The Nijmegen Breakage Syndrome Protein Is Essential for Mre11 Phosphorylation upon DNA Damage

(Received for publication, April 12, 1999, and in revised form, May 14, 1999)

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The Nijmegen breakage syndrome (NBS), a chromosomal instability disorder, is characterized in part by cellular hypersensitivity to ionizing radiation. Repair of DNA double-strand breaks by radiation is dependent upon a multifunctional complex containing Rad50, Mre11, and the NBS1 gene product, p95 (NBS protein, nibrin). The role of p95 in these repair processes is unknown. Here it is demonstrated that Mre11 is hyperphosphorylated in a cell cycle-independent manner in response to treatment of cells with genotoxic agents including γ irradiation. This response is abrogated in two independently established NBS cell lines that have undetectable levels of the p95 protein. NBS cells are also deficient for radiation-induced nuclear foci containing Mre11, while those with Rad51 are unaffected. An analysis of the kinetic relationship between Mre11 phosphorylation and the appearance of its radiation-induced foci indicates that the former precedes the latter. Together, these data suggest that specific phosphorylation of Mre11 is induced by DNA damage, and p95 is essential in this process, perhaps by recruiting specific kinases.

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The abbreviations used are: NBS, Nijmegen breakage syndrome; FBS, fetal bovine serum; MMS, methylmethane sulfonate; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; PBS, phosphate-buffered saline; CIP, calf intestine phosphatase.

* This work was supported by a grant from the Susan G. Komen Foundation (to P. L. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The membranes were then probed with alkaline phosphatase-conjugated rabbit anti-mouse IgG and visualized by incubation with colorigenic substrates. In some of the experiments, p84, a nuclear matrix protein, served as the internal control for immunoblots.

**Immunostaining—** T24 cells grown on coverslips in tissue culture dishes were irradiated as above and collected at various time points as indicated. The cells were washed in PBS and fixed for 30 min with 4% formaldehyde in PBS with 0.1% Triton X-100. After five PBS washes, the cells were permeabilized with 0.05% saponin at room temperature for 30 min, followed by five PBS washes. The cells were blocked with 10% goat serum in PBS, 0.5% Nonidet P-40 at room temperature for 30 min. After one PBS wash, the cells were incubated with the primary mAb at 4 °C overnight. After five PBS washes, the cells were incubated with the fluorescein isothiocyanate-conjugated secondary antibody (Fisher) for 30 min. After washing extensively in PBS, 0.5% Nonidet P-40, cells were further stained with 4,6-diamidino-2-phenylindole (1 μg/ml in H₂O, Fisher) and mounted in Permafluor (Lipshaw-Immunonon, Inc., Pittsburgh, PA). Images obtained from cells with a standard fluorescence microscope (Axioskop Photomicroscope, Zeiss) were recorded with a Hamamatsu CCD camera and processed for presentation using Adobe Photoshop. JS cells were centrifuged (800 rpm) onto coverslips using Cytospin 3 (Shandon) prior to processing for presentation using Adobe Photoshop. JS cells were centrifuged (800 rpm) onto coverslips using Cytospin 3 (Shandon) prior to processing for presentation using Adobe Photoshop.

**RESULTS AND DISCUSSION**

Since Mre11 is postulated to have specific biochemical activities important for DNA repair, we began with its analysis under conditions of DNA damage. For this purpose, T24 cells, derived from a human bladder carcinoma, were exposed to several genotoxic agents, including UV radiation, MMS, γ radiation, adriamycin, or cis-DDP. In Western analyses of Mre11 in the extracts obtained from cells treated with these agents, the presence of two or more slower migrating bands was noted (Fig. 1A, lanes 2–6, arrowheads, compared with lane 1), suggestive of post-translational modification. To examine the relationship of these damage-induced, slower migrating species in the context of the Mre11-Rad50 complex, α-Rad50 mAb 13B3 immunoprecipitates from cells treated with UV, MMS, and γ radiation were fractionated by SDS-PAGE and probed with α-Rad50 mAb and α-Mre11 antibody. Fig. 1B shows that the same pattern of slower migrating Mre11 bands (arrowheads) is present in the Rad50 immunoprecipitates prepared from treated cells (compare lanes 3–5 with lane 2). In contrast to Mre11, Rad50 does not demonstrate any alterations in its mobility subsequent to treatment of the cells (Fig. 1B, top panel).

A common post-translational modification that can result in altered gel mobility of proteins is phosphorylation. To investigate this possibility, α-Rad50 immunoprecipitates from lysates prepared from UV-exposed or UV-irradiated cells that had been metabolically labeled with ³²P-phosphoric acid were either untreated or treated with CIP. The autoradiograms of each fractionated metabolically labeled immunoprecipitates revealed that Mre11 has a moderate level of phosphorylation under these conditions and that UV treatment results in a slower mobility band that is clearly labeled (Fig. 1C, arrowheads). Whether the moderate level of phosphorylation of Mre11 is constitutive or due to metabolic labeling of the cells is not known. Consistent with Mre11 hyperphosphorylation, CIP treatment eliminated the ³²P-labeled Mre11 bands (Fig. 1C, arrowheads) and results in a single α-Mre11 immunoreactive band in Western analysis of immunoprecipitates, which has a mobility equal to the α-Mre11 band in untreated samples (Fig. 1D, compare lanes 2, 3, and 4). Hyperphosphorylation of Mre11 also demonstrated a dose- and exposure-dependent response for MMS and γ radiation treatments, respectively (Fig. 1E).

These data firmly indicate that Mre11 is hyperphosphorylated upon treatment of cells with DNA-damaging agents, suggesting that this modification may play a role in the biochemistry of repair complexes. It is also apparent in Fig. 1C that both Rad50 and p95, in addition to Mre11, also undergo DNA-damage-induced phosphorylation under conditions of metabolic labeling (Fig. 1C, compare lanes 5, 6, and 7, which appear to retard the mobility of the p95 bands, but not Rad50 (Fig. 1C)).

To determine whether the phosphorylation of Mre11 is cell cycle-dependent, T24 cells were density-arrested (14), subsequently released, and then either mock-exposed or exposed to 0.05% MMS for 1 h. At various times subsequent to release, cells were harvested and the lysates analyzed by immunoblotting with α-Mre11 and α-Rad50 mAbs. The cell cycle sta-
tus of the lysates at each of the time points was assessed on the immunoblots by probing with α-RB antibodies (Fig. 2B; Ref. 14). Fig. 2A shows an approximately equal level phosphorylation of Mre11 at each of time points, indicating that this DNA damage-responsive hyperphosphorylation of Mre11 is cell cycle-independent. Rad50 does not demonstrate any changes in its gel mobility in the lysates of treated cells at any of the time points subsequent to release from arrest (Fig. 2A). These data indicate that the Mre11-Rad50-p95 complex is the target of DNA damage-induced phosphorylation of each component protein.

To investigate potential signaling systems that could be involved in Mre11 hyperphosphorylation, a panel of cell lines carrying specific genetic defects involved in the DNA repair processes were used. These included: ATM GM09607A cells, which are deficient for the product of the ataxia telangiectasia gene, a kinase involved in signaling pathways that regulate the response of cells to normal proliferative stimuli and the response to DNA damage (16); M059J and M059K cells (17), which are deficient and proficient, respectively, for DNA-PK, a serine-threonine protein kinase important for DSB (double strand break) repair (18); HCC1937 cells that express a C-terminally truncated BRCA1 protein and are hypersensitive to DNA damage (11); Capan-1 cells (12) that express a C-terminally truncated BRCA2 protein and are hypersensitive to MMS (19); and Saos2 cells, which are deficient for both the retinoblastoma protein, important in cell cycle regulation, and p53, a critical protein in DNA damage checkpoint control (20, 21). Lysates obtained from post-irradiated cells were analyzed by immunoblotting using α-Mre11 antibody. Retarded bands indicative of Mre11 hyperphosphorylation similar to that observed in lysates from treated T24 cells were observed in the lysates prepared from irradiated GM09607A (ATM), Capan-1 (BRCA2), M059K and M059J (DNA-PK), HCC1937 (BRCA1), and Saos2 (RB/p53) cells (Fig. 3A, lanes 1–18 compared with Fig. 1). In striking contrast to these lines, lysates prepared from two independent NBS cell lines (JS and WG) that are deficient for p95 (Fig. 3B, compare lanes 1–7 with 8 and 9) did not have any slower migrating bands subsequent to treatment of the cells with γ irradiation (Fig. 3A). Since two separate, independent NBS lines did not demonstrate retarded bands indicative of Mre11 phosphorylation upon DNA damage, there is a strong suggestion that p95, a component of the Rad50-Mre11-p95 complex, is important for phosphorylation of Mre11 during cellular responses to genotoxic events. Consistent with the Western data, metabolically labeled extracts from JS analyzed by α-Rad50 immunoprecipitation showed that labeled Mre11 and Rad50 bands do not increase upon UV treatment (data not shown). Interestingly, in the ATM-deficient cell line, GM09607A, a small amount of the hyperphosphorylated form of Mre11 can be found, which is increased significantly after DNA damage treatment (Fig. 3A, lane 1, compare with lanes 2 and 3). These results indicate that the ATM kinase may not be responsible for Mre11 hyperphosphorylation.

To further investigate potential outcomes of Mre11 hyperphosphorylation, the integrity of the Rad50-Mre11-p95 complex was assayed in JS and WG cells. Lysates prepared from untreated and MMS-treated T24, JS, and WG (NBS) cells were immunoprecipitated with α-Rad50 mAb 13B3, and Western blots of the immunoprecipitates were probed with p95, Mre11, and Rad50 antibodies. As observed previously, the Mre11 and p95 immunoreactive bands were readily detected in the Rad50 immunoprecipitates from T24 cells regardless of treatment (Fig. 4A, lanes 1 and 2). Despite undetectable levels of p95 in JS and WG cells, Mre11, apparently not hyperphosphorylated, was present in the Rad50 immunoprecipitates (Fig. 4A, lanes 3–6). These results suggest that an absence of DNA damage-induced phosphorylation of Mre11 has no apparent effect on its association with Rad50.

We then examined the kinetics of phosphorylation in T24 cells and tested for any possible phosphorylation of Mre11 in JS cells at longer times after radiation exposure than that used in Fig. 3A and 4A. The analysis of the JS immunoblots using α-Mre11 antibodies showed no retardation of the Mre11 bands at the longer time periods up to 24 h subsequent to radiation exposure. The analysis of the T24 lysates prepared at the indicated times showed that the intensity of the retarded bands peaked 1–3 h subsequent to treatment (Fig. 4B).

A marker of cellular responses to DNA damage involving the Rad50-Mre11-p95 complex is radiation-induced foci that can be immunostained by antibodies for each of the constituent proteins (22), a response that is abrogated in NBS cells (2). To investigate the temporal relationship of Mre11 phosphorylation and appearance of the Rad50-Mre11-p95 foci, T24 and JS cells were immunostained with the α-Mre11 antibody at various times subsequent to exposure to γ irradiation. As observed previously (2), the NBS cell line had no detectable α-Mre11 immunoreactive nuclear foci in treated cells, while T24 cells

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**Fig. 2. Mre11 hyperphosphorylation upon DNA damage is cell cycle-independent.** A, T24 cells synchronized at G1 by density arrest were released into the cell cycle and treated with MMS at the times indicated (G10 = G1, G24 = S, G33 = G2). After a 1-h treatment with no recovery, lysates were analyzed by immunoblotting using α-Rad50 and α-Mre11 mAbs. p84 served as internal control. B, the cell cycle status of the lysates in A was verified by the pattern of RB phosphorylation (14). US, unsynchronized.
treated identically had readily detectable foci using this antibody (Fig. 4C, compare the last two panels in rows one and three, from top to bottom). Interestingly, α-Rad51 antibodies2 were effective in immunostaining radiation-induced foci in both T24 and JS cells (Fig. 4C, panels one and two of rows one and three). These data further support the notion that the pathways for subnuclear partitioning of the Rad51 protein and Rad50-Mre11-p95 complex in response to DNA damage are distinct.2 The number of cells containing Mre11 foci at each of time points from 0.5 to 24 h was determined, and the percentages were plotted on the x axis as a function of time in the graph illustrated in Fig. 4D. A similar plot of the relative intensity of the slower migrating Mre11 bands shows that the hyperphosphorylation of Mre11 precedes Mre11 foci formation subsequent to treatment of cells with genotoxic agents (Fig. 4D).

These data indicate that Mre11 hyperphosphorylation subsequent to DNA damage is dependent upon intact p95 protein. The structure of p95 does not suggest a kinase role for this protein, indicating that it may be responsible for recruiting a putative kinase to phosphorylate Mre11. In fact, from Fig. 1C, it is apparent that both Rad50 and p95 are also phosphorylated upon DNA damage. These results suggest that the Rad50-Mre11-p95 complex may be a substrate for the kinase(s). Identification of this kinase(s) will be important toward understanding the DNA damage-responsive signaling pathways that are crucial for cellular responses to genomic insults. Although the consequence of Mre11 hyperphosphorylation does not appear to involve Rad50-Mre11 association in cells, the absence of radiation-induced Mre11 foci in NBS cells suggests that Mre11 hyperphosphorylation may have a role in subnuclear partitioning of the Rad50-Mre11-p95 complex to nuclear volumes containing DNA double-strand breaks (10). The strong correlations between 1) p95 and DNA-damage-induced Mre11 phosphorylation, 2) p95 and the radiation-induced Rad50-Mre11-p95 nuclear foci, and 3) p95 deficiency and the chromosomal instability and radiation-sensitive phenotype seen in NBS patients all point to the biological significance of the presence of p95 in the Rad50-Mre11 complex. The DNA damage-induced phosphorylation mediated by p95 is likely to be an important event in the DNA repair process.

Acknowledgments—We thank Drs. V. M. Der Kaloustian, K. Sullivan, and J. Allalunis-Turner for providing cell lines; Paula Garza for antibody preparation; and Dr. Tom Boyer for critical reading the manuscript. We are very grateful to both Drs. Wen-Hwa Lee and Z. D. Sharp for their encouragements and critical comments throughout the course of this study.

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J. Biol. Chem. 1999, 274:19513-19516.
doi: 10.1074/jbc.274.28.19513

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