressing wild-type NBS1, the FHA or BRCT domain mutants, or the other deletion mutants did (Fig. 4C). These data thus indicate that the FHA and BRCT domains are not required for the inhibition of DNA synthesis after irradiation. Consistent with previous observations (9–12), only the ATM phosphorylation sites and the MRE11 binding domain of NBS1 appear to be required for S phase checkpoint control. Cells expressing the S343A or S278A/S343A mutants exhibited normal CHK2 activation despite manifesting the RDS phenotype, consistent with the notion that phosphorylation of NBS1 and that of CHK2 by ATM function in different pathways (20, 37). Together, our results thus suggest that cell viability after exposure to IR is correlated with the IR-induced formation of NBS1-MRE11-RAD50 foci but not with S phase checkpoint control.

### Discussion

Given the important role of the NBS1-MRE11-RAD50 complex in DNA damage-induced checkpoint control and DNA repair, we examined the functional domains of NBS1 required for ATM-dependent responses to DNA damage. We showed that the time-dependent phosphorylation of CHK2 in response to low-dose IR was defective in both AT and NBS cells and that the ability of IR to induce CHK2 phosphorylation in NBS cells was restored by ectopic expression of NBS1, suggesting that the activation of CHK2 by IR requires both NBS1 and ATM. The phosphorylation of CHK2 was apparent in NBS cells at late time points after irradiation, suggesting that NBS1 is specifically required for the rapid phosphorylation of CHK2. High doses of IR also activate CHK2 independently of ATM and NBS1 (32), suggesting that regulation of CHK2, especially in response to high-dose IR, is also mediated by a distinct mechanism. The IR-induced phosphorylation of CHK2 in NBS cells was also not restored by expression of NBS1 mutants that lack an intact MRE11 binding domain, consistent with the notion that the activation of CHK2 by low-dose IR requires formation of the NBS1-MRE11-RAD50 complex.

Consistent with the results obtained for CHK2 activation, the phosphorylation of p53 on Ser20 was also found to be dependent on formation of the NBS1-MRE11-RAD50 complex. We have previously shown that ATM activation, G1 checkpoint control, and phosphorylation of p53 on Ser15 by ATM appear normal in primary NBS cells (9). The phosphorylation by ATM of p53 on Ser15 and of MDM2 on Ser395 may therefore be sufficient for the induction of G1 checkpoint control in NBS cells despite the defect in p53 phosphorylation on Ser20.

The ATM-dependent phosphorylation of NBS1 on Ser343 is essential for S phase checkpoint control. The defect in S phase checkpoint control apparent in NBS cells was not comple-
mented by expression of the S343A or S278A/S343A mutants of NBS1, even though these mutants restored normal phosphorylation of CHK2 and formation of nuclear foci in response to IR as well as normal radiation resistance. These results are consistent with the notion that the radiation sensitivity of NBS cells is independent of the S phase checkpoint defect (9, 33, 38). The phosphorylation of SMC1 by ATM has been shown to be required for S phase checkpoint control (15, 16), although expression of the S343A mutant of NBS1 in NBS cells complemented phosphorylation of SMC1 by ATM in one study (15), whereas the S278A/S343A mutant of NBS1 failed to do so in another study (16). Taken together, these observations support the proposal that ATM–NBS1-MRE11-RAD50–SMC1 and ATM-CHK2-CDC25A pathways function independently in control of progression into or through S phase after exposure of cells to IR (37).

We also showed that the FHA and BRCT domains of NBS1, but not phosphorylation of NBS1 by ATM, were required for both nuclear focus formation and radiation resistance. However, in contrast to our results, a previous study found that neither the FHA domain nor the BRCT domain of NBS1 contributed to radiation resistance (35), and another study showed that both formation of nuclear foci and radiation resistance require an intact MRE11 binding domain as well as the FHA and BRCT domains of NBS1 (39). Although the reasons for these discrepancies are not clear, our observation that both formation of nuclear foci and radiation resistance require an intact MRE11 binding domain as well as the FHA and BRCT domains of NBS1 may provide insight into the radiation sensitivity of NBS cells. The fact that the NBS1-MRE11-RAD50 complex relocates to the vicinity of DNA strand breaks and forms nuclear foci in response to IR suggests a role for this complex in sensing or repair of such strand breaks (21, 23, 26). Mutation of either the MRE11 binding, FHA, or BRCT domains of NBS1 might therefore impede the localization of NBS1-MRE11-RAD50 to sites of DNA damage and the consequent repair of strand breaks and thereby increase radiation sensitivity. A role for the NBS1-MRE11-RAD50 complex in DNA repair has also been suggested by the observations that NBS1 modulates the nuclease activity of MRE11 in vitro (40, 41) and is essential for DNA repair by homologous recombination in vertebrate cells (42). In addition, studies showing that the FHA and BRCT domains of NBS1 are required for the association of NBS1 with chromatin and γ-H2AX (39, 43) also suggest a functional role

FIGURE 3. Requirement for NBS1-MRE11-RAD50 complex formation in the ATM-dependent phosphorylation and activation of CHK2 in response to IR. NBS-ILB1 cells stably expressing the indicated NBS1 proteins were exposed to the indicated doses of IR and then incubated for 30 min, after which cell lysates were subjected to immunoblot analysis with antibodies to CHK2 (A) or with antibodies either to p53 or to p53 phosphorylated on Ser20 (B).
for these domains of NBS1 in nuclear focus formation. Consistent with a role for NBS1 in DNA repair, NBS1-MRE11-RAD50 forms a complex that also contains BRCA1 and SMC1 (16, 44). The ATM-dependent phosphorylation of SMC1 requires NBS1 and BRCA1, and cells expressing SMC1 with mutated ATM phosphorylation sites exhibit increased radiation sensitivity (15). These observations thus raise the possibility that the ATM–NBS1-MRE11-RAD50–BRCA1–SMC1 signaling pathway is a major contributor to cell survival after exposure to IR.

NBS1 does not mediate IR-induced signaling upstream of ATM, given that activation of ATM and phosphorylation of ATM target sites (Ser15 of p53, Thr68 of CHK2) appears normal in NBS cells (9) (data not shown). However, previous studies (16, 32, 34) and our present observations have shown that regulation of ATM target proteins such as CHK2 and SMC1 in response to DNA damage requires the NBS1-MRE11-RAD50 complex as well as ATM. We therefore propose that NBS1 is not only an ATM substrate but also functions either as a mediator of ATM signaling or as a scaffold protein in ATM-dependent responses to DNA damage, thereby promoting phosphorylation by ATM of its many downstream substrates.

Materials and Methods

Cell Culture

EBV-immortalized lymphoblastoid cell lines, including normal cells (GM0536; NIGMS Human Mutant Cell Repositories), AT cells (GM1526), and NBS cells (GM7078B), were cultured in RPMI 1640 supplemented with 15% fetal bovine serum (HyClone Laboratories, Logan, UT). The SV40-transformed NBS fibroblast cell line NBS-ILB1 (kindly provided by M. Zdzienicka, Leiden University) was grown in DMEM supplemented with 10% fetal bovine serum.

Antibodies

Mouse polyclonal antibodies to the Flag epitope, rabbit polyclonal antibodies to the HA, and rabbit polyclonal antibodies to CHK2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies to NBS1 were from Novus Biologicals (Littleton, CO). Mouse monoclonal antibodies to MRE11 (12D7) were from GeneTex (San Antonio, TX). Mouse polyclonal antibodies to p53 and to p53 phosphorylated on Ser20 were from Cell Signaling (Beverly, MA).
**Plasmids and Cell Transfection**

A full-length cDNA for wild-type human NBS1 was generated and cloned into the pSG5 vector (Stratagene, La Jolla, CA). The cDNAs for various NBS1 mutants were generated by site-directed mutagenesis with the use of a QuickChange kit (Stratagene) and were subcloned into pMVC5-puro (Clontech Laboratories, Inc., Palo Alto, CA) for the generation of retroviral expression vectors. NBS-ILB1 cell lines that stably express HA-tagged wild-type or mutant NBS1 proteins were generated by retroviral infection as described (9). Transient transfection of 293T cells with vectors for HA-tagged NBS1 mutants and Flag-tagged MRE11 was performed with the use of calcium phosphate.

**Immunoprecipitation and Immunoblot Analysis**

Cells were lysed in NP40 lysis buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM MgCl₂, 1% NP40] supplemented with protease inhibitors [aprotinin (10 μg/ml), leupeptin (10 μg/ml), 1 mM phenylmethylsulfonyl fluoride] and phosphatase inhibitors (1 mM NaF, 1 mM Na₃VO₄). For immunoprecipitation, equal amounts of cell lysate (2 mg of protein in 900 μl) were incubated at 4°C for first 2 h with 5 μg of antibodies to Flag and then for 1 h in the additional presence of 30 μl of protein G-agarose (Oncogene). The resulting immunoprecipitates or cell lysates were fractionated by SDS-PAGE on a 7.5% gel and then subjected to immunoblot analysis.

**Colony Survival Assays**

For determination of radiation sensitivity, cells were plated in triplicate at limiting dilutions in six-well plates after exposure to 0, 1, 2, or 4 Gy of IR. After incubation at 37°C for 2 weeks, the cells were fixed with 95% methanol and stained with crystal violet. A population of >80 cells was counted as one colony, and the number of colonies was expressed as a percentage of the value for nonirradiated control cells. The mean ± SD values of the triplicates for each radiation dose were determined.

**Immunostaining of NBS1-MRE11-RAD50**

For determination of the effect of IR on the subcellular localization of the NBS1-MRE11-RAD50 complex, cells (1 × 10⁴ to 2 × 10⁴) were plated on eight-well glass chamber slides, cultured for 24 h, and exposed to 10 Gy of IR. The cells were then incubated for an additional 30 min or 8 h, fixed for 20 min with PBS containing 3% paraformaldehyde and 2% sucrose, and permeabilized for 20 min with PBS containing 0.2% Triton X-100. After washing with PBS, the cells were incubated overnight at 4°C with PBS containing 2% goat serum, rabbit antibodies to NBS1, and mouse antibodies to MRE11. The cells were washed with Tris-buffered saline and then incubated with FITC-conjugated goat antibodies to mouse IgG and rhodamine-conjugated goat antibodies to rabbit IgG (Oncogene Science, Uniondale, NY).

**RDS Assay**

Transient inhibition of DNA synthesis after irradiation was assessed as described (45). In brief, cells in the logarithmic phase of growth were cultured in the presence of [³H]thymidine (10 nCi/ml) for ~24 h to provide an internal control for cell number (by allowing normalization for total DNA content of samples). The cells were then cultured for an additional 24 h in normal culture medium, irradiated, incubated for 30 min, and then labeled for 15 min with [¹⁴C]thymidine (2.5 μCi/ml). After harvesting and washing twice with PBS, the cells were fixed for at least 30 min at 4°C with 70% methanol, transferred to Whatman filters, and exposed sequentially to 70% and 95% methanol. The filters were air-dried and the amount of associated radioactivity was determined with a liquid scintillation counter. The [³H]:[¹⁴C] ratio for each sample was calculated and expressed as a percentage of the value for nonirradiated control cells.

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**References**


