NBS1 and TRF1 Colocalize at Promyelocytic Leukemia Bodies during Late S/G2 Phases in Immortalized Telomerase-negative Cells

IMPLICATION OF NBS1 IN ALTERNATIVE LENGTHENING OF TELOMERES*

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Nijmegen breakage syndrome, a chromosomal instability disorder, is characterized in part by cellular hypersensitivity to ionizing radiation. The NBS1 gene product, p95 (NBS1 or nibrin) forms a complex with Rad50 and Mre11. Cells deficient in the formation of this complex are defective in DNA double-strand break repair, cell cycle checkpoint control, and telomere length maintenance. How the NBS1 complex is involved in telomere length maintenance remains unclear. Here we show that the C-terminal region of NBS1 interacts directly with a telomere repeat binding factor, TRF1, by both yeast two-hybrid and in vivo DNA-coimmunoprecipitation assays. NBS1 and Mre11 colocalize with TRF1 at promyelocytic leukemia (PML) nuclear bodies in immortalized telomerase-negative cell lines, but rarely in telomerase-positive cell lines. The translocation of NBS1 to PML bodies occurs specifically during late S to G2 phases of the cell cycle and coincides with active DNA synthesis in these NBS1-containing PML bodies. These results suggest that NBS1 may be involved in alternative lengthening of telomeres in telomerase-negative immortalized cells.

Telomeres comprise tracts of noncoding chromosomal hexanucleotide repeat sequences that, in combination with specific proteins, prevent degradation, rearrangement, and chromosomal fusion events (1). Telomere length is maintained by the de novo addition of telomere repeats by telomerase (2). In mammals, telomerase expression is ubiquitous in embryonic tissues and down-regulated in somatic adult tissues. There are, however, exceptions such as in regenerative tissues or tumor cells (3).

Recombination can lengthen telomeres in the absence of telomerase. For example, when the yeast telomerase RNA component, Terc1, is deleted, telomeres become shortened and most cells die (4). However, gene conversion mediated by the Rad52 pathway subserves telomere shortening in rare surviving cells (5). Genetic studies in yeast have also implicated the Rad50-Mre11-Xrs2 complex in telomere length maintenance, aside from its additional roles in homologous and nonhomologous recombinational repair, DNA damage assessment, and/or cell cycle checkpoint regulation (6). The Rad50-Mre11-Xrs2 complex has been proposed to function in the preparation of DNA ends for telomerase-mediated replication and is therefore implicated in telomerase-dependent telomere length maintenance, rather than in protection of telomeric ends.

In mammalian cells, Mre11 and Rad50, together with NBS1 (p95) form a complex (7–9) comparable in mass to a similar assemblage in Saccharomyces cerevisiae containing Rad50, Mre11, and Xrs2. The NBS1 gene mutated in Nijmegen breakage syndrome, a chromosomal instability disorder, encodes a 95-kDa protein (NBS1) with two functional modules found in cell cycle checkpoint proteins, a forkhead-associated domain and an adjacent BRCA1 C-terminal (BRCT) repeat (10). Rad50 is a coiled coil SMC (for structural maintenance of chromosomes)-like protein with ATP-dependent DNA binding activity (11). Mre11 has been proposed to have both structural (end-holding) and catalytic activities including double-stranded DNA 3’ to 5’ exonuclease and single-stranded endonuclease activity (9, 12–15). Despite their similar size (95 kDa), NBS1 and Xrs2p are quite dissimilar in sequence, although it remains possible that they will be functional analogues or early related homologues (16).

The function of NBS1 is unknown although there is speculation that it might recognize signals from a DNA damage-sensing complex that could be in the form of phosphorylation of serine or threonine residues that are, in turn, recognized by the forkhead-associated domain in NBS1 (16). Mre11 colocalizes to subnuclear regions containing DNA breaks within 30 min after irradiation of normal human diploid fibroblasts (17). In NBS1 cells, a deficiency of NBS1 is correlated with an inability to form Mre11-Rad50 nuclear foci in response to ionizing radiation (8). Together, these observations point to a major role for the Mre11-Rad50-NBS1 complex in repair of DNA double-strand breaks. The NBS1 protein is essential for Mre11 phosphorylation upon DNA damage (18). In addition, NBS1 function has been linked to ATM by the observation that phosphorylation of NBS1 in response to radiation exposure is ATM-dependent (19–21). Whether the mammalian Rad50-
Mre11-NBS1 complex also plays a role in the maintenance of telomere length has not yet been demonstrated. To address this issue, we have undertaken to identify cellular interacting partners of NBS1 by yeast two-hybrid screening. Here we report the identification of a telomere repeat binding factor, TRF1, as an interaction partner of NBS1. We show that NBS1 and Mre11 colocalize with TRF1 in PML nuclear bodies in telomerase-negative immortalized cells during G2 phase of the cell cycle. Significantly, the NBS1/TRF1 foci undergo active BrdUrd incorporation during late S/G2 transition, thus suggesting a novel role for NBS1 in telomere lengthening in telomerase-negative immortalized cell lines.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Interaction System**—Full-length NBS1 cDNAs (minus the first 12 amino acids) were fused to the DNA-binding domain of GAL4 in the pAS2–1 vector and used as the bait in two-hybrid screens using human lymphocyte cDNA library. Detailed screening procedures were described previously (22). Similarly, a cDNA fragment containing Rad50 (amino acid 753 to 1312), Mre11 (amino acid 1–680), to BRCA1 (amino acid 1–1315) was fused to the DNA-binding domain of GAL4 in the pAS2–1 vector. To map the binding regions, various truncated forms of TRF1 and NBS1 were generated and fused to the GAL4 activation domain of pGAD10 and DNA-binding domain of pAS2–1, respectively, and cotransformed into Mav203 with full-length NBS1 and TRF1, respectively. β-Galactosidase activity was quantified with chlorophenol red-β-galactopyranoside (CPRG) as the substrate (22).

**Cell Lines, Culture Conditions, Synchronization, and BrdUrd Labeling**—T24, a human bladder carcinoma cell line, MCF7, a human breast cancer cell line, HeLa, a human cervical carcinoma cell line, Saos2, a human osteosarcoma cell line, and IMR90, a human fetal lung primary fibroblast are from ATCC (America Type Culture Collection). SV40-immortalized human fibroblast cell lines, VA13, GM847, and LM217, were kindly provided by J. Shay and J. P. Murnane. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (10% CO2). Cell synchronization was performed by double thymidine block in G1/S boundary was performed as described with modification (23). Cells blocked at G1/S transition were released and fixed at different time points (0–13 h). For an additional arrest at early G2, by Hoechst 33342, cells were released from double thymidine block for 2 h, incubated with Hoechst 33342 at the final concentration of 0.2 μg/ml for 11 more h, washed with PBS, fixed, and processed for staining (24). For BrdUrd pulse labeling, cells were released from double thymidine block for 13 h, labeled with BrdUrd for 30 min with a cell proliferation kit (Amersham Pharmacia Biotech, RPN20), fixed, and stained as described.

**DNA Coimmunoprecipitation Assay**—The procedure was performed essentially as described (25). Briefly, cell nuclear extracts were prepared as described previously (26). A telomeric DNA-containing TTAGGG11 repeat sequence was labeled with 32P-dCTP by Klenow filling and purified in 5% polyacrylamide gel. For each reaction, 50 μg of nuclear extract was incubated with the telomere probe (30, 000 cpm) in a binding buffer (20 mM HEPES, pH 7.9, 150 mM KC1, 1 mM MgCl2, 5% glycerol, 4% Ficoll, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) at room temperature for 30 min. The protein-DNA complex was then immunoprecipitated by specific antibody and protein A beads and washed 3 times with binding buffer. The complex was digested by proteinase K and extracted with phenol/chloroform. The telomere probe was then precipitated by ethanol and electrophoresed in 5% native polyacrylamide gel and autoradiographed.

**Immunostaining**—This immunostaining procedure was adapted from the previous published work of Durfee et al. (27). Briefly, cells were grown on coverslips in tissue culture dishes and collected at various time points as indicated (random populated or synchronized). The cells were washed with PBS and fixed for 30 min in 3.7% formaldehyde in PBS plus 0.1% Triton X-100. Cells were then permeated with 0.05% Saponin at room temperature for 30 min, followed by washing five times with PBS. After being blocked with 10% goat serum in PBS/0.5% Nonidet P-40 at room temperature for 30 min, cells were incubated with primary antibodies at 4°C for overnight. After five PBS washes, cells were incubated with FITC or Texas Red-conjugated secondary antibodies (Southern Biotechnology Associates, Inc.) for 30 min. Cells were then washed extensively in PBS/0.5% Nonidet P-40, further stained with DAPI (1 μM/mL in H2O, Fisher), and mounted in Permafluor (Lipshaw-Immunocon, Inc.). Immunofluorescence microscopy was performed with a Nikon Eclipse TE300 immunofluorescence microscope. Images captured were processed with Adobe Photoshop.

**Antibodies**—Rabbit polyclonal antibodies specific for NBS1 and Mre11 were obtained from Novus Biologicals (Littleton, CO). FML mAb PG-M3 was from Santa Cruz Biotechnology (Santa Cruz, CA). 12D7 mAb specific for Mre11 has been described before (28). Mouse α-TRF1 was used as a control.
polyclonal antibodies were obtained by using bacterially expressed and purified GST-TRF1 fusion protein as antigen.

Isolation of GM847 Cells with Inducible Expression of the GFP-TRF1—To establish cell clones that express a GFP-TRF1 fusion protein, we have used the inducible expression system controlled by a tet-responsive promoter. A cDNA fragment containing GFP-TRF1 fusion protein was subcloned into an expression plasmid (pUHD10–3) driven by a core cytomegalovirus promoter linked to a tet operator heptad (pUHD10–3-GFP-TRF1) (29). This plasmid was cotransfected into GM847 cells with pCHTV, bearing a hygromycin-resistance gene and a cytomegalovirus-controlled tetracycline repressor-VP16 fusion transcription unit, and hygro-resistant clones were subsequently isolated. Several cell clones that express the GFP-TRF1 fusion protein upon removal of tetracycline were obtained.

RESULTS AND DISCUSSION

To explore the potential biological function(s) of NBS1, we used near full-length NBS1 as the bait in a yeast two-hybrid screen to recover interacting proteins encoded by a human lymphocyte cDNA library. One of the clones thus isolated corresponded to a near full-length cDNA for the telomeric DNA-binding protein TRF1. TRF1 consists of an acidic N terminus followed by a dimerization domain and a Myb-like DNA-binding domain at its C terminus (30). To determine the specific domain(s) of TRF1 required for binding to NBS1, various regions of TRF1 fused to the GAL4-transactivation domain were individually tested for their respective abilities to interact with NBS1 fused to the GAL4-DNA-binding domain by yeast two-hybrid assay. As shown in Fig. 1A, none of the isolated TRF1 fragments exhibited significant binding activity, suggesting that full-length TRF1 is required for efficient binding to NBS1. Reciprocally, a series of NBS1 deletion mutants fused to the GAL4-DNA-binding domain were tested for their abilities to interact with full-length TRF1. Interestingly, the C-terminal region of NBS1 (amino acids 534 to 754) is required for TRF1 binding, and its binding activity appears much stronger than that of the near full-length clone (Fig. 1B). It is possible that one or more inhibitory motifs reside N-terminally of amino acid 534 within NBS1 and that the NBS1-TRF1 interaction is enhanced by release of this inhibitory function by post-translational modification such as phosphorylation. In this regard, we note that there are multiple potential phosphorylation sites in this region, some of which are subject to regulation by the ATM kinase during the DNA damage response (19–21).

Because NBS1 forms a complex with Mre11, Rad50, and BRCA1 (28), we next tested whether TRF1 binds to any of these proteins in a yeast two-hybrid assay. As shown in Fig. 1C, TRF1 exhibits very little binding activity toward any one of these three proteins. These results suggest that TRF1 may bind to the Rad50-Mre11-NBS1 triplex through NBS1.

To determine whether the complex of TRF1 and NBS1 exists in human cells, telomeric DNA repeats were radioactively labeled...
and incubated with nuclear extracts. The labeled telomeric DNA repeats were specifically coimmunoprecipitated by antibodies against either TRF1 or NBS1 but not control antibodies against GST (Fig. 1D). These results further suggest that NBS1 binds to TRF1 in human cells and, furthermore, that the complex has the ability to bind the telomeric DNA repeats.

It has been well documented that the Rad50-Mre11-NBS1 triplex is involved in DNA double-strand break repair and, moreover, that specific nuclear foci positive for Rad50-Mre11-NBS1 are formed after ionizing radiation (8). Previous reports have suggested that, in the absence of DNA damage, no evident NBS1/Mre11 foci can be detected. Interestingly, we found that NBS1/Mre11 foci could be detected in about 5% of an asynchronously growing culture of human GM847 cells even in the absence of DNA damage treatment. These DNA damage-independent NBS1/Mre11 foci colocalize with TRF1 by immunostaining (Fig. 2), further supporting an in vivo association of NBS1 and TRF1 in human cells.

Yeager et al. (31) reported that TRF1 is associated with PML bodies in several telomerase-negative immortalized cell lines. Telomere length maintenance in this type of cells requires a telomerase-independent mechanism, previously designated as alternative lengthening of telomeres (ALT) (32). Coincidentally, the cell lines in which we observed DNA damage-independent NBS1/Mre11 foci are deficient in telomerase activity and immortalized through the ALT pathway. We therefore reasoned it likely that NBS1 might associate with PML bodies in these ALT cells. To determine whether this is the case, we examined a panel of telomerase-negative and -positive cell lines for the colocalization of NBS1 with PML bodies. As shown in Fig. 3, NBS1 colocalized with PML bodies in telomerase-negative immortalized cell lines including human GM847, Saos2, LM217, and VA13. However, in either telomerase-positive immortalized cell lines, such as T24 and MCF7, or human primary fibroblasts (IMR90), whereas PML bodies were detected, neither NBS1 foci nor NBS1 colocalization with PML bodies could be detected (Fig. 3).

Because only a small fraction of cells within a randomly growing telomerase-negative cell culture contain NBS1/Mre11 foci, it is likely that NBS1 foci formation is cell cycle stage-specific, perhaps during G2 phase. To test this possibility, cells synchronized at the G1/S boundary by a double thymidine block were released into the cell cycle and then fixed at specific time points post-release for immunostaining with NBS1-specific antibodies. As shown in Fig. 4, NBS1 foci formation in telomerase-negative immortalized cells occurs during late S/G2 phase. This cell cycle-specific formation of NBS1 foci was not observed in telomerase-positive cell lines. To more accurately establish the point at which NBS1 foci formation occurs during S/G2 phase, cells released from a double thymidine block were subsequently treated with Hoechst 33342, which specifically arrests cells at G2 phase. A significantly higher percentage of NBS1 foci-containing cells were observed in ALT cell lines but not in T24, MCF7, or HeLa cells (Fig. 4). These results suggest that NBS1 specifically translocates to PML bodies in ALT cells at G2 phase.

Telomere repeat sequences have been found in TRF1-containing PML bodies (31) and, as demonstrated above, NBS1 is localized to such PML bodies. It is therefore very likely that these PML bodies represent sites for maintaining telomere length in ALT cells. To test this possibility, a GFP-TRF1-expressing GM847 cell line was enriched for late S/G2 phase cells, pulse-labeled with BrdUrd for 30 min, and immunostained with an anti-BrdUrd antibody. GM847 cells were also subjected to an identical procedure and immunostained with anti-BrdUrd and anti-NBS1 antibodies. As shown in Fig. 5, the PML bodies in a fraction of GM847 cells, as indicated by NBS1 immunostaining or GFP-TRF1 immunofluorescence, underwent active BrdUrd incorporation at late S/G2 phase. By contrast, no BrdUrd incorporation could be observed in MCF7 cells following a similar labeling procedure. These results imply that the telomere lengthening process in ALT cells that requires DNA synthesis occurs in PML bodies at G2 phase of the cell cycle.

NBS1 is an apparent multi-functional protein, with demonstrated roles in DNA double-strand break repair and S-phase checkpoint control (8, 19). The interaction between TRF1 and the NBS1 complex suggests that NBS1 may be involved in telomere length maintenance in ALT cells. A homologous recombination-mediated mechanism has been proposed for telomere length maintenance in telomerase-negative cells in organisms ranging from human to yeast (32). Interestingly, Rad51, Rad52, RPA, and NBS1/Mre11 are localized to PML bodies (see Ref. 31 and this report). Given that multiple key players in homologous recombination are localized to PML
shortening at the telomere ends for this purpose. It remains to be shown that the NBS1-Mre11-Rad50 triplex is located make telomere ends available for replication. However, it re-
duplex telomeric repeat array. It is possible that the association
loops be formed by invasion of the 3′ telomeric overhang into the
NBS is a disorder characterized in part by an aging pheno-
more telomere maintenance provides a plausible explanation for these phenomena.

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