Active Role for Nibrin in the Kinetics of Atm Activation

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The Atm protein kinase is central to the DNA double-strand break response in mammalian cells. After irradiation, dimeric Atm undergoes autophosphorylation at Ser 1981 and dissociates into active monomers. Atm activation is stimulated by expression of the Mre11/Rad50/nibrin complex. Previously, we showed that a C-terminal fragment of nibrin, containing binding sites for both Mre11 and Atm, was sufficient to provide this stimulatory effect in Nijmegen breakage syndrome (NBS) cells. To discriminate whether nibrin’s role in Atm activation is to bind and translocate Mre11/Rad50 to the nucleus or to interact directly with Atm, we expressed an Mre11 transgene with a C-terminal NLS sequence in NBS fibroblasts. The Mre11-NLS protein complexed with Rad50, localized to the nucleus in NBS fibroblasts, and associated with chromatin. However, Atm autophosphorylation was not stimulated in cells expressing Mre11-NLS, nor were downstream Atm targets phosphorylated. To determine whether nibrin-Atm interaction is necessary to stimulate Atm activation, we expressed nibrin transgenes lacking the Atm binding domain in NBS fibroblasts. The nibrin ΔAtm protein interacted with Mre11/Rad50, however, Atm autophosphorylation was dramatically reduced after irradiation in NBS cells expressing the nibrin ΔAtm transgenes relative to wild-type nibrin. These results indicate that nibrin plays an active role in Atm activation beyond translocating Mre11/Rad50 to the nucleus and that this function requires nibrin-Atm interaction.

The response to DNA double-strand breaks (DSBs) in mammalian cells involves an essential signaling cascade that ensures genomic integrity. The response is initiated by detection of DSBs, followed rapidly by transduction of the damage signal throughout the cell to effector proteins involved in apoptosis, cell cycle control, and DNA repair.

Considerable information has been obtained about transduction of the damage signal to downstream targets. For DNA DSBs, the Atm protein kinase, mutated in individuals with the radiosensitivity disorder ataxia-telangiectasia (A-T), is the primary signal transducer (37). Atm exists as inactive dimers in undamaged cells but rapidly undergoes autophosphorylation at serine 1981 after exposure to DSB-inducing agents and dissociates into active monomers (1). The active Atm monomers phosphorylate a collection of critical downstream effector molecules, including nibrin, Mre11, Brca1, MDC1, 53BP1, p53, Chk2, Smc1, and FANC D2 (2, 8, 14, 16, 17, 19, 29, 36, 39).

Phosphorylation of some of these downstream effectors by Atm occurs in the nucleoplasm, whereas others are phosphorylated at sites of DNA damage where Atm relocalizes via interaction with the C terminus of nibrin, a member of the Mre11/Rad50/nibrin (MRN) complex (15, 26, 32, 44).

Although the targets of Atm are well established, the mechanism by which DNA DSBs are detected and the Atm signal transduction cascade initiated is less well understood. In their initial report of Atm autophosphorylation, Bakkenist and Kas- tan (1) observed that chromatin alterations mediated by exposure to chloroquine, hypotonic conditions, or histone deacetylase inhibitors were sufficient to activate Atm in the absence of DNA DSBs. These findings led the investigators to suggest that changes in chromatin structure caused by DNA DSBs were responsible for Atm activation.

Several lines of evidence suggest a specific role for the MRN complex in Atm activation. The MRN complex has well-documented DNA repair and S-phase checkpoint functions in both yeast and mammalian cells (9). Mre11/Rad50 display nuclease activity and can bind free DNA ends, activities that are enhanced in the presence of nibrin (10, 33, 34). Whereas nibrin has no enzymatic activity, the C terminus of nibrin binds Mre11 directly and translocates Mre11/Rad50 to the nucleus (11). After irradiation, nibrin relocates to the sites of DNA damage within 5 min and, as mentioned above, binds and relocates Atm to these sites (15, 26, 44). Nibrin is phosphorylated by Atm in response to DNA damage, and this phosphorylation event is required for proper S-phase checkpoint activation (16, 25, 43, 46).

Hypomorphic mutations in nibrin and Mre11 result in the radiosensitivity disorders Nijmegen breakage syndrome (NBS) and A-T-like disorder (ATLD), which share many features with A-T (38, 42). Cell lines from patients with NBS or ATLD have delayed kinetics of Atm autophosphorylation at early times after low doses of irradiation or exposure to radiomimetic compounds (5, 18, 20, 41). Similarly, cells in which Mre11 has been degraded by adenovirus infection have deficient Atm activation (4). More recently, Difilippantonio et al. (12) reported that mouse B cells conditionally null for nibrin expression displayed little or no Atm activation.

Previously, we showed that nuclear expression of Mre11/Rad50 complexed with just a C-terminal fragment of nibrin was sufficient to stimulate Atm activation at early times after irradiation (5). In contrast, nuclear expression of a nibrin transgene lacking the C-terminal 100 amino acids was unable to stimulate Atm activation under the same conditions (5, 18).
Since the C-terminal 100 amino acids of nibrin contain separate but adjacent binding domains for Mre11 and Atm, nibrin may stimulate Atm autophosphorylation by translocating Mre11/Rad50 to the nucleus or by binding and relocating Atm to the sites of DNA damage (15, 44). The former hypothesis is appealing since the MRN complex can process DNA DSBs, an activity that would likely induce chromatin alterations (33, 34). Indeed, ATLD cells complemented with a nibrin-deficient mutant of Mre11 were deficient in Atm activation, and a Rad50 ATP-dependent DNA-unwinding activity mutant was unable to stimulate Atm autophosphorylation in vitro (24, 41). Whether nibrin-Atm interaction enhances Atm activation after irradiation is not clear from the published studies (15, 44). Using cell extracts, You et al. (44) found nibrin-Atm interaction stimulated Atm activation, whereas in an in vivo system, Atm autophosphorylation was found to be independent of nibrin-Atm interaction (15).

To discriminate between these two possible roles of nibrin in Atm activation, we expressed an Mre11 transgene with an artificial C-terminal NLS sequence in NBS fibroblasts in order to deliver Mre11/Rad50 to the nucleus in the absence of nibrin. We compared Atm activation after irradiation in these cells to NBS cells expressing nibrin transgenes lacking the Atm binding domain. Our results indicate that nibrin plays an active role in stimulating Atm autophosphorylation after irradiation beyond translocating Mre11/Rad50 to the nucleus and that this function requires nibrin-Atm interaction.

MATERIALS AND METHODS

**Cell lines.** The simian virus 40 transformed fibroblast cell line, NBS-ILB1, was established from an NBS patient homozygous for the 657del5 mutation (21). NBS-ILB1 cells infected with the pLXIN retroviral vector alone (BD Clontech, Palo Alto, CA) or with pLXIN expressing a wild-type nibrin cDNA (NBS1), a nibrin mutant with a C-terminal deletion of 100 amino acids (aa) (NBS652), or a 300-aa C-terminal fragment of nibrin (NbrFR5) have been described previously (5, 7, 11). D6089 is a primary fibroblast cell line established from an ATLD patient homozygous for the R633X nonsense mutation (38). GM0637 simian virus 40 transformed fibroblasts were used as controls. All cells were grown in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 15% fetal calf serum (HyClone, Logan, UT), 100 U of penicillin/ml, and 100 μg of streptomycin/ml (Invitrogen)/ml. Cells stably expressing pLXIN transgenes were also maintained in 900 μg of G418 (Invitrogen)/ml. For retroviral packaging, Phoenix A cells were grown in Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum with penicillin-streptomycin (www.stanford.edu/group/nolan).

**Retroviral gene expression.** Recombinant retroviruses constructed for these experiments included pLXIN Mre11, pLXIN Mre11-NLS, pLXIN NBS1 ΔAtm that lacked the Atm binding domain, and pLXIN NbrFR5 ΔAtm. A wild-type Mre11 cDNA previously cloned by PCR (11) was subcloned into pLXIN, upstream of the IRES-neomycin cassette. To construct Mre11-NLS, the stop codon of the Mre11 cDNA was changed to a serine residue (TGA→TCA) by using QuikChange site-directed mutagenesis (Stratagene). Transduction (Stratagene, Inc., La Jolla, CA), and a rabbit polyclonal antibody specific for nibrin, Mre11, Rad50, or Atm (Novus Biological, Littleton, CO). Mre11 protein was also detected by using a monoclonal anti-Mre11 antibody (a gift from T. Demaggio, Icos Corp., Bothell, WA). Nibrin phosphorylation was detected by using an anti-phosphoserine 343 monoclonal antibody (Upstate Cell Signaling Solutions, Lake Placid, NY), and Atm autophosphorylation was detected with an anti-phosphoserine 1981 monoclonal antibody (Abcam, Cambridge, MA). A monoclonal Chk2 antibody (a gift from D. Delia) was used to detect total Chk2 protein, and Chk2 phosphorylation was detected with a rabbit anti-phosphothreonine 68 antisera (Cell Signaling Technology, Beverly, MA). Total p53 protein was detected by using a monoclonal p53 antibody (Upstate), and a rabbit polyclonal phosphoserine 15 antibody was used to detect p53 phosphorylation (Cell Signaling Technology). The HA tag was used to detect a monoclonal anti-HA antibody (Roche). Primary antibodies were detected with horseradish peroxidase-coupled goat anti-rabbit immunoglobulin G (IgG) and goat anti-mouse IgG (BD Pharmingen, San Diego, CA). Immunoblot staining was detected by chemiluminescence (Perkin-Elmer Life Sciences, Wellesley, MA).

**Chromatin isolation.** Isolation of chromatin-bound proteins from cells was performed according to the method of Mendez and Stillman (30). Briefly, cells were harvested by trypsinization, washed, and resuspended in buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 1% triton, 1 mM dithiothreitol, protease inhibitors). After addition of 0.1% Triton-X-100, cells were incubated for 5 min on ice, and cytoplasmic proteins were isolated by centrifugation. The nuclear pellet was washed once in buffer A and lysed in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, and protease inhibitors) for 30 min on ice. Soluble nuclear proteins were isolated by centrifugation. The insoluble nuclear pellet was then washed sequentially in buffer B supplemented with 125 mM NaCl and then 250 mM NaCl to release proteins associated with chromatin with increasing avidity. The remaining insoluble nuclear pellet was lysed directly in LDS buffer (Innogen). Cellular fractions were sonicated, and immunoblot analysis was performed as described above. Rabbit antiserum specific for Hs90 was used as a cytoplasmic protein control (Cell Signaling Technology), and a monoclonal antibody to TRF2 was used as a nuclear protein control (EMD Biosciences, La Jolla, CA).

**Immunofluorescence.** Cell lines were grown on glass coverslips overnight (ViroCult, Minneapolis, MN), and the following day they were exposed to 0 or 12 Gy of irradiation. Cells were fixed for 6 h with 4% paraformaldehyde–0.1% Triton X-100 and were blocked overnight in 10% fetal calf serum in phosphate-buffered saline. To detect nibrin and Mre11 localization in the cells, unirradiated coverslips were costained with a monoclonal γ-H2AX antibody (Upstate) and a rabbit polyclonal antibody specific for Mre11 (Novus). The primary antibodies were detected with goat anti-rabbit IgG coupled to Alexa 568 and goat anti-mouse IgG coupled to Alexa 488 (Molecular Probes, Eugene, OR). Confocal microscopy was performed with a Nikon fluorescence microscope and a Bio-Rad confocal imaging system using LaserSharp 2000 (Bio-Rad). Individual fields were Z-planed, and images at 488 and 568 nm were stacked and merged.

**RESULTS**

The Mre11-NLS transgene interacts with Rad50 and localizes to the nucleus. The endogenous Mre11/Rad50 proteins do not contain any recognizable NLS sequences and, in the absence of nibrin, remain complexed in the cytoplasm (3, 13, 35). To determine whether Mre11/Rad50 alone are capable of stimulating Atm activation, we attached two artificial NLS sequences and an HA tag to the C terminus of an Mre11 cDNA cloned in the pLXIN retroviral vector. These additional se-
quences added 29 amino acids to the Mre11 protein, changing the predicted molecular weight from approximately 81 to 84 kDa. The Mre11-NLS transgene was expressed in NBS-ILB1 fibroblasts, which do not express full-length nibrin protein, and clonal cell lines expressing Mre11-NLS (Mre11), or an Mre11-NLS transgene with two artificial NLS sequences and an HA tag attached to the Mre11 C terminus. Individual clones of NBS cells expressing the Mre11-NLS transgene were isolated (NLS.8, NLS.11, and NLS.12). A total of 25 µg of total cellular protein per lane was separated on a 7% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The Western blot (WB) was probed with monoclonal antibodies specific for Mre11 or the HA tag. (B) Mre11 was immunoprecipitated (IP) from 250 µg of total cellular protein isolated from the above cell lines using a polyclonal anti-Mre11 antibody or a monoclonal anti-HA antibody. Immunoprecipitated proteins were separated on a 3 to 8% SDS-polyacrylamide gel, and the immunoblot was probed with a polyclonal Rad50 antiserum or a monoclonal anti-Mre11 antiserum. The arrows indicate the positions of endogenous Mre11 protein and the higher-molecular-weight Mre11-NLS protein.

FIG. 1. Expression of the Mre11-NLS transgene in NBS cells and interaction with Rad50. (A) Western blot analysis of GM0637 fibroblasts or NBS-ILB1 cells stably transduced with the pLXIN retroviral vector alone (LXIN), wild-type nibrin (NBS1), wild-type Mre11 (Mre11), or an Mre11-NLS transgene with two artificial NLS sequences and an HA tag attached to the Mre11 C terminus. Individual clones of NBS cells expressing the Mre11-NLS transgene were isolated (NLS.8, NLS.11, and NLS.12). A total of 25 µg of total cellular protein per lane was separated on a 7% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The Western blot (WB) was probed with monoclonal antibodies specific for Mre11 or the HA tag. (B) Mre11 was immunoprecipitated (IP) from 250 µg of total cellular protein isolated from the above cell lines using a polyclonal anti-Mre11 antibody or a monoclonal anti-HA antibody. Immunoprecipitated proteins were separated on a 3 to 8% SDS-polyacrylamide gel, and the immunoblot was probed with a polyclonal Rad50 antiserum or a monoclonal anti-Mre11 antibody. The arrows indicate the positions of endogenous Mre11 protein and the higher-molecular-weight Mre11-NLS protein.

FIG. 2. Subcellular localization of the Mre11-NLS protein in NBS cells. (A) Cytoplasmic and nuclear proteins were isolated from GM0637 control fibroblasts or NBS-ILB1 cells stably transduced with the pLXIN retroviral vector alone (LXIN), wild-type nibrin (NBS1), wild-type Mre11 (Mre11), or the Mre11-NLS transgene (NLS.8, NLS.11, and NLS.12). A total of 10^5 cell equivalents of protein per lane were electrophoresed on 7% SDS-polyacrylamide gels. Western blots (WB) were probed with a monoclonal anti-Mre11 antibody, a polyclonal antiserum specific for the cytoplasmic heat shock protein (Hsp90), or a monoclonal antibody specific for the telomeric protein, TRF2. The arrows indicate the positions of endogenous Mre11 protein and the higher-molecular-weight Mre11-NLS protein. (B) Immunofluorescence analysis was performed on NBS-ILB1 cells stably transduced with the pLXIN retroviral vector alone, wild-type nibrin (NBS1), or the Mre11-NLS transgene (NLS.8). Fibroblasts were stained for nibrin and Mre11 expression using a polyclonal anti-nibrin antibody (red) and a monoclonal antibody to Mre11 (green). The Mre11-NLS protein was specifically detected using a monoclonal antibody specific for the HA tag (HA, green). Immunofluorescence was detected by confocal microscopy at 488 and 568 nm. Individual fields were Z-planed and stacked. Magnification, ×600.

The Mre11-NLS transgene localized to the nucleus of NBS-ILB1 cells, as determined by Western blot analysis of cytoplasmic and nuclear proteins and by immunofluorescence. In NBS-ILB1 cells infected with vector alone, Mre11 was localized primarily in the cytoplasm, but expression of wild-type nibrin in NBS cells resulted in nuclear localization of Mre11 (Fig. 2A). The Mre11-NLS protein was also localized to the nucleus of
NBS-ILB1 cells, although approximately half of the transgenic protein remained in the cytoplasm, along with the endogenous Mre11 protein in these cells. By comparison, Mre11 was localized only in the cytoplasm of NBS cells infected with a wild-type Mre11 transgene lacking NLS sequences. Similar results were observed by immunofluorescence, detecting the Mre11-NLS protein with the anti-HA antibody (Fig. 2B). Thus, expression of an Mre11-NLS transgene in NBS cells resulted in substantial nuclear expression of Mre11/Rad50 in the absence of nibrin.

Atm activation and function are not stimulated by Mre11-NLS/Rad50.

To determine whether Mre11/Rad50 alone could stimulate the kinetics of Atm activation, as measured by serine 1981 autophosphorylation, NBS cells expressing Mre11-NLS protein were exposed to 3 Gy of ionizing radiation and harvested 15 min later. Atm was immunoprecipitated (IP) from 800 μg of protein using a polyclonal anti-Atm antibody and protein was separated on 3 to 8% SDS-polyacrylamide gels. Western blots (WB) were probed with a monoclonal phosphoserine 1981 antibody or a polyclonal Atm antibody. (B) Chk2 threonine 68 phosphorylation and p53 serine 15 phosphorylation by Atm were assessed in the above cells, as well as GM0637 control fibroblasts, 15 min after exposure to 0 or 3 Gy of ionizing radiation. A total of 20 μg of total cellular protein per lane was separated on 7% SDS-polyacrylamide gels. Immunoblots were probed with a phosphothreonine 68 antisera or a monoclonal anti-Chk2 antibody. For p53, the immunoblots were probed with phosphoserine 15 antisera or a monoclonal p53 antibody.

Mre11-NLS complements Atm activation and function in ATLD cells.

The C terminus of Mre11 encodes one of the two DNA-binding domains of the Mre11 protein (9). To rule out the trivial possibility that the attachment of the HA tag and NLS sequences to the C terminus of Mre11 interfered with the function of the protein, we tested the ability of the Mre11-NLS protein to complement ATLD cells, that lack full-length Mre11. A primary ATLD fibroblast cell line was infected with the empty pLXIN vector or pLXIN encoding wild-type Mre11 or Mre11-NLS. ATLD cells express low levels of endogenous nibrin protein that is stabilized in the presence of exogenous Mre11 (38). Therefore, exogenously expressed Mre11-NLS protein can potentially enter the nucleus by two routes, by binding nibrin and translocating to the nucleus via nibrin, as
FIG. 4. Atm activation and function after irradiation in ATLD cells expressing the Mre11-NLS protein. (A) Expression of the Mre11 NLS transgene in ATLD cells was assessed by Western blot analysis. Total cellular lysates were isolated from GM0637 control fibroblasts and ATLD D6809 cells stably transduced with the pLXIN retroviral vector (ATLD LXIN), wild-type Mre11 (ATLD Mre11), or the Mre11-NLS transgene (ATLD NLS). Then, 25 μg of protein per lane was electrophoresed on a 7% SDS-polyacrylamide gel and Western blotted (WB). The immunoblot was probed with a monoclonal anti-Mre11 antibody or a polyclonal antibody specific for Hsp90, as a loading control. The arrows indicate the endogenous Mre11 protein and the higher-molecular-weight Mre11-NLS protein. (B) Nibrin phosphorylation in the above cell lines was analyzed 15 min after exposure to 0 or 3 Gy of ionizing radiation (IR). Nibrin was immunoprecipitated (IP) from 250 μg of total cellular protein using a polyclonal antinibrin antibody and proteins were separated on a 3 to 8% SDS-polyacrylamide gel. Western blots were probed with a phosphoserine 343 monoclonal antibody or a polyclonal antibody specific for nibrin. To assess Mre11-NLS complex formation with nibrin, the blot was reprobed with a monoclonal anti-Mre11 antibody. (C) Atm autophosphorylation at serine 1981 was assessed in the above cell lines 15 min after exposure to 0 or 3 Gy of irradiation. Next, 25 μg of total cellular protein per lane was electrophoresed on a 3 to 8% SDS-polyacrylamide gel. The Western blot was probed with a monoclonal phosphoserine 1981 antibody or a polyclonal anti-Atm antisera. Chk2 threonine 68 phosphorylation was analyzed by probing a similar immunoblot with phosphothreonine 68 antisera or a monoclonal anti-Chk2 antibody. To detect p53 serine 15 phosphorylation, immunoblots were probed with phosphoserine 15 antisera or a monoclonal p53 antibody. (D) Irradiation-induced foci were examined 6 h after 12 Gy of irradiation in the above ATLD fibroblasts. Mre11 or Mre11-NLS foci were detected with Mre11 antisera (red) and γ-H2AX foci were detected with a monoclonal γ-H2AX antibody (green). Immunofluorescence was collected by using confocal microscopy at 488 and 568 nm. Individual fields were Z-planed and stacked. Magnification, ×1,000.
by Western blot analysis. As shown in Fig. 5A, Mre11 was
localizing to chromatin in the absence of nibrin, NBS-
expressing cells, a finding consistent with the inability
of Mre11 to translocate to the nucleus in the absence of nibrin.
Expression of wild-type nibrin in NBS cells resulted in a
decrease of Mre11 in the cytoplasmic fraction and a proportional
increase in the amount of Mre11 detected in nuclear fractions,
particularly the chromatin bound nuclear pellet (Fig. 5B).
Likewise, Mre11-NLS protein was detected in the nuclear frac-
tions, including the nuclear pellet, in NBS cells expressing the
Mre11-NLS transgene. Thus, the Mre11-NLS protein appeared
to be able to associate with chromatin in the absence of nibrin.

ATLD cells was sufficient to stimulate Atm autophosphoryla-
tion at 15 min after exposure to 3 Gy of ionizing radiation, sim-
ilar to levels observed with a wild-type Mre11 transgene
(Fig. 4C). This stimulatory effect on Atm activation resulted in
stimulation of Atm activation in our in vivo system, we intro-
duced into a full-length nibrin cDNA, as well as NbFR5, and
the Mre11-NLS transgene were unirradiated or exposed to 3 Gy of
ionizing radiation. After 30 min cells were harvested and fractionated
into cytoplasmic (lanes C), nuclear soluble (lanes NS), nuclear 125 mM
NaCl wash (lanes 125), nuclear 250 mM NaCl wash (lanes 250), and
the insoluble nuclear pellet (lanes NP). For comparison, whole-cell
lysate was included (lanes WC). A total of 10⁶ cell equivalents of
protein per lane was separated on 3 to 8% SDS-polyacrylamide gels
and Western blotted (WB). (A) Immunoblots of pLXIN cellular frac-
tions were probed with an anti-Mre11 monoclonal antibody to assess
the localization of Mre11 in the absence of full-length nibrin. The blot
was reprobed with anti-Hsp90 antisera as a control for cytoplasmic
proteins or with a monoclonal TRF2 antibody as a control for nuclear
proteins. (B) Localization of Mre11 in cellular fractions from LXIN,
NBS1, and Mre11-NLS expressing cells was detected by probing im-
umobLOTS with a monoclonal Mre11 antibody.

FIG. 5. Localization of the Mre11-NLS protein to chromatin in
NBS cells. NBS-ILB1 cells stably transduced with the pLXIN retroviral
vector alone (LXIN), wild-type nibrin (NBS1), or the Mre11-NLS
transgene (Mre11-NLS) were unirradiated or exposed to 3 Gy of
ionizing radiation. After 30 min cells were harvested and fractionated
into cytoplasmic (lanes C), nuclear soluble (lanes NS), nuclear 125 mM
NaCl wash (lanes 125), nuclear 250 mM NaCl wash (lanes 250), and
the insoluble nuclear pellet (lanes NP). For comparison, whole-cell
lysate was included (lanes WC). A total of 10⁶ cell equivalents of
protein per lane was separated on 3 to 8% SDS-polyacrylamide gels
and Western blotted (WB). (A) Immunoblots of pLXIN cellular frac-
tions were probed with an anti-Mre11 monoclonal antibody to assess
the localization of Mre11 in the absence of full-length nibrin. The blot
was reprobed with anti-Hsp90 antisera as a control for cytoplasmic
proteins or with a monoclonal TRF2 antibody as a control for nuclear
proteins. (B) Localization of Mre11 in cellular fractions from LXIN,
NBS1, and Mre11-NLS expressing cells was detected by probing im-
umobLOTS with a monoclonal Mre11 antibody.

ATLD cells sufficient to stimulate Atm autophosphoryla-
tion at 15 min after exposure to 3 Gy of ionizing radiation, similar
to levels observed with a wild-type Mre11 transgene
(Fig. 4C). This stimulatory effect on Atm activation resulted in
phosphorylation of nibrin, Chk2, and p53 in the complemented
samples as shown in Fig. 5A, Mre11 was
localized primarily in the cytoplasmic and nuclear soluble frac-
tions in NBS-ILB1 cells infected with the pLXIN vector alone.
The presence of Mre11 in the nuclear soluble fraction is likely
due to contamination of this fraction with cytoplasmic proteins,
as evidenced by reprobing the blot with antibodies to Hsp90, a cytoplasmic protein. Very little Mre11 protein was
localized to the nuclear pellet, or chromatin-bound, fraction from LXIN cells, a finding consistent with the inability of
Mre11 to translocate to the nucleus in the absence of nibrin.

Interaction between nibrin and Atm is necessary to stimulate
Atm activation is not stimulated by nibrin lacking the Atm
binding domain. The results presented above indicated that
nuclear expression of Mre11/Rad50 in the absence of nibrin
was insufficient to stimulate Atm autophosphorylation after
irradiation, despite the fact that the complex localized to chro-
matin. Thus, nibrin appears to play a more active role in
stimulating Atm autophosphorylation beyond simply directing
the translocation of Mre11/Rad50 to the nucleus. Recent stud-
ies have shown that the C-terminal 20 amino acids of nibrin
bind Atm and that deletion of this region is sufficient to disrupt
nibrin-Atm interaction and the recruitment of Atm to the sites
of DNA damage (15, 32, 44). However, contradictory results
obtained in different systems have failed to resolve whether
interaction between nibrin and Atm is necessary to stimulate
Atm activation at early times after DNA damage (15, 44).

To test the contribution of nibrin-Atm interaction to the
stimulation of Atm activation in our in vivo system, we intro-
duced a nonsense mutation (K735X) in nibrin that deleted the
C-terminal 19 amino acids. The K735X mutation was intro-
duced into a full-length nibrin cDNA, as well as NbFR5, and
the nibrin ΔAtm transgenes were expressed in NBS-ILB1 fi-
brablasts. Immunoprecipitation with nibrin antisera revealed
that the NBS1 ΔAtm and NbFR5 ΔAtm transgenes were ex-
pressed at similar levels as the parental full-length nibrin and
NbFR5 transgenes in NBS cells (Fig. 6A). Immunoblot anal-
ysis with Mre11 and Rad50 antisera demonstrated that both
NBS1 ΔAtm and NbFR5 ΔAtm proteins complexed with Mre11 and Rad50. The amount of Mre11/Rad50 coimmunoprecipitated with the Atm binding domain mutants was slightly less than the parent protein but, given the proximity of the Mre11 and Atm binding domains in nibrin, this result was not surprising. As expected, the Mre11/Rad50/nibrin Atm complex localized to the nucleus on Western blots of nuclear and cytoplasmic extracts (data not shown).

The ability of the nibrin ΔAtm proteins to stimulate the kinetics of Atm activation was assessed by exposing cells to 3 Gy of ionizing radiation and harvesting cells 15 min later. Atm protein was immunoprecipitated and serine 1981 phosphorylation was analyzed by Western blotting. As shown in Fig. 6B, NBS-ILB1 cells expressing wild-type nibrin (NBS1), nibrin lacking the C-terminal Atm binding domain (NBS1 ΔAtm), a 300-aa C-terminal fragment of nibrin (NbFR5), and NbFR5 lacking the C-terminal Atm binding domain (NbFR5 ΔAtm). Nibrin was immunoprecipitated (IP) from 250 μg of total cellular protein using polyclonal nibrin antisera and proteins were separated on a 3 to 8% SDS-polyacrylamide gel. The Western blot (WB) was probed with an anti-Mre11 monoclonal antibody, a polyclonal Rad50 antibody, or a monoclonal nibrin antibody. (B) Atm autophosphorylation at serine 1981 was assessed in the above cells 15 min after treatment with 0 or 3 Gy of ionizing radiation (IR). Atm was immunoprecipitated from 800 μg of total cellular protein using a polyclonal Atm antibody and proteins were separated on 3 to 8% SDS-polyacrylamide gels. Immunoblots were probed with a monoclonal phosphoserine 1981 antibody or a polyclonal Atm antibody. To detect Chk2 and p53 phosphorylation, 20 μg of total cellular protein isolated from the above cells 15 min after 0 or 3 Gy of irradiation was separated on 7% SDS-polyacrylamide gels. Immunoblots were probed with phosphothreonine 68 antisera or a Chk2 monoclonal antibody. For p53, Western blots were probed with phosphoserine 15 antisera or a monoclonal p53 antibody.

FIG. 6. Expression of nibrin ΔAtm transgenes in NBS cells and Atm activation after irradiation. (A) Expression and MRN complex formation was analyzed in GM0637 control fibroblasts and NBS-ILB1 cells stably transduced with the pLXIN retroviral vector alone (LXIN), wild-type nibrin (NBS1), nibrin lacking the C-terminal Atm binding domain (NBS1 ΔAtm), a 300-aa C-terminal fragment of nibrin (NbFR5), and NbFR5 lacking the C-terminal Atm binding domain (NbFR5 ΔAtm). Nibrin was immunoprecipitated (IP) from 250 μg of total cellular protein using polyclonal nibrin antisera and proteins were separated on a 3 to 8% SDS-polyacrylamide gel. The Western blot (WB) was probed with an anti-Mre11 monoclonal antibody, a polyclonal Rad50 antibody, or a monoclonal nibrin antibody. (B) Atm autophosphorylation at serine 1981 was assessed in the above cells 15 min after treatment with 0 or 3 Gy of ionizing radiation (IR). Atm was immunoprecipitated from 800 μg of total cellular protein using a polyclonal Atm antibody and proteins were separated on 3 to 8% SDS-polyacrylamide gels. Immunoblots were probed with a monoclonal phosphoserine 1981 antibody or a polyclonal Atm antibody. To detect Chk2 and p53 phosphorylation, 20 μg of total cellular protein isolated from the above cells 15 min after 0 or 3 Gy of irradiation was separated on 7% SDS-polyacrylamide gels. Immunoblots were probed with phosphothreonine 68 antisera or a Chk2 monoclonal antibody. For p53, Western blots were probed with phosphoserine 15 antisera or a monoclonal p53 antibody.

NBS1 ΔAtm and NbFR5 ΔAtm proteins complexed with Mre11 and Rad50. The amount of Mre11/Rad50 coimmunoprecipitated with the Atm binding domain mutants was slightly less than the parent protein but, given the proximity of the Mre11 and Atm binding domains in nibrin, this result was not surprising. As expected, the Mre11/Rad50/nibrin ΔAtm complex localized to the nucleus on Western blots of nuclear and cytoplasmic extracts (data not shown).

The ability of the nibrin ΔAtm proteins to stimulate the kinetics of Atm activation was assessed by exposing cells to 3 Gy of ionizing radiation and harvesting cells 15 min later. Atm protein was immunoprecipitated and serine 1981 phosphorylation was analyzed by Western blotting. As shown in Fig. 6B, NBS-ILB1 cells expressing wild-type nibrin or the NbFR5 transgenes stimulated robust Atm autophosphorylation early after irradiation, whereas Atm activation was dramatically reduced in NBS cells expressing NBS1 ΔAtm and NbFR5 ΔAtm. There appeared to be some residual ability of the NBS1 ΔAtm protein to stimulate Atm activation compared to NBS cells expressing the NbFR5 ΔAtm transgene or vector alone.

The reduction in Atm activation in the nibrin binding site mutants was reflected in decreased phosphorylation of downstream Atm targets (Fig. 6B). As expected, phosphorylation of Chk2 threonine 68 was reduced in NBS-ILB1 cells expressing the nibrin ΔAtm proteins, since this target is phosphorylated by Atm at the sites of DNA DSBs. More importantly, however, Atm phosphorylation of p53 serine 15 was also reduced in the nibrin binding site mutants. Since p53 serine 15 phosphorylation takes place in the nucleoplasm, this result confirms that nibrin-Atm interaction is necessary for stimulation of Atm activation after irradiation.

DISCUSSION

Studies using NBS cells complemented with various nibrin transgenes have revealed a role for the nibrin C terminus in the activation of the Atm protein kinase (5, 18). The contribution of nibrin to Atm activation is not an absolute requirement, but rather nibrin stimulates the kinetics of Atm activation. This stimulatory effect is evident primarily at early time points after low doses of irradiation (5, 18, 20). The recent finding that Atm activation is deficient using in vitro reactions lacking recombinant nibrin and in mouse cells conditionally null for nibrin expression raises the possibility that nibrin is actually required...
for Atm activation and that the quantitative effect on Atm activation seen in NBS cells is due to the hypomorphic nature of the 657del5 mutation in these cells (12, 24, 28).

The mechanism by which nibrin stimulates Atm autophosphorylation was addressed in the current study. Since the nibrin C-terminal 100 aa contains binding domains for Mre11 and Atm, we sought to determine whether the role of nibrin in Atm activation is to translocate Mre11/Rad50 to nuclear, or to interact with Atm. Using an Mre11 transgene with artificial NLS sequences attached to the C terminus, we were able to translocate Mre11/Rad50 to the nucleus in the absence of nibrin. Although the Mre11-NLS transgene was not as efficient at directing Mre11/Rad50 nuclear localization as intact nibrin, Mre11-NLS localized to chromatin and complemented Atm activation in ATLD cells. Despite this, Mre11-NLS/Rad50 failed to stimulate Atm after irradiation. These results indicate that whereas the role of nibrin in translocating Mre11 and Rad50 to the nucleus is necessary for normal Atm activation kinetics, it is not sufficient to restore this function.

What else is nibrin doing then? In vitro studies have demonstrated that nibrin stimulates the enzymatic activities of the Mre11/Rad50 complex (22, 34). Clearly, Mre11/Rad50 enzymatic activity is required for Atm activation, since ATLD cells complemented with a nuclease-deficient mutant of Mre11 fail to activate Atm after neocarzinostatin treatment and a Rad50 ATPase mutant fails to activate Atm in vitro (24, 41). These results suggest that DNA DSBs must be processed by Mre11/Rad50 complex (22, 34). Clearly, Mre11/Rad50 enzyme activity is required for Atm activation and that the quantitative effect on Atm activation was reflected in reduced phosphorylation of p53 serine 15, which is not dependent upon recruitment of Atm to DNA DSBs, clearly delineating the role of nibrin in facilitating Atm activation from recruitment of Atm to DNA DSBs by nibrin. The K735X nonsense mutation used here to delete the Atm binding domain of nibrin is adjacent to the A734X mutation made by Falck et al. (J. Falck and S. Jackson, personal communication) and is unlikely to explain the difference in our results. However, Falck et al. have now performed additional experiments using a different Atm phospho-serine 1981 antibody and have obtained results consistent with our findings (J. Falck and S. Jackson, personal communication). Thus, there now is a consensus on this issue, with all studies indicating that Atm activation is stimulated by nibrin-Atm interaction.

Although we found that interaction between the nibrin C terminus and Atm had a profound effect on Atm activation, we consistently observed that the NBS1 ΔAtm transgene had some residual ability to stimulate Atm activation after irradiation, compared to the NbFr5 ΔAtm transgene that contains only the C terminus of nibrin. These findings suggest that other regions of nibrin can also contribute to Atm activation. The obvious candidate for this interaction would be via the nibrin FHA and BRCT domains. We, and others, have previously reported that mutations in the nibrin FHA and BRCT domains interfere with Atm phosphorylation of nibrin (6, 45). The nibrin FHA/BRCT domains are also required for accumulation of nibrin at the sites of DNA damage via interaction with MDC1, which may enhance activation of recruited Atm (6, 17, 27, 40, 45).

In the context of the overall DNA DSB response from DNA damage to the activation of downstream effectors, nibrin appears to play multiple roles. To sense DNA damage nibrin plays a role by translocating Mre11/Rad50 to the nucleus, stimulating their enzyme activity, and binding Atm, leading to its activation. In transducing the damage signal to downstream targets, nibrin functions by directing the accumulation of Atm at the sites of DNA damage where some Atm targets are phosphorylated. MRN may also act as an adaptor for some phosphorylation events, increasing the avidity of Atm for its targets (5, 23). Lastly, nibrin contributes to the effector phase of the DNA damage response through its role in S-phase checkpoint activation and by stimulating the DNA repair enzymatic functions of Mre11/Rad50.

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