Nibrin Forkhead-associated Domain and Breast Cancer C-terminal Domain Are Both Required for Nuclear Focus Formation and Phosphorylation*

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The Mre11-Rad50-nibrin protein complex plays an essential role in the mammalian cellular response to DNA double-strand breaks. The disorder Nijmegen breakage syndrome (NBS) results from mutations in the NBS1 gene that encodes nibrin, and NBS cells are radiosensitive and defective in S-phase checkpoint activation following irradiation. In response to radiation, nibrin is phosphorylated by Atm, and the Mre11-Rad50-nibrin complex relocalizes to form punctate nuclear foci. The N terminus of nibrin contains a forkhead-associated (FHA) domain and a breast cancer C-terminal (BRCT) domain, the functions of which are unclear. To determine the role of the FHA and BRCT domains in nibrin function, we have performed site-directed mutagenesis of conserved residues in these motifs. Mutations in the nibrin FHA and BRCT domains did not affect interaction with Mre11-Rad50 or nuclear localization of the complex. However, mutation of conserved residues in either domain disrupted nuclear focus formation and blocked nibrin phosphorylation after irradiation, suggesting that these events may be functionally interdependent. Despite an effect on nibrin phosphorylation, expression of the FHA or BRCT mutants in NBS cells restored the downstream phosphorylation of Chk2 and Smc1, necessary for S-phase checkpoint activation. None of the mutations revealed separate functions for the FHA or BRCT domains, suggesting they do not function independently.

Nijmegen breakage syndrome (NBS) is a rare autosomal recessive disorder characterized by growth retardation, microcephaly, immunodeficiency, and an increased incidence of lymphoid cancers (1). Cells from NBS patients are sensitive to ionizing radiation, or other agents that induce DNA double-strand breaks, and display chromosomal instability, frequently involving the immune receptor loci on chromosomes 7 and 14 (1). In addition, NBS cells have defects in S-phase cell cycle checkpoint control following exposure to ionizing radiation (2). NBS is caused by mutations in the NBS1 gene on chromosome 8q21 that encodes a 754-amino acid protein, nibrin (3–5). Patients with NBS have truncating mutations in the NBS1 gene that result in premature termination of nibrin, and their cells fail to make detectable full-length protein (3, 5).

The phenotype of NBS cells suggests a defect in the detection, signaling, or repair of DNA double-strand breaks. Consistent with this possibility, nibrin is found in a complex with Mre11 and Rad50 in vivo, two proteins with established DNA repair functions (3, 6). Nibrin interacts directly with Mre11 via sequences in the C terminus of nibrin, and this interaction is required for the nuclear localization of the Mre11-Rad50-nibrin complex (7). Within the nucleus the Mre11-Rad50-nibrin complex is distributed unevenly, aggregating in nuclear foci that some reports divide into distinct classes (8, 9). Type I foci are observed in untreated cells where they co-localize with PML bodies (9, 10). Type II and type III foci are induced by agents such as ionizing radiation, which create DNA double-strand breaks, and arise at putative sites of DNA repair (8, 9, 11). Other cellular proteins involved in DNA damage responses also accumulate at these sites with varying timing and kinetics (12, 13).

In addition to nuclear relocalization, nibrin is phosphorylated upon exposure of cells to ionizing radiation. Nibrin phosphorylation is carried out by the Atm protein kinase, mutated in the radiation sensitivity disorder ataxia-telangiectasia (A-T), and occurs primarily on two residues, Ser-278 and Ser-343, although other nibrin residues may be targets as well (2, 14–16). The function of nibrin phosphorylation is unknown but has been reported to be required for radiation-induced nuclear focus formation by the Mre11-Rad50-nibrin complex (16) and for activation of the S-phase cell cycle checkpoint following radiation (2, 16). Recent investigations (17–20) have suggested that both arms of the S-phase checkpoint, involving Atm phosphorylation of either Chk2 or Smc1, are dependent on nibrin expression.

Despite this knowledge, the function of nibrin is the most poorly understood of the three components of the Mre11-Rad50-nibrin complex. NBS1 mutant alleles identified in NBS patients appear to be hypomorphic, likely obscuring essential functions of nibrin that result in embryonic lethality in mice carrying null mutations of the Nbs1 gene (21, 22). Unlike Mre11 and Rad50, mammalian nibrin displays only limited sequence similarity with its functional homologue in Saccharomyces cerevisiae, Xrs2 (3). Two potentially functional domains, a forkhead associated (FHA) domain (residues 24–100),
and a breast cancer C-terminal (BRCT) domain (residues 114–182) are readily discernible in the primary sequence of human nibrin (5). These domains are frequently observed in proteins involved in the DNA damage response, although the juxtaposition of an FHA and a BRCT domain appears unique to nibrin (23, 24). FHA domains have been shown to mediate phospho-protein interactions, such as that between Rad53 and phosphorylated Rad9 or the homodimerization of phosphorylated Chk2, whereas BRCT domains mediate direct protein-protein interactions, such as those involving 53BP1 and p53 (25–29). The availability of known ligands for these and other FHA and BRCT domains has facilitated the identification of critical residues for protein interactions mediated by these domains through the use of site-specific mutagenesis and x-ray crystallography.

The role of the FHA and BRCT domains in nibrin function has yet to be clearly defined. It is noteworthy that all known mutations in NBS patients truncate nibrin downstream of these motifs, suggesting the FHA and BRCT domains are indispensable for nibrin function (5, 30). We have previously demonstrated that a C-terminal 353-amino acid fragment of nibrin, lacking both the FHA and BRCT domains, failed to restore cell survival following irradiation and nuclear focus formation when expressed in NBS cells (7). N-terminal truncation of the nibrin FHA domain was also shown to be sufficient to disrupt nuclear focus formation, although the truncation mutant was characterized by protein instability, resulting in a low level of expression and several protein species of different molecular weights (31). There is also scant information regarding the proteins that interact with the nibrin FHA and BRCT domains. Maser et al. (32) demonstrated an interaction between the N-terminus of nibrin, including the FHA and BRCT domains, and the E2F1 transcription factor in a yeast two-hybrid analysis, although this interaction constituted only a small portion of endogenous nibrin. Moreover, yeast two-hybrid analysis is unable to detect phosphoprotein interactions such as those mediated by FHA domains.

To address the role of protein interactions mediated by the nibrin FHA or BRCT domains in nibrin function, we have performed site-directed mutagenesis of these targets. Targeted mutations were made at residues in the nibrin FHA and BRCT domains that were deemed critical for interaction with their ligands, based on analogy to other previously studied FHA- or BRCT-containing proteins. These mutated constructs were stably introduced into NBS cells by retroviral transduction, and the effects on the DNA damage response were analyzed. We find that disruption of interactions involving either the FHA or the BRCT domain blocks nuclear focus formation by the Mre11-Rad50-nibrin complex in irradiated cells. Surprisingly, these nibrin mutants are not phosphorylated in response to ionizing radiation exposure, but their expression restores the S-phase checkpoint.

**Site-specific Mutagenesis**—Five point mutations, R28A, H45A, D95N, G136E, G137E (labeled as GG136–137ERE in the figures), and Y176A, were introduced into the nibrin FHA and BRCT domains using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA). The corresponding nucleotide changes, 82–83AG → GC, 153–154CA → GC, 285G → A, 407G → A, 410G → A, and 526–527TA → GC, were made in individual NBS1 cDNAs using complementary oligonucleotide primers overlapping the nucleotides to be changed. The following primer sequences were utilized: R28A, 5′-CGTGTGACTGTTGTTGCGGCAAAATCGTCCATTTGG-3′; H45A, 5′-CATGTGACGCAAGGTTGC-3′; D95N, 5′-GCCAACATTTGAGTTCGTTGTATTCCATTTTTG-3′; G136E, 5′-GATATTGGCAACATGAATTTACTGATGAACTGGACAG-3′; and Y176A, 5′-GTCGCAATTTGAAAGCGAGCCTTGTATGTTTTATC-3′. QuikChange PCR was performed on an EcoRI fragment of the NBS1 cDNA that encompassed the FHA and BRCT domains, from 62 of the 5′ untranslated region to nucleotide 536, subcloned in pBluescript (Strategene). PCR reactions were performed according to the manufacturer’s specifications using 10–20 ng of double-stranded plasmid DNA; 125 ng each of the complementary forward and reverse mutagenic primers; 200 μM each of dATP, dCTP, dGTP, and dTTP; 10 mM KCl; 10 mM (NH4)2SO4; 20 mM Tris-HCl, pH 8.8; 2 mM MgSO4; 0.1% Triton X-100; 100 μg/ml bovine serum albumin; and 2.5 units of Pfu Turbo DNA polymerase (Stratagene) in a final volume of 25 μl. PCR was performed at 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 7 min for 16 cycles. The resultant PCR product was incubated with 10 units of DpnI (Roche Applied Science, Indianapolis, IN) at 37 °C for 1 h to digest the parental plasmid template, and 1 μl of the reaction was transformed into XL1-Blu supercompetent bacteria (Strategene). Resultant recombinant colonies were screened for the appropriate nucleotide mutation by fluorescent sequencing. Individual mutations were introduced into a full-length NBS1 cDNA by subcloning the mutated EcoRI fragment into an EcoRI digested full-length NBS1 cDNA in pBluescript (34).

**Retroviral Gene Expression**—For retroviral gene expression, a BanHI-NcoI fragment of a mutant NBS1 cDNA was cloned into the XhoI site of the pLXIN retroviral vector (BD Biosciences, San Diego, CA), upstream of the internal ribosome entry site-neomycin cassette. This fragment extended from –62 of the 5′ untranslated region to position 2286 of the NBS1 cDNA, 21 bp 3′ of the stop codon, and just upstream of the polyadenylation signal. Retroviral infection of mutant NBS1 cDNAs was performed as described previously (Ref. 34, see also www.stanford.edu/group/nolan). Briefly, 15 μg of retroviral plasmid DNA was introduced into Phoenix A cells using calcium phosphate transfection, and viral supernatants were harvested after 48 h. Following filtration through a 0.45-μm filter, viral supernatants were incubated with NBS-ILB1 target cells for 24 h. Stable bulk cell lines were selected with 1 mg/ml G418 starting 48 h after infection of NBS-ILB1 cells with the viral supernatant.

**Western Blot Analysis**—Protein expression or modification was analyzed by Western blotting. Total cell lysates were prepared by lysing fibroblast cell lines at a final concentration of 106 cells/ml in 1.1× LDS sample buffer (11% glycerol, 155 mM Tris base, 117 mM Tris HCl, 2.2% LDS, 561 μM EDTA, 242 μM Serva Blue G250, 189 μM phenol red) containing 1 mM sodium vanadate and a protease inhibitor mixture (Roche Applied Science). To assess protein phosphorylation following irradiation, cells were exposed to 10 μg of ionizing radiation and harvested after 1 h. Alternatively, immunoprecipitates were prepared by lysing 2×106 cells in 50 mM sodium phosphate, pH 7.2, 0.5% Triton X-100, 2 mM EDTA, 2 mM EGTA, 25 mM sodium fluoride, 25 mM glycerophosphate, 2 mM sodium orthovanadate, and 1 mM sodium vanadate, and then incubating with Roche Applied Science). Lysates were pre-cleared with normal rabbit IgG (Zymed Laboratories Inc., South San Francisco, CA) and GammaBind Plus-Sepharose (Amersham Biosciences, Piscataway, NJ) for 1 h and then were immunoprecipitated with rabbit polyclonal anti-nibrin antiserum (Novus Biologicals, Littleton, CO). Immunoprecipitates were washed with lysis buffer four times, resuspended in 20 μl of 1.1× LDS buffer, and boiled for 5 min. Prior to gel electrophoresis, 9 parts of cell lysate or immunoprecipitate were mixed with 1 part of 10× NuPAGE sample-reducing agent (Invitrogen) and heated for 10 min at 70 °C.

Detecting gel electrophoresis was performed using either 7% or 3–8% gradient NuPAGE Tris acetate gels (Invitrogen). 10% cell equivalents of total cell lysates or 106 cell equivalents of immunoprecipitates were loaded per lane and electrophoresed at 150 V in Tris acetate SDS running buffer (50 mM Tricine, 50 mM Tris base, 0.1% SDS) with...
Nibrin FHA and BRCT Domains

Mutation of Nibrin FHA and BRCT Domain-conserved Residues—To disrupt the function of the nibrin FHA and BRCT domains, we introduced mutations in amino acid residues that are conserved among FHA and BRCT domains and/or residues that have been demonstrated to be necessary for ligand binding by these domains in other proteins. As shown in Fig. 1A, two conserved amino acids in the nibrin FHA domain, arginine at position 28 and histidine at position 45, were changed to alanine by site-directed mutagenesis. In the x-ray crystal structure of the Rad53 FHA1 domain, these conserved amino acids are located in peptide loops that bind phosphorylated Rad9 (24, 35). The conserved arginine binds the phosphotyrosine residue and the phosphopeptide backbone directly, whereas the conserved histidine stabilizes the architecture of the phosphopeptide binding site (35). Mutation of these residues to alanine in Rad53 ablated binding of the FHA1 domain to phosphorylated Rad9 (26). As a control, we introduced a third mutation in the nibrin FHA domain, D95N. This sequence variant of nibrin was identified among normal individuals and is not associated with any pathologic phenotype (36). In the crystal structure of the Rad53 FHA1 domain, the residue corresponding to Asp-95 is located on a peptide loop facing away from the phosphopeptide and does not participate in peptide binding (24).

To disrupt protein interactions mediated by the BRCT domain of nibrin, mutations were introduced in the two conserved motifs identified in known BRCT sequences. Motif-1 consists of a highly conserved glycine/glycine-glycine-alanine pair located between the α1 helix and the β2 strand of the BRCT domain, based on the XRCC1 crystal structure (23). These residues provide the flexibility required for the sharp turn connecting the two structures. As shown in Fig. 1B, we changed the two corresponding glycines at position 136–137 of the nibrin BRCT domain to glutamic acid. Motif-2 of the BRCT domain is centered around a highly conserved aromatic residue, usually tryptophan (23). In nibrin, this residue is a tyrosine at position 176 that was changed to alanine (Fig. 1B). Motif-2 occurs within the α3 helix of the BRCT domain that participates in ligand binding, and the tryptophan residue likely stabilizes the conformation of the BRCT recognition motif (23, 28, 29). Mutation of the conserved tryptophan in the XRCC1 BRCT domain has been reported to abolish binding to DNA ligase III (23).

Point Mutations in the Nibrin FHA or BRCT Domains Do Not Affect Interaction with Mre11-Rad50—To assess the effects of the mutations made in the FHA and BRCT domains of nibrin, NBS1 cDNAs carrying individual mutations were cloned into the pLXIN retroviral vector and introduced into a
Nibrin FHA and BRCT Domains

Bulk cell lines stably expressing the nibrin transgenes were selected in G418. NBS-ILB1 cells are homozygous for the common Slavic mutation, 657del5, and produce undetectable levels of full-length nibrin protein (33). We have previously shown that introduction of a wild type NBS1 cDNA into NBS-ILB1 cells restored full-length nibrin expression and complemented radiation sensitivity (34). In response to ionizing radiation, exogenously expressed nibrin was phosphorylated on Ser-343 and relocated to form nuclear foci (14, 34).

Expression of the nibrin FHA and BRCT domain mutants in NBS-ILB1 cells was assessed by Western blot using an anti-nibrin antibody. All of the FHA and BRCT domain mutant cell lines expressed full-length nibrin protein when compared with NBS-ILB1 cells infected with wild type NBS1 or a normal fibroblast control (Fig. 2). Although nibrin was readily detectable in all cell lines, there was variation in the level of expression between the different FHA and BRCT mutants. The FHA R28A and D95N mutants expressed about 2-fold less nibrin than NBS-ILB1 cells expressing the wild type NBS1 cDNA, whereas the FHA H45A mutant and the BRCT G136E,G137E and Y176A mutants expressed 5- to 6-fold less nibrin as the NBS1 cell line.

To determine if the nibrin FHA and BRCT mutants could interact with the Mre11 and Rad50 proteins, total cell lysates were immunoprecipitated with anti-nibrin antisera and analyzed by Western blotting with antibodies to nibrin, Mre11, and Rad50 (Fig. 3). In comparison to NBS-ILB1 cells infected with the LXIN vector alone, readily detectable levels of Mre11 and Rad50 protein were immunoprecipitated from all the FHA and BRCT mutant cell lines. The levels of Mre11 and Rad50 protein detected varied slightly, according to the level of nibrin protein expressed in the different mutant cell lines. These results indicate that mutations in the FHA and BRCT domains of nibrin did not affect interaction with Mre11 and Rad50, consistent with the Mre11 interaction domain being located in the C terminus of the nibrin protein (7).

Radiation-induced Nuclear Focus Formation Is Disrupted by Point Mutations in the Nibrin FHA and BRCT Domains—Immunofluorescence staining was performed to examine the cellular localization of nibrin FHA and BRCT mutant proteins. Cells were co-stained with anti-nibrin antisera and a monoclonal antibody to Mre11 and analyzed by confocal microscopy. In all FHA and BRCT mutant cell lines, nibrin was correctly localized to the nucleus (Fig. 4). Consistent with Western blot results, the FHA and BRCT mutant cell lines showed lower levels of nibrin staining on a per cell basis than cells expressing wild type nibrin. We also observed that Mre11 was translocated to the nucleus in all the FHA and BRCT mutant cell lines, whereas Mre11 remained cytoplasmic in NBS-ILB1 cells infected with the LXIN vector alone (data not shown). These results confirm the immunoprecipitation data presented in Fig. 3, showing that the FHA and BRCT mutants complexed with Mre11 and Rad50.

To determine if nibrin FHA and BRCT mutant proteins were capable of forming nuclear foci in response to ionizing radiation, cells were exposed to 12 Gy of radiation and were fixed and stained after 8 h. sheep anti-nibrin antibody (red) and a monoclonal antibody specific for Mre11 (green). Immunofluorescence was examined by confocal microscopy at 488 and 568 nm. Magnification, ×1000.
Y176A mutant failed to form radiation-induced nuclear foci. These results were specific for mutations made in FHA and BRCT domains. Therefore, normal phosphorylation of nibrin FHA and BRCT point mutants following irradiation. NBS-ILB1 cells stably transduced with the empty retroviral vector (LXIN), wild type nibrin (NBS1), the nibrin S343A phosphorylation site mutant, the nibrin FHA point mutants (R28A, H45A, and D65N), or the BRCT point mutants (GG136–137EE and Y176A) were exposed to 10 Gy of ionizing radiation and total cell lysates were prepared 1 h later. An A-T fibroblast cell line, AT3Bl, was included as a negative control (A-T). Phosphorylation of nibrin at all potential sites was detected by mobility shift. 10^6 cell equivalents/lane were separated on 3–8% gradient SDS-PAGE gels and transferred to nylon membranes. Western blots were probed with anti-nibrin polyclonal antisera. To detect phosphorylation at the Ser-343 site in nibrin, cell lysates were immunoprecipitated with a polyclonal antibody to nibrin and 10^6 cell equivalents/lane were separated on 3–8% gradient SDS-PAGE gels and transferred to nylon membranes. Western blots were probed with polyclonal antisera specific for Mre11 as a loading control. Blots were then stripped and probed with polyclonal antisera specific for Mre11 as a loading control.

Phosphorylation of S-phase Checkpoint Proteins Is Restored in Cells Expressing Nibrin FHA or BRCT Domain Point Mutants—Phosphorylation of nibrin has been reported to be required for activation of the S-phase checkpoint following exposure to ionizing radiation (2, 16). Recent investigations have identified two arms of the radiation-induced S-phase checkpoint, involving Atm phosphorylation of either Chk2 or Smc1, which are dependent on nibrin function (17–20). To determine if point mutations in the nibrin FHA or BRCT domain that impair nibrin phosphorylation might also affect activation of the S-phase checkpoint, we tested for phosphorylation of Chk2 and Smc1 following exposure to ionizing radiation. NBS-ILB1 cells expressing wild type NBS1, the S343A phosphorylation site mutant, or the nibrin FHA and BRCT mutants were exposed to 10 Gy of radiation, and cell lysates were prepared 1 h later. An A-T cell line was included as a negative control. Chk2 phosphorylation was analyzed by Western blot using Chk2 polyclonal antisera. Consistent with a requirement for nibrin expression for Chk2 phosphorylation, NBS-ILB1 cells infected with empty vector did not display detectable Chk2 phosphorylation after irradiation, similar to the results obtained with A-T cells (Fig. 6). Expression of wild type NBS1 in NBS-ILB1 cells was sufficient to restore Chk2 phosphorylation. Under our assay conditions, phosphorylation of Chk2 did not require nibrin phosphorylation at Ser-343, because NBS-ILB1 cells expressing the phosphorylation site mutant S343A showed levels of Chk2 phosphorylation similar to wild type nibrin. Similarly, all the nibrin FHA and the BRCT point mutants showed normal levels of Chk2 phosphorylation, indicating that the FHA and BRCT domain of nibrin are not required for Chk2 phosphorylation by Atm.

Similar experiments were performed to assess Smc1 phosphorylation using a polyclonal Smc1 antibody and phosphopeptide antisera specific for one of the two reported Smc1 phosphorylation sites, Ser-957. As shown in Fig. 7, no Smc1 phosphorylation was detected in A-T cells, consistent with a requirement for the Atm protein kinase, however, a low level of Smc1 phosphorylation was evident in NBS-ILB1 cells and NBS-ILB1 cells infected with the plXIN vector. Introduction of

![Fig. 5](image5.png) **Phosphorylation of nibrin FHA and BRCT point mutants following irradiation.** NBS-ILB1 cells stably transduced with the empty retroviral vector (LXIN), wild type nibrin (NBS1), the nibrin S343A phosphorylation site mutant, the nibrin FHA point mutants (R28A, H45A, and D65N), or the BRCT point mutants (GG136–137EE and Y176A) were exposed to 10 Gy of ionizing radiation, and total cell lysates were prepared after 1 h. An A-T fibroblast cell line, AT3Bl, was included as a negative control (A-T). Phosphorylation of nibrin at all potential sites was detected by mobility shift. 10^6 cell equivalents/lane were separated on 3–8% gradient SDS-PAGE gels and transferred to nylon membranes. Western blots were probed with anti-nibrin polyclonal antisera. To detect phosphorylation at the Ser-343 site in nibrin, cell lysates were immunoprecipitated with a polyclonal antibody to nibrin and 10^6 cell equivalents/lane were separated on 3–8% gradient SDS-PAGE gels and transferred to nylon membranes. Western blots were probed with polyclonal antisera specific for Mre11 as a loading control. Blots were then stripped and probed with polyclonal antisera specific for Mre11 as a loading control.

![Fig. 6](image6.png) **Phosphorylation of Chk2 in the nibrin FHA and BRCT point mutants following irradiation.** NBS-ILB1 cells stably transduced with the empty retroviral vector (LXIN), wild type nibrin (NBS1), the nibrin S343A phosphorylation site mutant, the nibrin FHA point mutants (R28A, H45A, and D65N), or the BRCT point mutants (GG136–137EE and Y176A) were exposed to 10 Gy of ionizing radiation, and total cell lysates were prepared after 1 h. An A-T fibroblast cell line, AT3Bl, was included as a negative control (A-T). 10^6 cell equivalents/lane were separated on 3% SDS-PAGE gels and Western-blotted. Chk2 phosphorylation was detected by mobility shift on Western blots using a polyclonal antibody specific for Chk2. To control for loading, the blot was stripped and reprobed with polyclonal antisera specific for Mre11.
wild type NBS1 into NBS-ILB1 cells resulted in an increased level of Smc1 phosphorylation following irradiation, similar to levels observed in the control fibroblast cell line. Smc1 phosphorylation was also detectable in all the FHA and BRCT mutant cell lines, as well as in the S343A phosphorylation site mutant, although the level of Smc1 phosphorylation appeared to vary in some of the mutant cell lines compared with controls.

To determine if variation in the level of Smc1 phosphorylation was the result of FHA or BRCT point mutations, or simply due to differences in the level of mutant protein expressed, we quantitated the Smc1 phosphorylation-specific signal shown in Fig. 7 by densitometry and compared the level of phosphorylation to nibrin expression. This analysis showed no significant difference in Smc1 phosphorylation in the FHA or BRCT mutant cell lines compared with the controls (data not shown). As a second approach, individual clones were isolated from the H45A, G136E, G137E, and Y176A bulk cell lines that expressed higher levels of mutant protein than the parental lines. Smc1 phosphorylation was analyzed in the cloned lines 1 h after exposure to 10 Gy of ionizing radiation. As shown in Fig. 8, the level of Smc1 phosphorylation detected in the cloned cell lines was equivalent to cells expressing wild type nibrin, even though the clones expressed less nibrin protein than the NBS1 cell line. Chk2 phosphorylation was also normal in the cloned cell lines, as expected (data not shown). Taken together, these results indicate that point mutations in the nibrin FHA or BRCT domain do not affect activation of the Smc1 arm of the S-phase checkpoint following irradiation.

**DISCUSSION**

We have performed site-directed mutagenesis of the nibrin FHA and BRCT domains to elucidate the role of these protein motifs in nibrin function. In our previous study of the interaction between nibrin and Mre11, expression of a C-terminal fragment of nibrin containing the Mre11 binding domain but lacking the FHA and BRCT domains, demonstrated that nibrin functions as more than just a molecular chaperone for Mre11. In the absence of nibrin foci in this study or in our previous experiments, wild type nibrin and mutant cell lines were included as controls. 105 cell equivalents/lane were electrophoresed on 3–8% gradient SDS-PAGE gels and Western-blotted. Duplicate blots were probed with a polyclonal anti-Smc1 antibody, to control for loading, or with phosphopeptide antisera specific for the Ser-957 phosphorylation site in Smc1 to detect Smc1 phosphorylation.

Mutagenesis of the FHA and BRCT domains of nibrin affected the expression of several of the mutants. The FHA H45A mutant and the BRCT G136E, G137E and Y176A mutants were expressed at lower levels than wild type nibrin in NBS-ILB1 cells, although full-length nibrin protein was clearly detectable in all three cell lines. A previous study reported that mutation of the conserved histidine residue in the FHA1 domain of Rad53, analogous to residue His-45 in nibrin, resulted in reduced protein expression (37). Analysis of the x-ray crystal structure of the Rad53 FHA1 domain revealed the conserved histidine stabilizes the architecture of the phosphopeptide binding site, suggesting that disruption of this residue might alter the tertiary structure of the FHA domain, leading to protein instability (35). Thus, even under the more controlled conditions of site-directed mutagenesis, alterations within the FHA and BRCT domains of nibrin do not appear to be well tolerated. It is difficult to separate mutation of functional residues in FHA and BRCT domains from residues that play a structural role, given the three-dimensional nature of protein-ligand interactions. Despite the variation in levels of full-length nibrin protein expressed, however, all of the FHA and BRCT point mutants in these experiments were able to complex with Mre11 and Rad50 and translocate the Mre11-Rad50-nibrin complex to the nucleus.

Mutation of conserved residues in either the nibrin FHA or BRCT domain disrupted Mre11-Rad50-nibrin focus formation following irradiation, indicating that both of these domains mediate interactions necessary for recruitment of nibrin to type III foci. Moreover, nibrin is responsible for directing Mre11-Rad50 to foci. We did not observe Mre11 foci in the absence of nibrin foci in this study or in our previous experi-
Nibrin FHA and BRCT Domains

ment using a C-terminal fragment of nibrin containing only the Mre11 binding domain (7). The effect on focus formation was specific for FHA or BRCT residues predicted to participate in protein-ligand interactions, because mutation of a nonconserved amino acid in the nibrin FHA domain, D95N, did not affect the ability to form nuclear foci.

In addition to eliminating nuclear focus formation, the other clear effect of mutations in the nibrin FHA and BRCT domains was to impair phosphorylation of nibrin following cellular irradiation. We found that mutation of the His45 residue of the nibrin FHA domain, or the Gly-136, Gly-137, or Tyr-176 residue of the BRCT domain of nibrin, resulted in no apparent nibrin phosphorylation. There was no shift detectable by Western blot analysis, and a phosphopeptide antibody specific for the Ser-343 site of nibrin failed to show any reactivity with these three mutants. The assay conditions used here were sufficiently stringent to detect phosphorylation at other sites within nibrin as demonstrated by the residual shift detected in NBS cells expressing the S343A phosphorylation site mutant. Thus, both the FHA and BRCT domains of nibrin mediate interactions that are necessary for phosphorylation of nibrin by Atm. One possibility is that the nibrin FHA and BRCT domains interact with the Atm protein directly. Investigations have shown that nibrin and Atm interact and this association increases following exposure of cells to irradiation (14, 16). This model is appealing because Atm is capable of autophosphorylating itself, providing the potential for a phosphoprotein interaction with the FHA domain of nibrin (38). We are currently exploring the interaction of the FHA and BRCT point mutants with Atm.

The effect of point mutations in the nibrin FHA and BRCT domains on both phosphorylation and nuclear focus formation suggests a possible functional interdependence between these responses to irradiation. This is of interest because it bears on the position of nibrin in the DNA damage signaling cascade. Some models place nibrin as strictly a downstream phosphoacceptor in the DNA damage signaling cascade. The FHA and BRCT domains may serve as a DNA damage sensor and activating Atm kinase activity, and that mutant nibrin molecules may be able to serve this function, although not as robustly as wild type nibrin.

One interesting aspect of these experiments is that we were unable to assign distinct functions to either the FHA or the BRCT domain of nibrin. In general, mutations in both domains had similar effects on nibrin function. The nibrin orthologue in yeast, Xrs2, lacks any discernable BRCT domain. However, our results indicate that the corresponding region in human nibrin does constitute a functional BRCT domain. The inability to dissect the function of the FHA domain away from the function of the BRCT domain of nibrin may not be surprising, given the close proximity of the two domains. Upon formation of secondary structure, the primary sequence of each domain is likely to influence the secondary structure of the other domain and, hence, its function. Identification of the proteins that interact with the nibrin FHA and BRCT domains may help to clarify this result. In this regard, we have not been able to unambiguously demonstrate an interaction between wild type nibrin or the FHA and BRCT mutants and the E2F1 transcription factor by co-immunoprecipitation, as reported by Maser et al. (32). This may be due to the reduced level of expression of some of the mutants, because only a small fraction of endogenous nibrin was shown to interact with E2F1. It seems likely that many different proteins will be shown to interact with the nibrin FHA and BRCT domains depending on environmental conditions, as well as the position of cells in the cell cycle.

Note Added in Proof—Similar results have been reported recently by Zhao et al. (39).

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