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Cell, Vol. 93, 477–486, May 1, 1998, Copyright ©1998 by Cell Press

The hMre11/hRad50 Protein Complex and Nijmegen Breakage Syndrome: Linkage of Double-Strand Break Repair to the Cellular DNA Damage Response

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Summary

Nijmegen breakage syndrome (NBS) is an autosomal recessive disorder characterized by increased cancer incidence, cell cycle checkpoint defects, and ionizing radiation sensitivity. We have isolated the gene encoding p95, a member of the hMre11/hRad50 double-strand break repair complex. The *p95* gene mapped to 8q21.3, the region that contains the *NBS* locus, and p95 was absent from NBS cells established from NBS patients. p95 deficiency in these cells completely abrogates the formation of hMre11/hRad50 ionizing radiation-induced foci. Comparison of the *p95* cDNA to the *NBS1* cDNA indicated that the *p95* gene and *NBS1* are identical. The implication of hMre11/hRad50/p95 protein complex in NBS reveals a direct molecular link between DSB repair and cell cycle checkpoint functions.

Introduction

Nijmegen breakage syndrome (NBS) is a rare autosomal recessive disorder characterized by microcephaly, immunodeficiency, and an increased incidence of hematopoietic malignancy (Weemaes et al., 1981; van der Burgt et al., 1996). The clinical features of NBS overlap to some extent with those of ataxia telangiectasia (AT), and thus, NBS has been described as an AT variant syndrome. However, NBS and AT are genetically distinct (Saar et al., 1997), and NBS patients do not exhibit neurological abnormalities, telangiectasia, or increased α-feto protein levels observed in AT patients (reviewed in Shiloh, 1997).

Nonetheless, NBS and AT exhibit remarkably similar phenotypes at the cellular level, suggesting that the corresponding gene products function in the same pathway. Heterokaryons of AT and NBS cells showed unlinked noncomplementation of the chromosome instability phenotypes of each, leading to speculation that the respective gene products are physically associated (Stumm et al., 1997). Cells from both NBS and AT patients show increased sensitivity to ionizing radiation (IR) as well as increased levels of spontaneous and induced chromosomal fragility. In addition, NBS and AT cells fail to induce p53 at the G1/S checkpoint and fail to suppress DNA synthesis in response to ionizing radiation (Taalman et al., 1983; Young and Painter, 1989; Jongmans et al., 1997; Perez-Vera et al., 1997; Sullivan et al., 1997) (AT reviewed in Hoekstra, 1997; Shiloh, 1997). Together, these data suggest that the AT gene product, ATM, is a component of, or functions in close proximity to, the primary sensor of DNA damage. Accordingly, the AT phenotypes can be explained by the failure to signal the presence of DNA damage. Hence, IR sensitivity in AT cells is generally attributed to defects in the cellular DNA damage response. However, some data suggest that DNA repair functions in AT cells may also be affected (Bedford, 1985; Blocher et al., 1991; Pandita and Hittelman, 1992; Murnane, 1995; Cornforth and Maser et al., 1997). Consistent with this notion, cells established from AT patients exhibit increased rates of intrachromosomal DNA recombination (Meyn, 1993).

We have characterized a double-strand break (DSB) repair complex from human cells that includes hMre11 and hRad50 (Petrini et al., 1995; Dolganov et al., 1996). The role of this complex in DSB repair is supported by the finding that hMre11 and hRad50 colocalize in nuclear foci in response to treatment of cells with DSB-inducing agents. The formation of hMre11 and hRad50 nuclear foci is reduced in SV-40-transformed AT cells, suggesting that the complex may be regulated by the signaling pathway that includes ATM (Maser et al., 1997). Furthermore, in normal diploid fibroblasts, hMre11 (and presumably the hMre11/hRad50 protein complex) becomes associated with DSBs in irradiated cells within 30 min postirradiation (Nelms et al., 1998). Thus, the hMre11/hRad50 protein complex, like ATM, appears to function in close proximity to the sensor(s) of DNA damage.

hMre11 and hRad50 are highly conserved relative to their yeast counterparts, suggesting that some functions of the *Saccharomyces cerevisiae* complex are conserved in mammals (Petrini et al., 1997). The phenotypic features of *Scmre11* and *Scrad50* mutants include hyperrecombination, sensitivity to DNA-damaging agents, and DNA repair deficiency (Ajimura et al., 1993; Game, 1993). These features are reminiscent of chromosomal instability syndromes such as AT, NBS, Bloom syndrome, and others (Fukuchi et al., 1989; Gatti et al., 1991; Meyn, 1995; van der Burgt et al., 1996; Ellis, 1997).

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The conservation of Mre11 and Rad50 functions predicts that similar phenotypic outcomes would result from mutations in humans that affect the hMre11/hRad50 protein complex. However, deficiencies in hMre11 or hRad50 have not been associated with any known chromosomal instability syndromes. Recent attempts to create a null *mre11* mutant in murine embryonic stem cells indicate that the gene is essential, suggesting that homozygous null mutants of *hMRE11* and *hRAD50* will not be found in the human population (Xiao and Weaver, 1997).

Previously, we showed that the hMre11/hRad50 complex consists of five proteins: hMre11, hRad50, and three additional proteins of 95 kDa, 200 kDa, and 400 kDa. Since Xrs2, a member of the *S. cerevisiae* Mre11/Rad50/Xrs2 protein complex is a 95 kDa protein, the 95 kDa subunit of the human complex was presumed to be the human *XRS2* homolog (Dolganov et al., 1996). To test this hypothesis, we isolated and characterized the 95 kDa subunit, p95, by direct protein sequencing. We found that p95 is a novel protein, with only limited homology to the *S. cerevisiae* Xrs2.

The locus encoding p95 maps to human chromosome segment 8q21.3, a region that was recently reported to contain the *NBS* locus (Saar et al., 1997). We compared the *p95* cDNA to the gene defective in NBS, *NBS1* (Varon et al., 1998, this issue of *Cell*), and found that p95 is the product of the *NBS1* gene. We further showed that p95 protein is not present in cell lines established from NBS patients. We demonstrate that p95 is an integral member of the hMre11/hRad50 complex and that the function of the complex is impaired in cells from NBS patients. These data define a direct molecular link between proteins involved in DSB repair and the activation of cellular DNA damage responses.

Results

Purification of the hMre11/hRad50 Complex from HeLa Extract

The 95 kDa and 200 kDa components of the hMre11/hRad50 protein complex were purified from a HeLa cell extract for direct protein sequencing. A 20%–50% $(NH_4)_2SO_4$ precipitate was separated by anion-exchange chromatography, and fractions containing hRad50 and hMre11 were identified by Western blotting. The hRad50/hMre11 complex was further purified from peak fractions by immunoaffinity chromatography and fractionated by SDS-PAGE. Our previous characterization of the hMre11/hRad50 protein complex indicated that five proteins of 81 kDa, 95 kDa, 150 kDa, 200 kDa, and approximately 400 kDa were immunoprecipitable by hMre11 antiserum (Dolganov et al., 1996). As expected, these proteins were readily visible on a Coomassie-stained gel (Figure 1). Other proteins observed were not part of the hMre11/hRad50 complex as shown by immunoprecipitation with preimmune serum (data not shown). The 95 kDa and 200 kDa bands (referred to henceforth as p95 and p200) were excised from the gel and subjected to mass spectroscopic analysis.

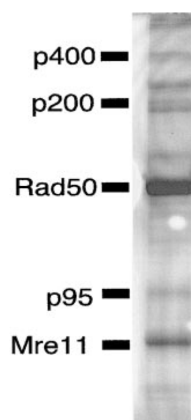

Figure 1. Purification of hRad50/hMre11 Complex

The eluent from the anti-hMre11 protein A-agarose beads (see Experimental Procedures) was separated by SDS-PAGE and stained with colloidal Coomassie blue. The five specific bands that purify with the complex are indicated.

Mass Spectrometry of Purified Proteins

The p95 protein was digested with trypsin and analyzed by LC/MS/MS to acquire tandem mass spectra for sequence analysis. A tandem mass spectrum for a peptide of molecular mass 1420.2 Da was interpreted to represent the sequence NPSGLNDDYGQLK. A tBLASTx search of dbEST found a match to the sequence NPSGLND DYGQLK in human EST 926991.

A similar analysis of the p200 band identified this protein as fatty-acid synthase (FAS) (Jayakumar et al., 1996). Since subsequent gel filtration chromatography indicated that FAS did not coelute with the hRad50/hMre11 complex (data not shown), we concluded that the presence of FAS among the immunoaffinity-purified proteins was an artifact of our isolation procedure. We have since confirmed that the presence of FAS is indeed an artifact and that this protein is not a component of the hRad50/hMre11 complex.

Cloning of the *NBS1* cDNA

The EST cDNA clone encoding p95 peptides (Table 1) was obtained from the IMAGE consortium (clone identification number 926991 [EST11]). This clone and a second overlapping human EST, 1083839 (EST30), were sequenced in their entirety. The combined DNA sequence spanned 4483 bp and contained a 2265 bp open reading frame (Figure 2A), sufficient to encode a protein with a predicted molecular mass of 85 kDa. The resulting cDNA has been designated *NBS1* (for Nijmegen breakage syndrome). Comparison of the open reading frame with tandem mass spectra obtained from purified p95 identifed 16 additional peptide matches (Table 1). Contrary to our expectations, the predicted p95 protein is essentially unrelated to the *S. cerevisiae* Xrs2 protein; the two proteins share only modest homology (28% identity) over the N-terminal 115 amino acids (Figure 2B).

Conservation of p95

The primary sequence conservation of hMre11 and hRad50, as well as their physical association, suggests that at least some functions of the *S. cerevisiae* Mre11/Rad50/Xrs2 protein complex are conserved in mammals. However, p95 appears to have replaced Xrs2 in the human complex. We asked whether p95 was conserved in other species to address whether substitution

Table 1. Peptides Obtained from Mass Spectrometry Analysis

Peptide[a]	Position[b]
-QPPQIESFYPPLDEPSIGSK-	189–209
-LSSAVVFGGGEAR-	238–251
-WIQSIMDMLQR-	289–299
-QGLRPIPEAEIGLAVIFMTTK-	300–320
-TTTPGPSLSQGVSVDEK-	335–351
-MLSQDAPTVKE-	395–404
-TSSNNNSMVSNTLAK-	409–423
-IPNYQLSPTKLPSINK-	426–441
-NYFQPSTKK-	458–465
-NKEQHLSENEPVDTNSDNNLFTDTDLK-	503–529
-EMDDVAIEDEVLEQLFK-	552–558
-MDIETNDTFSDEAVPESSK-	595–613
-ELKEDSWAK-	625–635
-KLLLTEFR-	653–660
-NPSGINDDYGQLK-[c]	671–683
-EESLADDLFR-	736–745

[a] Mass spectrometry is unable to distinguish L/I and Q/K. The indication of L, I, Q, and K residues in the peptide sequences is based on the p95 ORF.
[b] The numbering system is based on the ORF of the p95 cDNA.
[c] This peptide was sequenced by tandem mass spectrometry and used for EST database searches. The remaining peptides corresponded to the derived amino acid sequence from the p95 ORF.

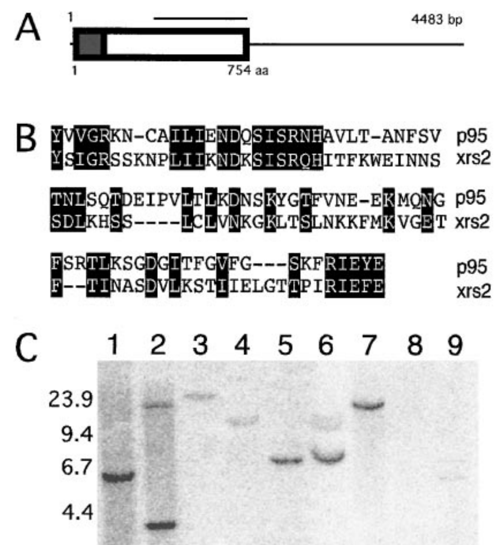

Figure 2. Structure and Conservation of the *p95* cDNA

(A) The schematic diagram represents the structure of the *p95* cDNA. The entire 4483 base pair (bp) cDNA is represented by the thin line, and the rectangular box is the 754 amino acid open reading frame (ORF). Within the ORF, the shaded box indicates the N-terminal region showing homology to *S. cerevisiae* Xrs2. The solid line above the ORF indicates the region cloned by two-hybrid screen utilizing hMre11 as bait.

(B) N-terminal alignment of p95 with ScXrs2. The shaded boxes indicate the regions of similarity. The two proteins show 28% identity and 46% similarity over the region displayed. The following amino acids were considered similar: (D, E, N, Q) (F, W, Y) (I, L, V) (K, R) (A, G) (S, T) (C) (H) (M) (P).

(C) Conservation of *NBS1*. A Zoo-Blot Southern blot (Clontech, Palo Alto, CA) of EcoRI-digested DNA from various species was probed with the *NBS1* cDNA. Lanes: 1, human; 2, monkey; 3, rat; 4, mouse; 5, dog; 6, cow; 7, rabbit; 8, chicken; 9, yeast. The positions of size markers (in kilobase pairs) are indicated on the left.

of p95 for Xrs2 in the Mre11/Rad50 complex was confined to humans. A Southern blot of DNA from various species probed with the *NBS1* cDNA revealed *NBS1* hybridization in monkey, rat, mouse, dog, cow, and rabbit, whereas no signal was observed in chicken or *S. cerevisiae* (Figure 2C). Rehybridization at lowered stringency did not reveal cross-hybridizing sequences in either chicken or *S. cerevisiae* (data not shown).

We used p95 antiserum to examine protein extracts from other species. As expected, cross-reacting material of the appropriate size was seen in monkey and cow extracts, but surprisingly, no cross-reactivity was observed in mouse and hamster (data not shown). We have similarly been unable to detect Rad50 in rodents with hRad50 antiserum (R. S. M., A. R. Bladl, M. S. Yao, and J. H. J. P., unpublished data). We also observed faint p95 antisera cross-reactivity in *C. elegans* and *X. laevis* extracts (data not shown). Hence, p95 appears to be conserved over a relatively broad phylogenetic spectrum.

Two-Hybrid Interaction of p95 and hMre11

In parallel with the approach described above, we undertook two-hybrid interaction screening to identify hMre11-interacting proteins. The *hMRE11* cDNA was cloned into the vector pAS1 as an in-frame fusion with the Gal4 DNA-binding domain (Durfee et al., 1993), and cDNAs encoding interacting proteins were isolated from a human B lymphoblastoid library by two-hybrid screening. DNA sequence and hybridization analyses revealed that 20 independent *NBS1* cDNA clones were among the interactors. The largest *NBS1* cDNA obtained in this screen (Figure 2A) began at amino acid position 363 of the p95 protein.

Expression of *NBS1*

Northern blot analysis with a *NBS1* cDNA probe revealed two *NBS1* mRNAs, a 4.4 kb transcript that was relatively abundant in all tissues and a 2.6 kb transcript that was present at high levels in testis (Figure 3A). The 4.4 kb mRNA, but not the 2.6 kb mRNA, was detected with a probe from the 3′ noncoding segment of the *NBS1* cDNA (Figure 3B), indicating that the two transcripts arise from the same locus but differ in the amount of 3′ untranslated sequence that they contain. The same Northern blot filter was hybridized to an *hMRE11* cDNA probe (Figure 3C). Two *hMRE11* mRNA species were detected; a 6.6 kb mRNA that was present in all tissues, and a 2.4 kb mRNA that was most abundant in testis. This pattern of expression is analogous to that of the murine *MRE11* gene (Petrini et al., 1995). The expression patterns of the *NBS1* 2.6 kb mRNA and the 2.4 kb *hMRE11* mRNA were identical, consistent with the observation that their respective protein products function together in the same complex.

Interaction of p95 with the hRad50/hMre11 Complex

To confirm the results of our immunoaffinity purification, we performed a series of immunoprecipitations from K562 cell extract with hMre11, hRad50, and p95 antisera (Dolganov et al., 1996). Immunoprecipitates were subsequently analyzed by Western blotting with the same

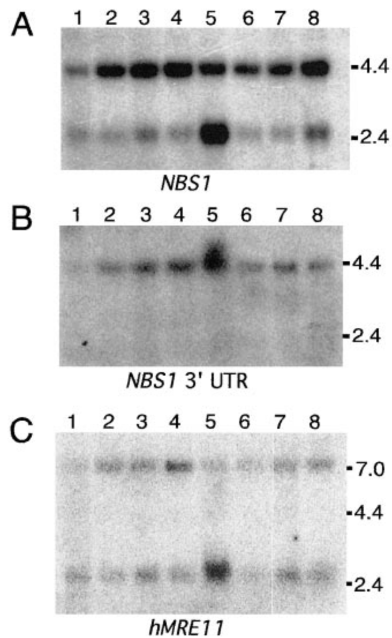

Figure 3. Northern Blot Analysis of *p95* and *hMRE11*

(A) A human multiple tissue Northern blot was probed with a cDNA fragment corresponding to bp 1–535 of the *NBS1* cDNA. Lanes: 1, peripheral blood lymphocytes; 2, colon; 3, small intestine; 4, ovary; 5, testis; 6, prostate; 7, thymus; 8, spleen. The positions of the size markers are indicated at right.

(B) The blot in (A) was stripped and reprobed with a cDNA fragment from the 3′-UTR of the *NBS1* cDNA corresponding to bp 3449–4385. The lanes and markers are identical to (A).

(C) The blot from (A) was stripped and reprobed with the human *MRE11* cDNA.

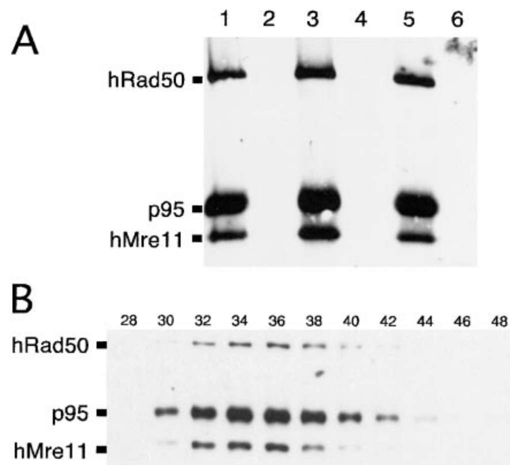

Figure 4. Interaction of p95 with the hRad50/hMre11 Complex

(A) Immunoprecipitations were carried out using K562 crude extract and subjected to Western blot analysis. The Western blot was probed with hRad50, hMre11, and p95 antisera. Lanes 1, 3, and 5 are immunoprecipitates with hRad50, hMre11, and p95 antisera, respectively, and lanes 2, 4, and 6 are the corresponding preimmune sera.

(B) Fractions from a Superose 6 FPLC separation of an aliquot of DEAE pool were subjected to Western blot analysis using hRad50, hMre11, and p95 antisera. The numbers at the top are the fraction numbers, and the positions of the three proteins are indicated on the left.

antisera. All three proteins (hRad50, hMre11, and p95) were precipitated with the three respective antisera but not with the corresponding preimmune sera (Figure 4A, lanes 1–6).

The association of these three proteins was also confirmed by gel filtration chromatography. hMre11/hRad50-containing fractions from the DEAE-sephacel column described above were pooled and separated on a Superose 6 gel filtration column. Western blotting of fractions from the sizing column with hMre11, hRad50, and p95 antisera was carried out. We found that the three proteins cochromatographed in a single peak corresponding to a molecular mass of approximately 1600 kDa (Figure 4B). hMre11, hRad50, or p95 proteins were not detected in later column fractions corresponding to lower molecular weight species. This observation suggests that the vast majority of hMre11, hRad50, and p95 in the cell are present in the high molecular weight complex.

Mutation of the *NBS1* Gene Is Responsible for Nijmegen Breakage Syndrome

Human metaphase cells were used for fluorescence in situ hybridization (FISH) with the *NBS1* cDNA. Cohybridization of probes EST11 and EST30 resulted in specific labeling only of chromosome 8 (Figure 5). Specific labeling of 8q21.2–22.1 was observed on four (2 cells), three (5 cells), two (12 cells), or one (6 cells) chromatid(s) of the chromosome 8 homologs in 25 cells examined. Of 61 signals observed, 53 (87%) were located at 8q21.2–22.1. Of these, 4 (7.5%) signals were located at 8q21.2, 46 (87%) signals were located at 8q21.3, and 3 (5.5%) signals were located at 8q22.1. Eight background signals were observed at other chromosomal sites. Six of these were single signals, and none of these chromosomal bands were labeled more than once. Double signals were observed once at 5q14. We observed specific signals at 8q21.3 in an additional hybridization experiment using this probe. These results suggest that the gene coding for p95 is localized to chromosome 8, band q21.3. Previous studies have demonstrated that the gene defective in the chromosome instability syndrome NBS maps to this locus (Matsuura et al., 1997; Saar et al., 1997).

We therefore tested whether p95 protein was present in cell lines established from NBS patients. Extracts were prepared from cell lines from five patients and subjected to Western blot analysis with p95 antiserum. p95 was not detected in any of the patient samples examined (Figure 6A). We showed that the levels of hMre11 and hRad50 were normal in NBS cells (data not shown). These data suggested that NBS is attributable to deficiency in the gene encoding p95. This hypothesis was confirmed by nucleotide sequence comparison of the *p95* cDNA with the positionally cloned *NBS1* gene isolated by Varon et al. (1998).

To assess whether hRad50 and hMre11 associate in the absence of p95, immunoprecipitations with hMre11 antiserum were carried out using crude extracts from a representative NBS cell line (JS; Figure 6B). We found

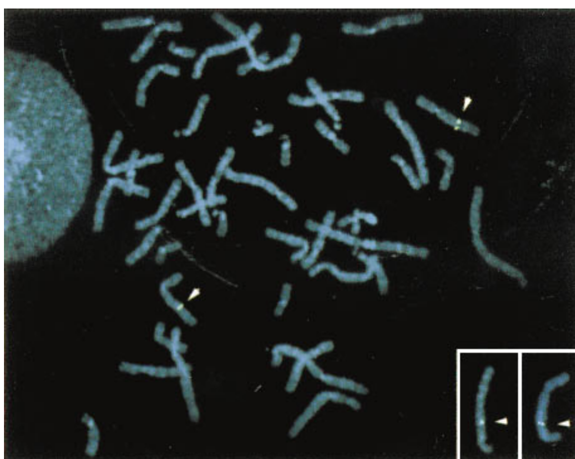

Figure 5. FISH Localization of *NBS1* to 8q21.3

Biotin-labeled *p95* cDNA probes were hybridized to human metaphase cells from phytohemagglutin-stimulated peripheral blood lymphocytes. The chromosome 8 homologs are identified with arrows; specific labeling was observed at 8q21.3. The inset shows partial karyotypes of two chromsome 8 homologs illustrating specific labeling at 8q21.3 (arrowheads). Images were obtained using a Zeiss Axiophot microscope coupled to a cooled charge coupled device (CCD) camera. Separate images of DAPI-stained chromosomes and the hybridization signal were merged using image analysis software (NU200 and Image 1.57).

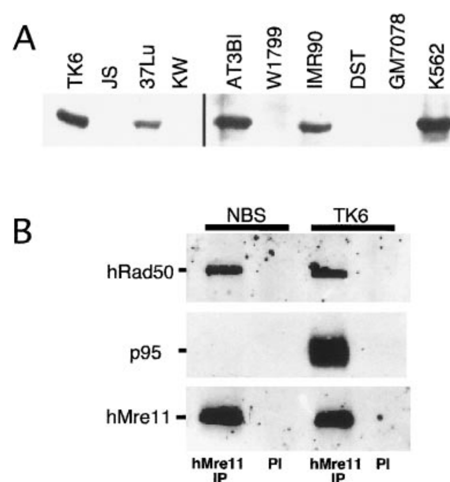

Figure 6. Analysis of p95 and the hRad50/hMre11 Complex in NBS Cells

(A) Crude extracts of normal cells (TK6, 37Lu, IMR90, and K562), an AT cell line (AT3BI), and NBS cells (JS, KW, W1799, DST, and GM7078) were subjected to Western blot with p95 antiserum.

(B) Immunoprecipitations were carried out on crude extracts from JS cells (NBS) and control cell line TK6 with hMre11 antiserum (hMre11 IP) or preimmune serum (PI). The resulting precipitates were subjected to Western blotting analysis with hMre11, hRad50, and p95 antisera. The positions of the three proteins are indicated on the left.

that similar amounts of hMre11 and hRad50 were coimmunoprecipitated by hMre11 antiserum from NBS extracts and control TK6 extracts (Figure 6B). The hMre11/hRad50 association is unaffected by the absence of p95, indicating that these two proteins interact directly.

Radiation-Induced Foci

We have previously shown that in human cells, hMre11 and hRad50 colocalize in large nuclear foci following treatment with agents that induce DSBs (Maser et al., 1997). To examine whether p95 is also present in ionizing radiation-induced foci (IRIF), 37Lu (normal diploid fibroblasts) were plated on glass slides, irradiated at a dose of 12 Gy, and doubly stained at 8 hr postirradiation with reagents to detect hMre11 and p95. hMre11 and p95 IRIF colocalized in irradiated cells, whereas diffuse nuclear staining for both proteins was observed in unirradiated control cells (Figures 7A–7H, 7K, and 7L). This observation is consistent with the notion that hMre11, hRad50, and p95 function in the same protein complex during the normal cellular response to DNA damage. As expected from Western blotting (Figure 6A), p95 was not detectable in NBS cell lines by immunofluorescence (Figures 7O–7T; data not shown).

Normal DNA damage responses such as cell cycle arrest, inhibition of DNA synthesis, and the induction of p53 are abrogated in NBS cells (Jongmans et al., 1997; Sullivan et al., 1997). Therefore, we asked whether the hMre11 IRIF response was intact in cell lines established from NBS patients. NBS fibroblasts (W1799 and KW) and normal control fibroblasts (IMR90 and 37Lu) were plated on slides as above, irradiated, and stained with hMre11 or p95 antiserum. The hMre11 and p95 IRIF responses of normal cells were consistent with previous studies (Maser et al., 1997), that is, 64%–85% of irradiated cells were positive for IRIF at 8 hr postirradiation. However, immunofluorescence analysis of over 1500 NBS cells showed that, in contrast to normal cells (Figures 7K–7N), the intranuclear levels of hMre11 and hRad50 were drastically reduced in both NBS cell lines, irrespective of prior irradiation (Figures 7U–7X and data not shown). Thus, hMre11-hRad50 IRIF do not form in the absence of p95.

Discussion

Nijmegen breakage syndrome is an autosomal recessive disorder characterized by developmental abnormalities, variable immune deficiency, and marked predisposition to malignancy. Cells established from NBS patients are sensitive to IR, exhibit chromosome fragility, and fail to activate cell cycle checkpoints in response to DNA damage (reviewed in Weemaes et al., 1994; van der Burgt et al., 1996; Shiloh, 1997). With respect to these features, the mutations that result in NBS and AT have essentially identical phenotypic outcomes. This suggests that the *NBS1* and *ATM* gene products mediate the same or closely related functions in the cellular DNA damage response (Shiloh, 1997).

In this study, we show that deficiency in a member of the hMre11/hRad50 protein complex, p95, is the cause of NBS. The evidence for this is 3-fold: (1) the *p95* locus maps to 8q21.3 (Figure 5), the region to which the *NBS* locus was previously localized (Matsuura et al., 1997; Saar et al., 1997); (2) the *NBS1* and *p95* cDNA sequences are identical (Varon et al., 1998); and (3) the p95 protein is absent from extracts of NBS cell lines

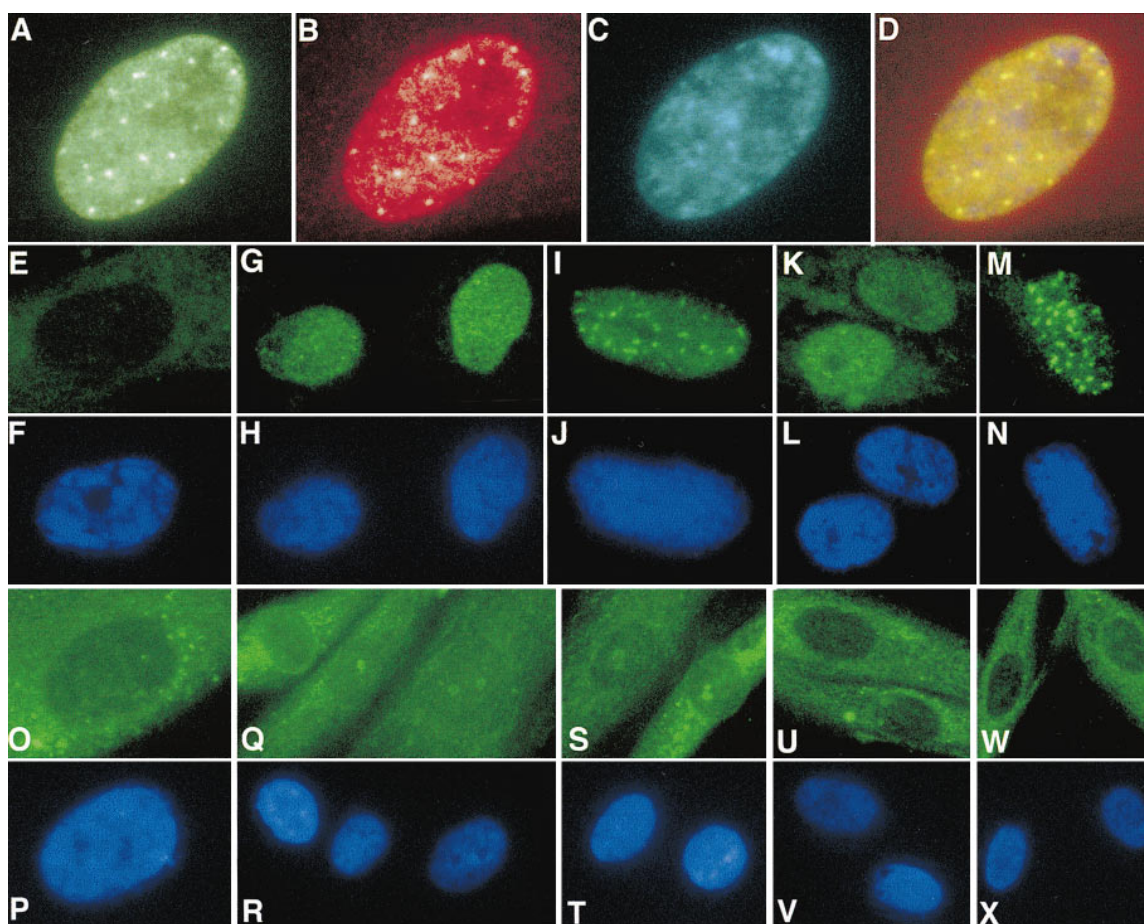

Figure 7. Ionizing Radiation-Induced Foci Formed by p95 and hMre11

(A–D) Colocalization of p95 and hMre11 IRIF. Irradiated 37Lu primary fibroblasts were harvested at 8 hr postirradiation, fixed, and probed with p95 antiserum and hMre11 mAb 8F3 as described in the Experimental Procedures. Images were captured of the same nuclei under FITC (A, green; p95 antiserum), Texas red (B, red; hMre11 mAb), and DAPI (C, blue; DNA) filters and merged (D) in Adobe Photoshop.

(E–X) IRIF formation in normal versus NBS cells. IMR90 normal fibroblasts (E–N) and W1799 NBS fibroblasts (O–X) were assessed for the ability to form IRIF. Unirradiated (E–H, K, L, O–R, U, and V) or irradiated (I, J, M, N, S, T, W, and X) cells were harvested at 8 hr posttreatment, fixed, and stained with DAPI and three different antibodies. (E) and (O) are p95 preimmune serum; (F) and (P) are the corresponding DAPI stains. (G), (I), (Q), and (S), are stained with p95 antiserum; (H), (J), (R), and (T) are the corresponding DAPI stains. (K), (M), (U), and (W) are stained with hMre11 antiserum; (L), (N), (V), and (X) are the corresponding DAPI stains.

that harbor *NBS1* mutations (Figure 6A). In light of the defects associated with NBS, these findings indicate that the hMre11/hRad50 protein complex is intimately involved in initiating the cellular DNA damage response.

The hMre11/hRad50 Protein Complex May Function as a Sensor of DNA Damage

We previously showed that the hMre11/hRad50 protein complex forms discrete nuclear foci (IRIF) following the induction of DSBs by ionizing radiation in normal cells (Maser et al., 1997). In contrast, IRIF do not form in NBS cells (Figure 7), suggesting that p95 is required for the relocalization of the hMre11/hRad50 protein complex to DSBs. This observation raises the possibility that p95 regulates the hMre11/hRad50 protein complex by transducing a signal originating from the site(s) of DNA damage. In normal cells, this signal leads to relocalization of the complex, whereas the signal is not transduced in NBS cells, and so movement of the complex does not occur. A similar, though less severe defect was observed in SV-40-transformed AT cell lines (Maser et al., 1997). These observations are consistent with the hypothesis that mutations in NBS and AT affect proximate functions in the DNA damage response (Shiloh, 1997).

However, the defect revealed by this aberrant IRIF response cannot account for the absence of DNA damage-dependent cell cycle checkpoints and diminished p53 responses observed in NBS cells (Jongmans et al., 1997; Shiloh, 1997; Sullivan et al., 1997). These aspects of the NBS phenotype indicate that p95, and by extension the hMre11/hRad50 protein complex, is an integral part of the signal that activates the cellular DNA damage response. In this regard, recent cytologic analyses of the hMre11/hRad50 protein complex in normal human cells are significant. DNA damage was induced in discrete subnuclear volumes, and DNA repair at those sites was monitored. hMre11 associated with DSBs within 30 min of their induction, and uniform distribution

of the protein was restored upon subsequent DSB repair (Nelms et al., 1998). The observed behavior of hMre11 at the sites of DNA damage is consistent with the notion that the hMre11/hRad50 protein complex functions as a DNA damage sensor and readily accounts for the DNA damage-dependent cell cycle checkpoint defects associated with NBS. The DNA repair functions of the hMre11/hRad50 protein complex are thus physically associated with the activation of other aspects of the cellular DNA damage response.

The hMre11/hRad50 Protein Complex in NBS Cells

The physical association of hMre11 and hRad50 is unaffected by the absence of p95 (Figure 6B). Similarly, the association of ScMre11 and ScRad50 does not appear to depend upon ScXrs2 (Johzuka and Ogawa, 1995; Ogawa et al., 1995). However, cytologic analyses reveal that the disposition of hMre11 and hRad50 is abnormal in NBS cells. The uniform nuclear distribution typical of hMre11 and hRad50 in unirradiated normal cells is not seen in NBS cells (Maser et al., 1997). Instead, the intranuclear abundance of hMre11/hRad50 appears to be reduced with a concomitant increase in the cytoplasmic level (Figures 7U–7X). Western blotting of fractionated NBS cell extracts shows that the protein is readily detectable in the nuclear fraction, though its level is somewhat diminished (data not shown). This is expected, since in murine embryonic stem cells, muMre11 is required for viability (Xiao and Weaver, 1997). Whereas loss of p95 may decrease the intranuclear abundance of hMre11 and hRad50, the levels of these proteins must be sufficiently high to support cell viability.

Divergence of the hRad50/hMre11 Protein Complex

hRad50 and hMre11 are human homologs of the *E. coli* proteins SbcC and SbcD (Sharples and Leach, 1995). SbcC and SbcD function in a protein complex, SbcCD, that possesses ATP-dependent double-stranded exonuclease activity and ATP-independent single-stranded endonuclease activity (Gibson et al., 1992; Connelly and Leach, 1996; Connelly et al., 1997). Genetic evidence from *S. cerevisiae* suggests that the Mre11/Rad50/Xrs2 protein complex functions as a nuclease in that organism (Ivanov et al., 1994, 1996; Tsubouchi and Ogawa, 1998). The biochemical activities of the hMre11/hRad50 protein complex have not been established, but the conservation of hMre11 and hRad50 suggests that they also encode nuclease activity.

Based on the conservation of hMre11 and hRad50, we hypothesized that p95 was the human Xrs2 homolog. This hypothesis is not supported by sequence comparison of p95 and Xrs2. The replacement of Xrs2 by p95 in humans may indicate that the function(s) mediated by these proteins is not conserved. This seems an unlikely possibility in light of the conservation of Mre11 and Rad50, particularly since neither Xrs2 nor p95 appears to function outside of its respective complex (Maser et al., 1997; Petrini et al., 1997). An alternative interpretation based on the NBS phentoype is that Xrs2 and p95 link the conserved activities of Mre11/Rad50 to the cellular DNA damage response in their respective organisms. In this sense, these divergent proteins could be considered functional analogs. The lack of similarity between p95 and Xrs2 would therefore reflect those features of the DNA damage response, and the roles of the Mre11/Rad50 nuclease within it, that are unique to each organism.

Phenotypic Similarity of *S. cerevisiae* and Human Mutants

The phenotypic features of *S. cerevisiae mre11/rad50/xrs2* mutants (Game, 1993; Petrini et al., 1997) are reminiscent of those observed in a number of human chromosomal instability syndromes such as NBS, AT, and Bloom syndrome (Chaganti et al., 1974; Bubley and Schnipper, 1987; Bigbee et al., 1989; Langlois et al., 1989; Meyn, 1993; Hojo et al., 1995). The chromosome fragility of NBS cells demonstrates that defects in the hMre11/hRad50 protein complex can result in similar phenotypic outcomes in mutant human cells.

Analogy of the *S. cerevisiae* and human phenotypes might also include DNA recombination defects, raising the possibility that immune dysfunction in NBS patients is attributable to DNA recombination defects. This interpretation is supported by the preponderance of chromosomal rearrangements involving chromosomes 7 and 14 in peripheral blood lymphocytes (reviewed in Weemaes et al., 1994). If the hMre11/hRad50 protein complex mediates single-stranded DNA endonuclease activity similar to that of SbcCD (Connelly and Leach, 1996), the complex might be important for the resolution of hairpin intermediates generated in the V(D)J recombination process (Gellert, 1997).

In this regard, it is noteworthy that similar fragility of chromosomes 7 and 14 is seen in AT patients, yet the immune defects observed in that disease do not appear to result from defects in V(D)J recombination (Hsieh et al., 1993). Immunoglobulin heavy chain rearrangements in the NBS lymphoblastoid cell line, GM7078, have been analyzed by DNA sequencing and found to be normal (Petrini et al., 1994). However, quantitative analysis of V(D)J recombination in NBS cells is required to address adequately the role of the hMre11/hRad50 protein complex in this process. Lymphocyte-specific recombination is also required for immunoglobulin class switching (reviewed in Stavnezer, 1996), and it is conceivable that this process is also affected in NBS patients. Furthermore, DNA recombination in other contexts, such as in meiotic recombination, may also be affected by mutation of *NBS1*.

p95 Links Recombinational DNA Repair to Cancer Predisposition

Genomic instability is frequently observed in human cancer predisposition syndromes (German, 1983; Timme and Moses, 1988; Cleaver, 1989; Jackson, 1995; Kolodner, 1995). Among such syndromes are congenitally acquired deficiencies in nucleotide excision repair and DNA mismatch repair. Since chromosomal rearrangements and changes in chromosome number are common features of malignant cells, errors in recombinational DNA repair are likely to play an important role in neoplasia (reviewed in Rabbitts, 1994; Rowley, 1994). The implication of p95

and the hMre11/hRad50 protein complex in NBS constitutes an important link between congenital recombinational DNA repair deficiency and genomic instability associated with the predisposition to malignancy.

Experimental Procedures

Cell Lines

HeLa S3 cells were obtained from the University of California–Berkeley Tissue Culture Center. Cell lines derived from patients with Nijmegan breakage syndrome were obtained from K. Sullivan (Children's Hospital of Philadelphia) and P. Concannon (Virginia Mason Research Center, Seattle, WA). Ataxia telangiectasia primary fibroblasts (AT3BI) were obtained from J. Murnane (University of California, San Francisco). IMR90, 37Lu, TK6, and K562 cell lines were grown as previously described (Maser et al., 1997). HeLa cells were grown in Joklik's MEM containing 5% newborn calf serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin. AT3BI and NBS primary fibroblasts (lines KW and W1799) were grown in DMEM/10%, FCS/5% Fetal Clone III (Hyclone, Logan UT), and NBS lymphoblasts (lines JS, GM7078, and DST) were grown in RPMI 1640/15% FCS.

Protein Purification

Crude HeLa extract was prepared essentially as described (Nishida et al., 1988). All procedures were carried out at 4°C. A 20%–50% $(NH_4)_2SO_4$ fraction was loaded on to a DEAE-sephacel column equilibrated in 50 mM Tris-HCl (pH 8.0), 1 mM DTT, 10% glycerol, and 50 mM NaCl (TDG + 50). The column was eluted with a 50 mM–500 mM NaCl gradient. hMre11 and hRad50 coeluted at 180 mM NaCl. Pooled fractions were concentrated by $(NH_4)_2SO_4$ precipitation and resuspended in TDG + 200.

hMre11 immunoaffinity reagent was constructed by cross-linking affinity-purified hMre11 antiserum with dimethylpimelimidate to protein A-agarose as described (Harlow and Lane, 1988). Pooled DEAE-sephacel fractions were incubated with the α-hMre11 beads in 300 mM NaCl. Beads were washed, and bound proteins quantitatively eluted with Acti-Sep elution media (Sterogene, Carlsbad, CA). Eluted proteins were dialyzed into 10 mM Tris-HCl (pH 8.0), 25 mM NaCl, and fractionated by SDS-PAGE. Proteins were visualized with colloidal Coomassie blue, and the unique 95 kDa band was excised for mass spectrometry analysis.

For gel filtration, pooled DEAE-sephacel column fractions derived as above were subjected to gel filtration on Superose 6 FPLC column (Pharmacia, Piscataway, NJ). Fractions were analyzed by Western blotting with p95, hMre11, and hRad50 antisera.

Mass Spectrometry

Proteins were subjected to in-gel trypsin digestion (Shevchenko et al., 1996). Microelectrospray columns were constructed from a 360 μm o.d. × 100 μm i.d. fused silica capillary with the column tip tapered to a 5–10 μm opening. The columns were packed with Perceptive Biosystems (Framingham, MA) POROS 10 R2, a 10 μm reversed-phase packing material, to a length of 10–12 cm. The flow from the HPLC pumps (typically 150 μl/min) was split precolumn to achieve a flow rate of 500 nL/min. The mobile phase used for gradient elution consisted of (A) 0.5% acetic acid and (B) acetonitrile/water 80:20 (v/v) containing 0.5% acetic acid. The gradient was linear from 0%–40% B in 50 min followed by 40%–80% B in 10 min or 0%–60% B in 30 min. Mass spectra were recorded on an LCQ ion trap mass spectrometer (Finnigan MAT, San Jose, CA) equipped with a microelectrospray ionization source (T. Gatlin, G. Kleeman, L. G. Hays, and J. R. Yates III, unpublished data). Electrospray was performed at a voltage of 1.6 kV. Tandem mass spectra were acquired automatically during the entire gradient run as previously described (Link et al., 1997). Tandem mass spectra of peptides from p95 were compared to the protein and gene sequence using the computer program SEQUEST (Eng et al., 1994; Yates et al., 1995). Sequences for potential contaminants such as human keratin and bovine trypsin were added to the database.

Two-Hybrid Interaction

hMre11 was expressed as a *GAL4* DNA-binding domain fusion protein from pAS1 (Durfee et al., 1993) in the yeast strain, PJ69-4A (James et al., 1996). Following introduction of a cDNA libary in the vector pACT derived from a human B-lymphoblastoid cell mRNA, cDNAs encoding hMre11 interactors were selected for by growth in the absence of adenine. Apparent adenine prototrophic colonies were retested on plates lacking histidine or adenine, and pACT cDNA clones were isolated from yeast exhibiting adenine and histidine prototrophy and analyzed by DNA sequencing.

Hybridizations

Multiple tissue Northern blots (Clontech, Palo Alto, CA) were probed as previously described (Dolganov et al., 1996) utilizing a *NBS1* or *hMRE11* cDNA labeled by random priming as the probe. The Zoo-Blot Southern blot (Clontech) was probed by standard procedures utilizing the *NBS1* cDNA as a probe.

Immunoblotting and Immunoprecipitation

p95 antiserum was raised in a rabbit against a fusion protein comprising amino acids 399–751 of human p95 fused to glutathione S-transferase (GST). Affinity purification of anti-p95 antiserum was performed as described (Dolganov et al., 1996) over GST (to remove GST reactivity from the antiserum) and GST-p95 columns constructed with Actigel resin (Sterogene, Carlsbad, CA). The hMre11 monoclonal antibody was derived from a mouse immunized with a 6X-His-hMre11 fusion protein in the University of Wisconsin Hybridoma facility.

Whole-cell extracts (from 3×10^5 cells) were prepared as described (Dolganov et al., 1996) and fractionated in 7.5% SDS-PAGE gels. Proteins were transferred to nitrocellulose, and immunoblots were performed (Dolganov et al., 1996) with p95, hMre11, and hRad50 antisera on the same filter in succession. Immunoprecipitations were performed on K562 lysates with p95, hMre11, hRad50, or the respective preimmune antisera as described (Dolganov et al., 1996). Immunoprecipitates were fractionated, transferred to nitrocellulose, and immunoblotted as above.

Chromosomal Localization of p95

Metaphase chromosomes were prepared from phytohemagglutin-stimulated peripheral blood lymphocytes from a normal human subject. The *NBS1* probes (clones 926991 and 1083839) were Biotin-labeled by nick translation using Bio-16-dUTP (Enzo Diagnostics, Farmingdale, NY), and fluorescence in situ hybridization was performed as described previously (Rowley et al., 1990). Hybridization was detected with fluorescein-conjugated avidin (Vector Laboratories, Burlingame, CA), and chromosomes were identified by staining with 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI).

Immunofluorescence

Primary fibroblasts were grown on glass slides, irradiated, and fixed as described (Maser et al., 1997). For double immunolabeling, cells were fixed in 3.5% paraformaldehyde and permeabilized as described (Scully et al., 1997). Cells were incubated with affinity-purified rabbit p95 antiserum (as above) and a 1:50 dilution of anti-hMre11 monoclonal ascites (line 8F3) for 1 hr at room temperature. After washing in PBS, cells were incubated with FITC-conjugated goat anti-rabbit and Texas red–conjugated donkey anti-mouse antisera (Jackson Immunoresearch, West Grove, PA) for 1 hr at room temperature. Cells were then washed, counterstained with DAPI, and mounted as described (Maser et al., 1997).

In IRIF assays, a minimum of 200 nuclei were analyzed for each cell line, treatment, and antibody examined (Maser et al., 1997). Unirradiated samples were fixed and processed as above along with irradiated samples. Mutant NBS cells and normal controls were processed simultaneously and treated identically.

Acknowledgments

We thank the members of our laboratories for helpful discussions during the course of this work, P. Concannon and K. Sperling for sharing results prior to publication, P. Concannon and K. Sullivan for NBS cell lines, D. Wilson for EST clones, and S. Broedel for FAS antisera. This work was supported by NIH Grant GM56888, the March of Dimes, the Milwaukee Foundation, the American Cancer Society (ACS grant NP-918), by a grant to the University of Wisconsin

Medical School under the Howard Hughes Medical Institute Research Resources Program for Medical Schools (J. H. J. P.), the Office of Health and Environmental Research, US Department of Energy, Contract 4459-011542 (W. F. M.), NIH Grant GM 52095, in part by NIH grant 5P41RR11823-02 and National Science Foundation Science and Technology Center BIR9214821 (J. R. Y. III), and NIH Grant CA40046 (M. L.). J. P. C was supported by NIH Postdoctoral Training grant CA09215-12, and R. S. M. was supported by NIH predoctoral training grant 5T32GM07133. Paper number 3512 from the University of Wisconsin–Madison Laboratory of Genetics.

Received March 16, 1998; revised March 31, 1998.

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GenBank Accession Number

The accession number for the sequence reported in this paper is AF058696.