Nuclear Export of NBN Is Required for Normal Cellular Responses to Radiation

Christine S. Vissinga, Tiong C. Yeo, Sarah Warren, James V. Brawley, Jennifer Phillips, Karen Cerosaletti, and Patrick Concannon

Molecular Genetics Program, Benaroya Research Institute, Seattle, Washington 98101-2795; Department of Immunology, University of Washington School of Medicine, Seattle, Washington 98195; and Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, Virginia 22908-0733

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Nijmegen breakage syndrome arises from hypomorphic mutations in the NBN gene encoding nibrin, a component of the MRE11/RAD50/nibrin (MRN) complex. In mammalian cells, the MRN complex localizes to the nucleus, where it plays multiple roles in the cellular response to DNA double-strand breaks. In the current study, sequences in mouse nibrin required to direct the nuclear localization of the MRN complex were identified by site-specific mutagenesis. Unexpectedly, nibrin was found to contain both nuclear localization signal (NLS) sequences and a nuclear export signal (NES) sequence whose functions were confirmed by mutagenesis. Both nuclear import and export sequences were active in vivo. Disruption of either the NLS or NES sequences of nibrin significantly altered the cellular distribution of nibrin and Mre11 and impaired survival after exposure to ionizing radiation. Mutation of the NES sequence in nibrin slowed the turnover of phosphorylated nibrin after irradiation, indicating that nuclear export of nibrin may function, in part, to downregulate responses after damage.

Exposure to ionizing radiation (IR) results in a spectrum of damage to cells that includes the induction of DNA double-strand breaks (DSBs). In mammalian cells, sensing of DNA DSBs is extremely rapid, occurring within seconds of exposure to IR, and very sensitive, responding to as little as a single DSB in a cell. The sensitivity and speed of this response require immediate access to genomic DNA and raise the possibility that nuclear localization of key components of the damage-sensing or signaling cascade could play an important regulatory role in the process.

The earliest measurable cellular response to DNA DSBs is phosphorylation of the protein kinase ATM on serine 1981. ATM exists normally in cells as an inactive dimer which, upon the induction of DNA DSBs, undergoes a transphosphorylation reaction and dissociates into active monomers (1). ATM is recruited to the sites of DNA DSBs via an interaction with the C-terminal end of the nibrin protein, amino acids 735 to 754 (9, 23), and subsequently phosphorylates nibrin (7, 10, 17, 21, 24) and other substrates. Phosphorylated nibrin then plays two key roles, one as a transducer of signals necessary to activate the S-phase checkpoint and the other as a scaffold for the recruitment and phosphorylation of other ATM substrates.

The MRE11/RAD50/nibrin (MRN) complex, of which nibrin is a component, has well-defined DNA repair functions, including DNA binding and nuclease activity. Consistent with these functions, hypomorphic mutations in nibrin and Mre11 result in radiation sensitivity disorders, Nijmegen breakage syndrome (NBS) and ataxia telangiectasia-like disorder, respectively. MRE11 interacts with a conserved binding site at the C-terminal end of nibrin, adjacent to the binding site for ATM (6, 9, 23). In NBS cells, where full-length nibrin is absent, MRE11 and RAD50 lose their nuclear localization and are distributed randomly throughout the cell, indicating a requirement for nibrin to maintain the correct subcellular localization of the MRN complex (3). Similar effects are observed in ataxia telangiectasia-like disorder cells, which have mutations in MRE11 that impair its binding to nibrin (20). Nibrin mutants lacking the C-terminal 100 amino acids that include the MRE11 binding site localize to the nucleus when expressed in NBS cells but fail to relocalize either MRE11 or RAD50 or to complement the cellular radiosensitivity associated with NBS (6, 15). These results suggest that sequences mediating nuclear localization of nibrin are located 5’ of the C-terminal 100 amino acids.

Given the critical role that nuclear localization plays in the function of the MRN complex, and hence the mammalian DNA DSB response, in the current study we used in vitro mutagenesis to map and identify sequences in mouse nibrin that affect the nuclear localization of the MRN complex. We demonstrate that the nuclear localization of nibrin and MRE11 represents an equilibrium state in a dynamic process of active import and export mediated by specific sequences in nibrin. Maintenance of this equilibrium by nibrin-mediated shuttling between the cytoplasm and the nucleus is required for normal cellular responses to DNA DSBs and may play a role in downregulating responses after damage.

MATERIALS AND METHODS

Construction of Nbs1 expression constructs and mutants. Nbn-green fluorescent protein (GFP) and all mutants were generated using Image clone 656105 encoding mouse nibrin (Research Genetics, Huntsville, AL) as the template in amplification reactions using Turbo Pfu polymerase (Stratagene, La Jolla, CA). Digested PCR products were ligated into BamHI-digested pEGFP-N1 (Clontech, Palo Alto, CA). The orientation of the gene and the accuracy of the

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Genetics, University of Virginia, P.O. Box 800733, Charlottesville, VA 22908-0733. Phone: (434) 982-3288. Fax: (434) 982-1815. E-mail: patcon@virginia.edu.

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sequence were confirmed by restriction mapping as well as nucleotide sequencing. Nbn-GFP truncation mutants were generated by introducing a BamHI restriction site, as well as an ATG initiation codon in the case of amino-terminal truncations, at the locations indicated in Fig. 1. Specific point mutations were generated using QuikChange site-directed mutagenesis (Stratagene). Full-length expression constructs for Nbs1, either wild type or containing inactivating mutations in the nuclear localization signal (NLS) or nuclear export signal (NES) sequences, were expressed from a plasmid containing a mouse genomic fragment extending from 2 kb upstream of the Nbn gene through exon 2. cDNA sequences, either wild-type or mutant forms of nibrin, were fused in frame in exon 2 of the plasmid construct. The resulting vectors express Nbn from the native promoter contained in the 2-kb upstream region.

Cell culture and transfection. NIH 3T3 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 1 mM sodium pyruvate, 100 μg/ml streptomycin, and 10 U/ml penicillin (Invitrogen, Carlsbad, CA). In experiments to inhibit nuclear export, leptomycin B (LMB; Sigma Chemical Corp., St. Louis, MO) was added at 20 ng/ml penicillin (Invitrogen), 1 mM penicillin (Invitrogen), and 10 U/ml penicillin (Invitrogen, Carlsbad, CA). To inhibit nuclear export, leptomycin B (LMB; Sigma Chemical Corp., St. Louis, MO) was added.

RESULTS

Nuclear localization of nibrin is directed by multiple independent NLS sequences. Examination of the amino acid sequence of mouse nibrin revealed four putative NLS sequences, KRER468 (NLS1), KRKR550 (NLS2), KKPR592 (NLS3), and KKKF567 (NLS4), one of which (NLS1) had been previously identified as a binding site for KPN1A2, which mediates nuclear import (19) (Fig. 1). A series of C-terminal and N-terminal truncation mutants of mouse nibrin were fused in frame with GFP, transiently transfected into NIH 3T3 cells, and examined for their cellular distribution by confocal microscopy. C-terminal nibrin truncation mutants encoding residues 1 to 527 or 1 to 562 and containing one or two putative NLSs, respectively, localized predominantly to the nucleus. Among the N-terminal truncation mutants, (463–752)-GFP, containing all four putative NLSs, displayed nuclear localization, whereas (529–752)-GFP, lacking NLS1, resulted in a minor but discernable increase in cytoplasmic GFP fluorescence. Nibrin mutant (587–752)-GFP, containing only the two most C-terminal NLSs, showed a further, and more marked, increase in cytoplasmic GFP fluorescence. Nibrin mutant (594–752)-GFP displayed no nuclear targeting, despite containing the most C-terminal putative NLS sequence. Results obtained with truncation mutants were extended by transiently transfecting full-length nibrin expression constructs containing site-specific NLS-inactivating point mutations into NIH 3T3 cells. The single mutants K465T and K548T displayed nuclear localization indistinguishable from that of wild-type nibrin, indicating that either of these sequences alone was sufficient to mediate nuclear import of nibrin (Fig. 1). In the double mutant K465T/K548T, cytoplasmic GFP fluorescence increased substantially, resulting in a homogeneous cellular distribution of GFP. The triple mutant K465T/K548T/K589T was excluded from the nucleus, as was the comparable truncation mutant (594–752)-GFP, confirming that NLS1, NLS2, and NLS3 function to direct nuclear localization of mouse nibrin.

Nibrin contains a conserved nuclear export sequence. Nuclear exclusion of the triple mutant K465T/K548T/K589T suggested the unexpected possibility of an active export mechanism operating on these nibrin mutants. The best-characterized pathway for protein export from the nucleus to the cytoplasm, the CRM1/exportin pathway, is sensitive to the antibiotic LMB (12). To determine whether the nibrin mutants lacking NLS function were subject to nuclear export via this pathway, NIH 3T3 cells expressing the truncation mutant (594–678)-GFP and the full-length triple point mutant K465T/K548T/K589T were treated with LMB. Wild-type nibrin tagged with GFP, as well as GFP alone, was unaffected by

Heterokaryon assay. Nuclear export was assessed by heterokaryon analysis essentially as described previously (2). NIH 3T3 mouse cells were transiently transfected with GFP-tagged wild-type nibrin, GFP-tagged NES mutant nibrin, or the GFP vector alone as described above. After 24 h, transfected NIH 3T3 cells were replated with an equal number of human HeLa cells on glass coverslips overnight. The following day, the cells were fused with 50% polyethylene glycol 1500 (Roche Applied Bioscience, Indianapolis, IN) for 30 s and then were washed and plated in 10-μg/ml cycloheximide for 2 to 4 h. Cells were fixed and blocked as described above, and nuclei were stained with Hoechst 33258 dye for 30 min. Heterokaryons were identified by phase-contrast and fluorescence microscopy based on the brightly staining centromeres in the mouse nuclei, which were absent in human nuclei. Heterokaryons containing ≤3 nuclei of each cell type were chosen for analysis.

Western blot analysis. Total cellular protein was isolated from cells with or without IR treatment by lysing cells in EBC buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM Na3VO4, 1 mM β-mercaptoethanol, 0.5% NP-40, protease inhibitors). Nuclear and cytoplasmic proteins were isolated using the NPER kit (Pierce Biotechnology, Rockford, IL). For direct Western blots, 20 μg of total cellular protein or 105 cell equivalents of nuclear or cytoplasmic fractions was electrophoresed per lane in NuPage Tris-acetate gels (Invitrogen) and transferred to Immobilon P membranes (Millipore, Billerica, MA). For immunoprecipitation, 500 μg of total cellular protein was immunoprecipitated with specific antibodies and protein G magnetic beads (Invitrogen) and then separated by electrophoresis and Western blotted. Immunoblots were probed with primary antibodies specific for nibrin (rabbit polyclonal; Cell Signaling Technology, Danvers, MA), Mre11 (monoclonal; gift from T. DeMaggio), Hsp90 (rabbit polyclonal; Cell Signaling Technology), Trf2 (monoclonal; ENM Chemicals, Gibbstown, NJ), Atm (rabbit polyclonal; Novus Biologicals, Littleton, CO), ATM phospho-S1981 (rabbit polyclonal; Abcam, Cambridge, MA), and nibrin phospho-S343 (rabbit polyclonal; Abcam). Primary antibodies were detected with either goat anti-rabbit immunoglobulin or goat anti-mouse immunoglobulin coupled to horseradish peroxidase (BD Biosciences, San Jose, CA), and signals were detected by chemiluminescence. Western blot signals were quantitated by densitometry using ImageQuant software version 5.2.

Clonogenic survival assay. Mouse ear fibroblast lines expressing various nibrin transgenes were tested for radiation survival using a standard clonogenic survival assay. Fibroblasts were exposed to 0, 1, 2, 3, or 4 Gy of IR and then plated at clonal dilution in triplicate. After 14 days, cells were fixed and stained with Coomassie blue stain (Bio-Rad, Hercules, CA). Colonies were enumerated, and the mean and standard deviation at each radiation dose were calculated. Radiation survival was expressed as the percentage of the unirradiated-control value and graphed using Prism GraphPad version 4.03.
exposure to LMB. However, both of the mutant proteins re-localized to the nucleus upon LMB treatment, consistent with their being a target of active nuclear export (Fig. 2A).

NESs are leucine-rich regions that conform loosely to a consensus sequence [L-X(2-3)-LIVFM-X-(2-3)-L-X-L/I] (13). Scanning mouse nibrin, Mre11, and Rad50 for this consensus sequence (14) revealed only a single potential site at positions 653 to 662 of nibrin, LLTEFRSLVV. The key residues of this sequence matching the consensus were highly conserved among nibrin sequences from a range of vertebrate species, and these residues were targeted for alanine substitution mutagenesis in the truncation mutant (594–678)-GFP that displayed nuclear exclusion. All point mutations made in the putative NES sequence of this construct resulted in increased nuclear fluorescence when transfected into NIH 3T3 cells (Fig. 2A). Mutants with increasing numbers of alanine substitutions displayed exclusively nuclear localization that could not be further enhanced by treatment with LMB.

Nibrin actively shuttles between the nucleus and cytoplasm by an NES-dependent mechanism. To determine whether the identified NES sequence was active in vivo, a heterokaryon assay (2) was performed. Full-length expression constructs for wild-type or NES-mutated mouse nibrin tagged with GFP were transiently transfected into NIH 3T3 cells, resulting in the expected nuclear fluorescence. These cells were then fused with HeLa cells in the presence of cycloheximide to block new synthesis of nibrin. Any appearance of GFP fluorescence in HeLa nuclei in these heterokaryons requires export of the fusion protein from the NIH 3T3 cell nuclei. As shown in Fig. 2B, at 2 h postfusion, GFP was readily detectable in the HeLa nuclei in heterokaryons formed with NIH 3T3 cells expressing wild-type nibrin but not in those expressing the NES-mutated form (AATEFRSAVA). When heterokaryons were scored for the presence of GFP fluorescence in the HeLa nuclei, a significant difference was observed between wild-type mouse nibrin and the NES-mutated version (P < 0.0001) (Table 1), confirming the NES dependence of the process.

**Fig. 1.** Nibrin contains three active NLS sequences. NIH 3T3 cells were transiently transfected with full-length mouse nibrin-GFP, nibrin-GFP mutants containing N-terminal or C-terminal truncating mutations, or full-length nibrin-GFP containing threonine substitutions at the indicated critical lysine residues. The cellular distribution of nibrin-GFP and nibrin-GFP mutants was assessed by confocal microscopy (magnification, ×400).

Nuclear localization of Mre11 requires both nuclear import and export of nibrin. In order to determine what phenotypes might depend upon the presence of the NLS or NES sequences in nibrin, it was necessary to express mutants in cells that lacked endogenous Nbn. Cells derived from NBS patients are hypomorphic, express a C-terminal fragment of nibrin containing both NLS and NES sequences, and thus were not suitable for such studies. Therefore, full-length nibrin constructs with mutations in either the three active NLS sequences or the NES sequence were introduced and stably expressed in a spontaneously immortal fibroblast cell line derived from mice with a conditional knockout of the Nbn gene (5). The endogenous Nbn locus was then inactivated by transient transfection of a plasmid expressing the cre recombinase, and cloned cell lines were established. Subcellular fractionation confirmed the expected alterations in cellular distribution of nibrin in these cells; nibrin was largely cytoplasmic in the NLS mutant cell line and largely nuclear in the NES mutant line (Fig. 3A). The cellular distribution of Mre11 in NLS mutant cells was comparable to that of nibrin, with only a small fraction observed in the nucleus. In the NES mutant, the distribution of Mre11 differed from that of nibrin; total Mre11 protein levels were decreased, and a significant fraction of Mre11 was found in the cytoplasm. Nibrin and Mre11 could still be coimmunoprecipitated from either NLS or NES mutant cells, although in decreased amounts, indicating that the mutations do not prevent their interaction (Fig. 3B).

Nuclear-cytoplasmic shuttling of nibrin is required for normal cellular responses to radiation. Expression of either the full-length NLS or NES mutants in cells lacking endogenous nibrin reduced the amount of intact MRN complex that is in the nucleus and that is potentially available to participate in cellular responses to DNA DSBs compared to cells expressing wild-type mouse nibrin. The NLS mutant was highly sensitive to IR in a clonogenic survival assay (Fig. 4), consistent with the significantly reduced levels of both nibrin and Mre11 in the nuclei of these cells. The NES mutant also displayed increased

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sensitivity to IR compared to undeleted cells or cells expressing wild-type nibrin but at a level intermediate between the controls and the NLS mutant.

The autophosphorylation of ATM on S1987 was assayed to determine if the radiosensitivity observed in NLS or NES mutated cells could be ascribed to effects on ATM activation (Fig. 5A). The NES mutant cells supported autophosphorylation of Atm at a level comparable to that of the wild type despite the reduced levels of Mre11 in the nuclei of these cells. The NLS mutant was impaired in its ability to stimulate Atm autophosphorylation when exposed to 4 Gy of IR but achieved normal levels of phosphorylated Atm when exposed to 12 Gy, comparable to the phenotype of human NBS cells.

The phosphorylation of mouse nibrin by Atm on serine 343 in response to radiation exposure was also examined in cells expressing either the NLS or NES mutants (Fig. 5B). The phosphorylated form of nibrin was present predominantly in the nucleus and was not detected in cells expressing only the NLS mutant, which could not be imported to the nucleus. The NES mutant was phosphorylated at levels comparable to those of the endogenous protein. Given that the NES mutant is impaired in its ability to exit the nucleus, we considered the possibility that this mutant might maintain nibrin phosphorylation over an extended period of time if nuclear-cytoplasmic shuttling was serving as a passive mechanism for eliminating activated nibrin from the nucleus after a cellular DNA damage response. A time course experiment indicated that the levels of nibrin phosphorylated on S343 began to diminish approximately 24 hours after irradiation.

### Table 1. Nuclear export of GFP-tagged mouse nibrin in NIH 3T3 × HeLa heterokaryons

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<th>NIH 3T3 transfactant</th>
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<tr>
<td></td>
<td>GFPNIH3T3</td>
<td>GFPHeLa</td>
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<tr>
<td>GFP-wild-type nibrin</td>
<td>24</td>
<td>23 (96; 0.79–1.0)</td>
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<tr>
<td>GFP-NES mutant nibrin</td>
<td>25</td>
<td>6 (24; 0.09–0.45)*</td>
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* P < 0.0001.

CI, confidence interval.

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**FIG. 2.** Nibrin contains an active NES sequence. (A) NIH 3T3 cells expressing wild-type nibrin-GFP, a nibrin-GFP fragment lacking any functional NLS (nibrin-594-678-GFP), a full-length nibrin-GFP mutant containing NLS inactivating point mutations (nibrin-K465T/K548T/K589T-GFP), GFP alone (vector), or nibrin-594-678-GFP containing the indicated mutations at conserved residues (L653, L654, L660, V661, and V662) within the putative NES of mouse nibrin were untreated (−) or treated with LMB (+; 20 μg/ml for 6 h). The cellular distribution of nibrin-GFP and nibrin-GFP mutants was assessed by confocal microscopy (magnification, ×600). (B) NIH 3T3 cells were transiently transfected with full-length constructs of either GFP-tagged wild-type mouse nibrin or GFP-tagged NES mutant nibrin. After 24 h, transfected NIH 3T3 cells were coplated with HeLa cells on glass coverslips overnight. On the following day, cells were fused and 2 hours later fixed and permeabilized. Cells were stained with Hoechst 33258 dye to distinguish mouse from human nuclei. Heterokaryons were identified by phase-contrast and fluorescence microscopy based on the brightly staining centromeres in the mouse nuclei, which were absent in human nuclei (magnification, ×945).

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**FIG. 3.** Subcellular localization of nibrin and Mre11 in NLS and NES mutant cell lines. (A) Nuclear (N) and cytoplasmic (C) extracts were prepared from the indicated cell lines separated on polyacrylamide gels, transferred to nylon membranes, and probed with antibodies to nibrin, Mre11, Hsp90 as a cytoplasmic control, and Trf2 as a nuclear control. Extracts were prepared from mouse fibroblasts containing a conditional knockout of the endogenous Nbn gene. These cells were transfected with a full-length Nbn expression construct in the vector pNA bearing the indicated mutations, and the endogenous Nbs1 gene was inactivated by cre deletion. NLS3X cells express only a full-length nibrin with the three functional NLS sequences mutated (K465T, K548T, and K589T). NLS3X cells express only full-length nibrin with the NES mutation AATEFRSAVA. Wild-type (WT) cells express only full-length wild-type nibrin, pNA cells have been transfected with the expression vector alone but have not been treated with cre and retain expression of the endogenous Nbn gene. (B) Whole-cell lysates from the above cell lines were immunoprecipitated using an anti-Mre11 monoclonal antibody, and immunoprecipitates were Western blotted. Immunoblots were probed with antibodies to nibrin or Mre11.

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mately 5 h following exposure to 12 Gy (Fig. 6). At 5 hours and earlier, there was no discernible difference in the ratios of phosphorylated nibrin to total cellular nibrin in comparing NES mutant cells with wild-type controls. However, at later time points, the NES mutants retained increased amounts of phosphorylated nibrin. At 10 h, the NES mutant cells retained twice as much nibrin S343 phosphorylation as wild-type nibrin, suggesting that the NES sequence in mouse nibrin may function, in part, to downregulate posttranslationally modified nibrin after DNA damage responses are complete.

FIG. 4. Clonogenic survival of NLS and NES mutant cell lines after exposure to IR. Mouse fibroblast cell lines with the endogenous Nbn gene disrupted and expressing the indicated full-length Nbn transgenes were irradiated with 0 to 4 Gy, plated in triplicate, and assayed for colony formation after 14 days. Results for 1 to 4 Gy were expressed as the percentage of the unirradiated-control value for each cell line. Each line represents the mean of two independent experiments. WT, wild type.

FIG. 5. Radiation damage response signaling in NLS and NES mutant cell lines. (A) Atm phosphorylation on S1987 following irradiation of NLS and NES mutant cell lines. Mouse fibroblast cell lines with the endogenous Nbn gene disrupted and expressing either a full-length wild-type (WT) or NES-mutated nibrin were mock irradiated or treated with 4 Gy or 12 Gy and harvested at 30 min. Nuclear (Nuc) and cytoplasmic (Cyt) extracts prepared from these cells were probed with an antibody directed against human ATM phosphorylated on S1981, which cross-reacts with mouse Atm phosphorylated on S1987 as well as antibodies to mouse nibrin and Mre11. Hsp90 and Trf2 serve as controls for the subcellular fractionation. (B) Nibrin phosphorylation on S343 following irradiation of NLS and NES mutant cell lines. The above cell lines were either mock irradiated or treated with 4 Gy and harvested at 60 min. Nuclear and cytoplasmic extracts prepared from these cells were probed with an antibody directed against mouse nibrin phosphorylated on S343 as well as antibodies to mouse nibrin and Mre11. Hsp90 and Trf2 serve as controls for the subcellular fractionation. Abbreviations are as defined for panel A.

FIG. 6. Time course of nibrin S343 phosphorylation following irradiation. Mouse fibroblast clones with the endogenous Nbn gene disrupted and expressing full-length transgenes for either wild-type (WT) or NES-mutated nibrin were treated with 12 Gy and harvested from 3 to 10 h later. Cell extracts were prepared and probed with an antibody to nibrin phosphorylated on S343. Nibrin S343 phosphorylation was quantitated by densitometry using ImageQuant version 5.2 and expressed relative to total nibrin detected in the same sample.
DISCUSSION

The MRN complex plays a critical role in the response to DNA DSBs in vertebrate cells. Given the rapidity with which such responses are mounted, it is not surprising that MRN displays a predominantly nuclear localization. In the current study, we examined sequences in mouse nibrin that could impact its subcellular localization and that of the MRN complex. Two NLS sequences in nibrin were identified that were equally capable of directing normal nuclear localization of nibrin, as well as a third site with a more modest effect. A fourth putative NLS motif, KKFK, had no apparent function. This region of mouse nibrin overlaps and shares critical residues with the site of Mre11 binding. The strong interaction of nibrin and Mre11 may mask the NLS4 sequence from the nuclear import machinery.

Our finding here of multiple redundant NLS sequences in mouse nibrin highlights the importance of maintaining nuclear localization of the MRN complex. The absence of recognizable NLS motifs in either Mre11 or Rad50 and the comparable effects of NLS mutations in nibrin on the subcellular localization of either nibrin or Mre11 suggest that the sequences mapped here control not just nibrin localization but also that of the entire MRN complex. Inactivation of these sequences resulted in a significant impairment of survival after irradiation, but cells containing these mutations were viable. Since the Mre11, Nbn, and Rad50 genes are essential in mice, the viability of these cells suggests either that the essential functions of these genes are not all nuclear or that there are alternative mechanisms for nuclear import of their protein products.

An unexpected finding from these studies was that mouse nibrin contained a putative NES sequence in addition to sequences directing its nuclear import. The presence of both an NLS and an NES in the same protein is not unique. There are precedents in other proteins such as p53, BRCA1, or APC for subcellular localization being determined either by competition between active NLS and NES sequences or by blocking access to NLS or NES sequences through posttranslational modification or protein interactions (8, 11, 22). Our data suggest that the former possibility is more likely in the case of nibrin since both NLS and NES activity could be demonstrated in heterokaryon assays. The strong bias toward nuclear localization of nibrin most likely represents an equilibrium point between actively competing processes of import and export.

While the need for nuclear import of nibrin and the other members of the MRN complex is clear, the role of nuclear export in the function of MRN is less obvious. That nuclear export does provide an important function was indicated by clonogenic survival studies in which cells expressing nibrin with mutations in the NES had impaired survival after irradiation relative to cells expressing wild-type mouse nibrin. This radiosensitivity is likely due, in part, to the impaired nuclear localization and reduction in levels of Mre11 that we observed in these cells. Whether these phenotypes result from an actual dependence on NES function for Mre11 stability or localization or whether mutations in the region of the NES act indirectly, perhaps by destabilizing MRN complexes, is unclear. In cell lines from human NBS patients, where only small amounts of a truncated nibrin protein are produced, nuclear localization of MRE11 and RAD50 is lost and their protein levels are also reduced, consistent with coordinate regulation of the levels and location of MRN components. Similar connections between protein turnover and nuclear export have been observed for a number of other DNA damage- or stress-responsive proteins such as p53 and BRCA1 (8).

We also considered the possibility that nibrin-mediated nuclear export might participate in the downregulation of radiation responses by redistributing MRN complex components that had been posttranslationally modified as part of the DNA damage response. Time-lapse confocal imaging of DNA DSB responses in living cells indicates that human nibrin transiently associates with breaks, during which time it is phosphorylated on S343 by ATM. The phosphorylated form of nibrin leaves the site of the break and accumulates in the nucleoplasm (18). Upon downregulation of ATM kinase activity, continuing nuclear export of nibrin and either dephosphorylation or turn-over in the cytoplasm could provide a simple mechanism to eliminate activated MRN components, helping to restore normal cell cycling. Time course data for NES mutant cells were consistent with this model. Up to 5 h postirradiation, levels of S343 phosphorylated nibrin were comparable in control and NES mutant cells. At later time points, the levels of phosphorylated nibrin declined in control cells but not in NES mutant cells, suggesting that nuclear export acts, in part, to eliminate phosphorylated mouse nibrin from the nucleus.

Finally, nuclear export of nibrin may also be required for some as-yet-recognized cytoplasmic functions for nibrin or other MRN complex members. In this regard, it has been reported that overexpression of human nibrin can result in activation of phosphatidylinositol (PI) 3-kinase, possibly resulting in increased cell survival (4). This activation may involve interaction between nibrin and subunits of the PI 3-kinase, which would be expected to occur in the cytoplasm. We were unable to demonstrate a similar interaction between nibrin and PI 3-kinase by coimmunoprecipitation in mouse fibroblasts (data not shown), but such interaction has been reported in neuronal (PC12) cells (16).

In summary, our results suggest that the localization of nibrin and the other MRN complex components in the cell is considerably more dynamic than static images or fractionation studies might suggest. Although the bulk of the MRN complex at any given time is localized to the nucleus, active NLS and NES sequences on nibrin direct its shuttling between the nuclear and cytoplasmic compartments. Disruption of either the import or export arms of this pathway redistributes MRN complex components within the cell and impairs the ability of cells to survive exposure to agents such as IR that induce DNA DSBs.

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