Nijmegen Breakage Syndrome Disease Protein and MRE11 at PML Nuclear Bodies and Meiotic Telomeres

David B. Lombard and Leonard Guarente

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Abstract

Nijmegen breakage syndrome is a disease characterized by immunodeficiency, genomic instability, and cancer susceptibility. The gene product defective in Nijmegen breakage syndrome, p95, associates with two other proteins, MRE11 and RAD50. Here we demonstrate that in the absence of DNA damage, a portion of p95 and MRE11 is concentrated in PML nuclear bodies (NBs); MRE11 localization to the NBs is p95-dependent. In mammalian meiocytes, these proteins are specifically found at the telomeres. These results implicate the NBs in the maintenance of genomic stability and suggest that p95 and MRE11 may have roles in telomere maintenance in mammals, analogous to the role their homologues play in yeast.

Introduction

NBS is a rare autosomal recessive disease. Affected individuals demonstrate delayed growth, microcephaly and mental retardation, variable immunodeficiency, gonadal dysfunction, and heightened susceptibility to cancer (1). Cells explanted from individuals with this disease show genomic instability and sensitivity to IR, and lack an appropriate G1-S checkpoint in response to DNA damage (2–6). The gene product defective in NBS is p95/Nibrin (hereinafter referred to as p95; Refs. 7–9). p95 is present in a complex with at least two other proteins, MRE11 and RAD50 (7). Immunofluorescent localization of MRE11, RAD50, and p95 in mammalian cells provides additional evidence that these proteins respond to DNA damage (7, 10). In the absence of damage, these proteins have been reported to be diffusely spread throughout the nucleoplasm. After treatment of cells with IR, all three proteins colocalize in numerous nuclear dots, termed IR-induced foci. This response is absent in cells derived from NBS patients (7). Experiments in yeast have implicated MRE11p and RAD50p in telomere maintenance (11, 12), although no telomeric role for these proteins has yet been demonstrated in mammals. Here we demonstrate that in the absence of irradiation, a fraction of p95 and MRE11 proteins is present at PML NBs in human mitotic cells. In addition, in mammalian meiocytes, these proteins are associated with telomeres.

Materials and Methods

Cells. The wild-type human fibroblast WI38 and the NBS mutant cell line GM07166 were obtained from the Coriell Institute for Medical Research. Cells were grown in DMEM/15% heat-inactivated fetal bovine serum. GM07166 were obtained from the Coriell Institute for Medical Research. Cells derived from NBS patients are grown under standard culture conditions. Slides were prepared using the CSK and PFA fixation method as described previously (14, 15). Briefly, slides were washed once in PBS, washed for 30 s in CSK [100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 10 mM PIPES (pH 6.8)], incubated for 2 min in CSK containing 0.5% Triton X-100, washed briefly in CSK, and then fixed in PBS containing 4% PFA (Electron Microscopy Sciences) for 10 min.

Primary antibodies and their dilutions were as follows: rabbit anti-p95 and rabbit anti-MRE11 (gifts of J. Petrini, University of Wisconsin; a subsequent aliquot was the gift of D. Hill, Oncogene Research, Boston, MA), both 1:200 dilutions; mouse monoclonal anti-PML (Santa Cruz), 1:100 dilution; rabbit anti-SCP3 (gift of Christa Heyting), 1:200 dilution; H3 mouse anti-SCP3 (gift of Christa Heyting), 1:2 dilution; PX1 mouse anti-MRE11 (GeneTex), 1:50 dilution; mouse anti-TRF1 (gift of Titia deLange, Rockefeller University, NY), 1:5000 dilution. Slides were preincubated in blocking buffer [PBS containing 0.2% cold water fish skin gelatin (Sigma), 5% goat serum, and 0.2% Tween 20] for 30 min and then incubated for 1 h in blocking buffer containing the indicated concentration of primary antibody (or antibodies) at 37°C in a humidity chamber. Slides were then washed three times for 5 min each in PBS containing 0.2% Tween 20 and then incubated in blocking buffer plus secondary antibody. All secondary antibodies were purchased from Vector and were used at 1:100 dilution with the exception of Cy3 antirabbit and Cy3 antimouse, which were purchased from Amersham-Pharmacia and used at 1:200 dilution. Typical results are shown. In all cases, control experiments were performed using each primary alone and both secondaries to verify the results obtained in the double-labeling experiments.

Results

p95 and MRE11 at PML NBs in Somatic Fibroblasts. The p95 protein previously has been shown to be present throughout the nucleus of nonirradiated cells. However, if cells are initially subject to a hypotonic preextraction prior to fixation, which removes some of the diffuse nucleoplasmic p95, a fraction of p95 is concentrated in discrete nuclear structures in nonirradiated WI38 primary human fibroblasts (Fig. 1A). Using different preextraction protocols, others have also observed concentration of p95 in nuclear foci in nonirradiated cells. The appearance of these p95 foci is similar to PML NBs. Costaining of WI38 fibroblasts with rabbit anti-p95 and mouse anti-PML demonstrated that these p95 foci are NBs and that the p95 and PML staining patterns are virtually coincident in nonirradiated cells. Identical results were obtained using a human antisemur derived from a patient with primary biliary cirrhosis containing a high titer of antibodies directed against NBs (data not shown). Under these conditions, numerous other antigens, including Rad51, do not localize to NBs. With previously published immunofluorescence methods (7, 10), these p95 foci are nearly totally obscured by diffuse nuclear p95 staining (data not shown).

1 The abbreviations used are: NBS, Nijmegen breakage syndrome; IR, ionizing radiation; NB, nuclear body.
DNA damage previously had been demonstrated to cause relocalization of RAD50, MRE11, and p95 to IR-induced foci (7, 10). WI38 fibroblasts were subjected to 10 Gy of γ-irradiation and then fixed and stained 8 h later for the presence of p95 and PML (Fig. 1B). Some overlap was observed between p95 and PML in irradiated cells; however, a substantial amount of p95 was now observed adjacent to NBs (Fig. 1B, closed arrow) or not associated with any apparent NBs (Fig. 1B, open arrow). Overall, the p95 and PML patterns overlapped much less well in irradiated than in nonirradiated cells. Thus γ-irradiation apparently causes redistribution of NB-associated p95 from NBs to larger domains in the nucleus, some of which abut NBs and some of which do not. Costaining with DAPI does not reveal any

Fig. 1. Fractions of p95 and MRE11 localize to NBs. Shown are immunofluorescence images of wild-type primary human fibroblasts (WI38; A–C) or primary fibroblasts derived from an individual with NBS (GM07166; D) stained with anti-95 and anti-PML (A and B) or anti-MRE11 and anti-PML (C and D). In B, cells were irradiated with 10 Gy of IR from a 137Cs γ-source and then allowed to recover for 8 h prior to fixation and staining. Extensive colocalization of focal p95 and NB staining are observed in WI38 cells (A). After γ-irradiation, p95 appears in foci adjacent to some NBs (B, closed arrow) and also appears at additional foci not associated with NBs (B, open arrow). MRE11 is also present at NBs (C) in a p95-dependent manner (D).
obvious relationship between these larger p95-containing domains and heterochromatic regions (Fig. 1B).

The localization of MRE11 was also assessed. A fraction of MRE11 also concentrated in NBs (Fig. 1C). In NBS cells, this focal MRE11 staining was absent, indicating that p95 is necessary for MRE11 localization to NBs in the absence of DNA damage (Fig. 1D).

**p95 and MRE11 Proteins at Meiotic Telomeres.** Localization of p95 and MRE11 was assayed in both mouse and human meiotic spreads. Immunofluorescence was performed on mouse meiotic spreads using anti-p95 and a monoclonal antibody (3H3) directed against SCP3, a proteinaceous component of the presynaptic axial elements that remains associated with the synaptonemal complex following synapsis (16). p95 labeling was predominantly seen associated with the very distal ends of the chromosomes (Fig. 2A). This labeling was seen in meiotic spreads in late leptonema, zygonema, and early pachynema (data not shown). To confirm these results, MRE11 localization was also determined in these preparations using a commercially available monoclonal antibody against MRE11 (PX1; GeneTex) and a polyclonal antiserum directed against SCP3 (Fig. 2B). MRE11 is seen to be concentrated at the ends of chromosomes, although some chromosomes are also coated by MRE11 protein. We have observed MRE11 present on the synaptonemal complex of zero to four chromosomes per meiotic spread (Fig. 2B). We typically observe this MRE11 staining in late leptonema, but we do not know whether it correlates with the precise stage of the spread within meiosis.

The localization of p95 and MRE11 to the ends of chromosomes during meiosis suggests that these proteins may be preferentially associated with telomeres during this process. To confirm this hypothesis, p95 and the telomere binding protein TRF1 were colocalized...
in human meiotic spreads using rabbit anti-p95 and mouse anti-TRF1. The use of human meioses was necessitated by the fact that the anti-TRF1 antibody did not react with the murine protein. We confirmed that the p95 staining seen at the ends of chromosomes was at the telomeres (Fig. 2C). This pattern of p95 staining was never observed in mitotic cells. Similar overlap was observed when rabbit anti-MRE11 and anti-TRF1 were used (data not shown).

Discussion

It previously had been observed that in the absence of genomic damage, the p95/MRE11/RAD50 complex is present throughout the nucleoplasm (7, 10). Using a preextraction technique to remove loosely bound p95 and MRE11, we now show that in the absence of irradiation, a fraction of these proteins is concentrated in NBs. After DNA damage, the NB-associated p95 and MRE11 partially relocalize from NBs into larger domains, many of which include the NBs. We also examined p95 and MRE11 localization in meiocytes. p95 and MRE11 proteins were found to be specifically present at the telomeres in mammalian meiosis.

Although NBs have been reported to be sites of accumulation of recently synthesized RNA (17) and of DNA replication (18), a full description of their function is lacking. Several DNA viruses replicate preferentially near NBs, and many of these viruses encode proteins that disrupt NBs (19, 20). The recent demonstration that several proteins with roles in DNA recombination are present in NBs in cells that maintain their telomeres via a non-telomerase-dependent mechanism (21) suggests that NBs may, under some circumstances, play a role in recombination and in the maintenance of genome stability as well. The data presented in this work, together with our recent observation that two helicases of the RecQ family, BLM and RECQL, are highly expressed during meiosis (23), this is the first demonstration that p95 and MRE11 localized at NBs. The data presented in this work, together with our recent observation that two helicases of the RecQ family, BLM and RECQL, are highly expressed during meiosis (23), this is the first demonstration that p95 and MRE11 localized at NBs. The data presented in this work, together with our recent observation that two helicases of the RecQ family, BLM and RECQL, are highly expressed during meiosis (23), this is the first demonstration that p95 and MRE11 localized at NBs.

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