Independent Roles for Nibrin and Mre11-Rad50 in the Activation and Function of Atm*

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The Atm protein kinase and Mre11-Rad50-nibrin (MRN) complex play an integral role in the cellular response to DNA double-strand breaks. Mutations in Mre11 and nibrin result in the radioresistance disorders ataxia-telangiectasia-like disorder (ATLD) and Nijmegen breakage syndrome (NBS), respectively. Cells from ATLD and NBS patients are deficient in activation of the Atm protein kinase and phosphorylation of downstream Atm targets following irradiation. However, the roles of individual MRN complex proteins in Atm function are not clear, because the mutations in NBS and ATLD cells result in global effects on the MRN complex. Previously we showed that the C-terminal 100 amino acids of nibrin were necessary and sufficient to translocate the MRN complex to the nucleus. Here we have taken advantage of the feature of nibrin to create isogenic cell lines lacking either nibrin or Mre11-Rad50 in the nucleus. We found that nuclear expression of Mre11-Rad50, but not nibrin, stimulated Atm activation at early times after low doses of radiation. At later times or higher doses of irradiation, Atm activation was independent of Mre11-Rad50 or nibrin. The requirement of MRN complex proteins for downstream Atm phosphorylation events following irradiation was more complex. Phosphorylation of nibrin and Chk2 by Atm required Mre11-Rad50 expression in the nucleus at early times after irradiation, reflecting the stimulation of Atm activation by Mre11-Rad50. By contrast, autophosphorylation of Smc1 at Ser-957 was dependent on the MRN complex 60 min after irradiation, even though Atm was activated at that time point. These results indicate an independent role for Mre11-Rad50 in the activation of Atm and suggest nibrin and/or Mre11-Rad50 also act as adaptors for some downstream Atm phosphorylation events.

The cellular response to DNA double-strand breaks (DSBs) involves detection of damaged DNA and subsequent signal transduction to the cellular machinery for apoptosis, cell cycle checkpoint control, and/or DNA repair. The coordination of these processes ensures the integrity of the genome and prevents mutations and chromosomal aberrations that can lead to malignant transformation. Central to the DSB response in mammalian cells is the Atm protein kinase that transduces the damage response signal to downstream effectors. Atm exists as an inactive dimer in the nucleus but undergoes autophosphorylation at Ser-1981 in response to DSBs and dissociates into active monomers (1). Among the many downstream Atm targets is the Mre11-Rad50-nibrin (MRN) complex. Both nibrin and Mre11 are phosphorylated by Atm in response to ionizing radiation (2–6). In vitro studies have shown Mre11-Rad50 can bind DNA and that Mre11 possesses a nuclease activity that can process these ends (7, 8). Nibrin stimulates the DNA binding and nuclease activity by Mre11-Rad50 (9, 10). In vivo, nibrin is responsible for translocating the MRN complex to the nucleus and relocating the complex to the sites of DSBs following irradiation (11, 12). The MRN complex is also required for activation of the S-phase checkpoint following DNA damage (13).

The interconnection between Atm and the MRN complex in the DSB response is underscored by the existence of radiosensitivity disorders caused by mutations in the genes encoding these proteins. Mutations in the Atm gene result in ataxia-telangiectasia (A-T), MRE11 gene mutations cause A-T-like disorder (ATLD), and mutations in the NBS1 gene are responsible for Nijmegen breakage syndrome (NBS) (14–16). These autosomal recessive disorders share some common features including immunodeficiencies and a predisposition to lymphoid cancers (16–18). Cells derived from A-T, ATLD, and NBS patients are hypersensitive to ionizing radiation and display spontaneous chromosomal instability, frequently involving chromosomes 7 and 14, as well as induced chromosomal instability (16–18). In response to ionizing radiation, A-T, ATLD, and NBS cells have cell cycle checkpoint defects, notably in the S-phase checkpoint as measured by radioresistant DNA synthesis (19).

Some Atm functions appear to be mediated through the MRN complex. Cells from ATLD and NBS patients have been reported to be deficient in Atm phosphorylation of p53, Chk2, Smc1, and FANCD2 following irradiation (16, 20–26). These observations led to the hypothesis that the MRN complex functions downstream of Atm as a mediator of Atm-induced signals. Recently, Uziel et al. (26) demonstrated that Atm activation following treatment with low doses of the radiomimetic drug necazarinostatin is deficient in NBS and ATLD cells, suggesting a second role for the MRN complex in the activation of Atm. The contribution of individual MRN complex proteins to Atm activation and function is not clear from these studies, however, because the mutations in NBS and ATLD cells have global effects on all members of the MRN complex proteins to Atm activation and function is not clear from these studies, however, because the mutations in NBS and ATLD cells have global effects on all members of the MRN complex.
complex. The truncating mutations in the NBS1 gene that are an invariant feature of NBS eliminate the production of full-length nibrin, but they also result in mislocalization of Mre11-Rad50 to the cytoplasm in NBS cells (27). Similarly, mutations in the MRE11 gene in ATLD cells significantly reduce the levels of Mre11 in the cells but also reduce the levels of nibrin and Rad50 (16).

We have used a retroviral expression system to assess the differential functions of nibrin and Mre11-Rad50 in the DSB response. Previously, we showed (11) that the C-terminal 100 amino acids of nibrin are necessary to bring the MRN complex to the nucleus. In the current study, a deletion mutant of nibrin that lacked the C-terminal Mre11 binding domain or an N-terminal nibrin deletion mutant that contained only the Mre11 binding domain were expressed in NBS fibroblasts to create cells lacking Mre11-Rad50 or nibrin, respectively, in the nucleus. The activation of Atm following exposure of the mutant cells to ionizing radiation was assessed, as well as the phosphorylation of downstream Atm targets including nibrin and the S-phase checkpoint proteins Smc1 and Chk2. We show that activation of Atm is stimulated by Mre11-Rad50 at early times after low doses of ionizing radiation. The requirement of nibrin or Mre11-Rad50 for downstream Atm phosphorylation events was more complex, suggesting that nibrin and/or Mre11-Rad50 may also act as adaptors for some Atm targets.

EXPERIMENTAL PROCEDURES

Cell Lines—NBS-ILB1 is an SV40-transformed fibroblast cell line established from an NBS patient homozygous for the common founder mutation 657del5 (28). NBS-ILB1 cells were grown in DMEM (Invitrogen) supplemented with 15% fetal calf serum (HyClone Laboratories, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). NBS-ILB1 cells stably expressing nibrin transgenes were maintained in the above medium with 500 μg/ml G418 (Invitrogen). An SV40-transformed A-T fibroblast cell line, AT3BI, and an SV40-transformed fibroblast cell line (11). Mre11-Rad50 remain cytoplasmic in the absence of Mre11, but the C-terminal 100 amino acids of nibrin from amino acids 1–400, including the conserved FHA and BRCT domains and the Atm phosphorylation site, was cloned into the pLXIN vector. In the current study, we took advantage of this finding to construct nibrin deletion mutants that, when expressed in NBS cells, created cells specifically lacking Mre11-Rad50 or full-length nibrin in the nucleus. As shown in Fig. 1A, the Nb652 fragment lacks the Mre11 binding domain of nibrin, whereas the NbFR5 construct retains the Mre11 binding domain but lacks the N terminus of nibrin from amino acids 1–400, including the conserved FHA and BRCT domains and the Atm phosphorylation sites at Ser-278 and Ser-343 (3, 6, 15). These mutant NBS1 cDNAs were cloned in the pLXIN retroviral vector and introduced into the NBS fibroblast cell line, NBS-ILB1, by retroviral infection. NBS-ILB1 cells fail to make full-length nibrin protein (28, Fig. 3). As controls, NBS-ILB1 cells were infected with retroviruses encoding wild type NBS1, a nibrin phosphorylation mutant, S278A/S343A, in which Ser-278 and Ser-343 were mutated to alanine, or with the empty pLXIN vector.

Localization of Nibrin or Mre11-Rad50 Can be Regulated by Mutagenesis of Nibrin—We have previously shown that the C-terminal 100 amino acids of nibrin bind to the N terminus of Mre11 and that this interaction is both necessary and sufficient to translocate the Mre11-Rad50 proteins to the nucleus of the cell (11). Mre11-Rad50 remain cytoplasmic in the absence of full-length nibrin, such as in NBS cells, or as a result of expression of a nibrin mutant lacking the C terminus (11, 27). In the current study, we took advantage of this finding to construct nibrin deletion mutants that, when expressed in NBS cells, created cells specifically lacking Mre11-Rad50 or full-length nibrin in the nucleus. As shown in Fig. 1A, the Nb652 construct lacks the Mre11 binding domain of nibrin, whereas the NbFR5 construct retains the Mre11 binding domain but lacks the N terminus of nibrin from amino acids 1–400, including the conserved FHA and BRCT domains and the Atm phosphorylation sites at Ser-278 and Ser-343 (3, 6, 15). These mutant NBS1 cDNAs were cloned in the pLXIN retroviral vector and introduced into the NBS fibroblast cell line, NBS-ILB1, by retroviral infection. NBS-ILB1 cells fail to make full-length nibrin protein (28, Fig. 3). As controls, NBS-ILB1 cells were infected with retroviruses encoding wild type NBS1, a nibrin phosphorylation mutant, S278A/S343A, in which Ser-278 and Ser-343 were mutated to alanine, or with the empty pLXIN vector.
Fig. 1B shows the expression of the nibrin transgenes and their interaction with Mre11-Rad50 as determined by immunoprecipitation with nibrin antisera. All of the constructs were expressed at levels comparable with endogenous nibrin in the control fibroblasts. Expression of wild type NBS1 or the S278A/S343A phosphorylation mutant in NBS-ILB1 cells resulted in production of full-length nibrin protein that coimmunoprecipitated with Mre11 or Rad50. NBS-ILB1 cells transduced with the Nb652 mutant expressed a smaller nibrin species that failed to coimmunoprecipitate Mre11 or Rad50, consistent with the deletion of the C-terminal Mre11 binding domain in this mutant. In contrast to Nb652, the NbFR5 mutant coimmunoprecipitated Mre11 and Rad50 at levels indistinguishable from wild type.

Analysis of nibrin and Mre11 localization by immunofluorescence in NBS-ILB1 cells expressing the nibrin transgenes supported these findings (Fig. 1C). Parental NBS-ILB1 cells infected with the vector alone displayed background levels of nibrin staining, and Mre11 was observed only in the cytoplasm. Expression of wild type nibrin or the S278A/S343A nibrin mutant in NBS-ILB1 cells restored nuclear localization of full-length nibrin and Mre11, apparent from the yellow colocalization of nibrin (Fig. 1C, red) and Mre11 (Fig. 1C, green). In cells expressing Nb652, nibrin staining was nuclear, whereas Mre11 remained cytoplasmic because of the deletion of the Mre11 binding domain. NBS cells expressing NbFR5 displayed nuclear staining for both the C-terminal nibrin fragment and Mre11. Thus, the subcellular localization of nibrin or Mre11-Rad50 is regulated by expression of nibrin mutants in NBS cells.

Mre11-Rad50 Stimulate Atm Activation Early after Irradiation—Atm activation, as measured by autophosphorylation at Ser-1981, is reduced in response to neocarzinostatin treatment in both ATLD and NBS cells (26). To assess whether nibrin, Mre11-Rad50, or the intact MRN complex are required for full Atm activation, we exposed cells expressing Nb652, NbFR5, or controls to 3 Gy of ionizing radiation and assessed Atm Ser-1981 phosphorylation after 15 and 60 min. As shown in Fig. 2A, Atm phosphorylation was dramatically increased in control fibroblasts 15 min after irradiation compared with parental NBS-ILB1 cells or NBS cells with vector alone. Restoration of nibrin and Mre11 localization by expression of wild type NBS1 or the S278A/S343A phosphorylation mutant in NBS-ILB1 cells resulted in an increase in Atm autophosphorylation in response to irradiation. Atm activation was also increased in NBS-ILB1 cells expressing the NbFR5 deletion mutant, indicating that nuclear expression of Mre11-Rad50 stimulated Atm phosphorylation in the absence of full-length nibrin. Conversely, nuclear expression of Nb652 in NBS-ILB1 cells in the absence of Mre11-Rad50 failed to stimulate Atm phosphorylation.
lysates were prepared after 15 or 60 min (A) or the nibrin mutants Nb652, NbFR5, and S278A/S343A were unirradiated or exposed to 3 Gy of ionizing radiation, and total cellular lysates were prepared after 15 (A) or 60 min (B). GM637 normal fibroblasts (control) and parental NBS-ILB1 cells were included as controls. Atm was immunoprecipitated (IP) from 500 µg of protein/cell line using a polyclonal anti-Atm antibody, and immunoprecipitates were separated on 3–8% SDS-polyacrylamide gels and Western-blotted (WB). Immunoblots were probed with a monoclonal antibody specific for the phospho-Ser-1981 (P-S1981) residue and then were stripped and reprobed with the polyclonal anti-Atm antibody. Quantitation of Ser-1981 phosphorylation in individual cell lines relative to total Atm protein was performed by densitometry and is shown below each lane.

Mre11-Rad50 and Nibrin Are Required for Chk2 Phosphorylation—Phosphorylation of the S-phase checkpoint proteins Smc1 and Chk2 by Atm is deficient in NBS and ATLD cells, indicating a requirement for the MRN complex for these phosphorylation events (20–22, 24–26). The activation of Chk2 by phosphorylation is a multistep process that is initiated by Atm phosphorylation of Chk2 at Thr-68, and possibly other sites within the Ser-Gln-rich region of Chk2, followed by oligomerization of Chk2 and autophosphorylation at several additional sites within the Chk2 molecule (31, 34). To determine whether nibrin or Mre11-Rad50 is necessary for nibrin phosphorylation at early times after irradiation.
Nibrin and Mre11-Rad50 in Atm Activation and Function

Phosphorylation was also restored by expression of NbFR5 in NBS cells, consistent with the requirement for Mre11-Rad50 to stimulate Atm activation at early time points. By contrast, Thr-68 phosphorylation was barely detected in NBS cells expressing Nb652 at 15 min after irradiation, demonstrating that nibrin alone was insufficient to induce Chk2 phosphorylation. At 60 min after 3 Gy of ionizing radiation (Fig. 4B), Chk2 Thr-68 was detectable in all the NBS cell lines, at somewhat variable levels, reflecting activation of Atm independent of the MRN complex at later times. We also detected slower migrating forms of Chk2 at 60 min in several of the cell lines, indicative of additional phosphorylation events on the Chk2 protein. The slower migrating forms of Chk2 were more evident after exposure of cells to a higher dose of ionizing radiation, 10 Gy (Fig. 5). Under these conditions, Chk2 autophosphorylation was detected in control cells, NBS cells expressing wild type nibrin, the S278A/S343A mutant, and to a lesser extent in cells expressing NbFR5. We failed to detect these slower migrating forms of Chk2 in parental NBS cells, NBS cells infected with vector alone, and in cells expressing the Nb652 mutant. Thus, whereas Chk2 Thr-68 phosphorylation is dependent upon Mre11-Rad50 at early times, Chk2 autophosphorylation is dependent on nuclear expression of Mre11-Rad50, and to a lesser extent nibrin, at later time points.

Smc1 Phosphorylation by Atm Requires Mre11-Rad50—We also examined the requirement for Mre11-Rad50 or nibrin for Atm phosphorylation of the S-phase checkpoint protein Smc1. NBS cells expressing Nb652, NbFR5, or controls were exposed to 3 Gy of ionizing radiation, and Smc1 Ser-957 phosphorylation was analyzed by Western blot analysis. Unlike phosphorylation of nibrin and Chk2 by Atm, which occurred readily after exposure to ionizing radiation, we found Smc1 was phosphorylated with slower kinetics as reported by others (data not shown) (24). Thus, we examined Smc1 Ser-957 phosphorylation at 60 min after irradiation. As expected, we observed a significant increase in Smc1 Ser-957 phosphorylation in control fibroblasts upon irradiation, which was not detected in A-T fibroblasts, NBS-ILB1 cells, or NBS cells infected with vector alone, consistent with a requirement for both Atm and the MRN complex for Smc1 phosphorylation (Fig. 6). Expression of wild type nibrin or the S278A/S343A mutant in NBS-ILB1 cells restored Smc1 phosphorylation. Robust Smc1 phosphorylation was also observed in NBS-ILB1 cells expressing NbFR5, indicating that nuclear expression of Mre11-Rad50 in the absence of full-length nibrin was sufficient to restore Smc1 phosphorylation. Conversely, Ser-957 phosphorylation was not detected in the Nb652 cell line that expresses only nibrin in the nucleus, even though Atm activation was detected in these cells at 60 min. Thus, Atm phosphorylation of Smc1 Ser-957 is dependent on Mre11-Rad50 at later times following irradiation.

Fig. 4. Phosphorylation of Chk2 following irradiation in NBS cells expressing nibrin mutants. NBS-ILB1 cells stably transduced with the pLXIN vector alone, wild type nibrin (NBS1), or the nibrin mutants Nb652, NbFR5, and S278A/S343A were unirradiated or exposed to 3 Gy of ionizing radiation. GM637 normal fibroblasts (control), AT3B1 A-T fibroblasts (A-T), and parental NBS-ILB1 cells were included as controls. Total cellular lysates were prepared after 15 (A) or 60 min (B), and 20 μg of protein/lane was separated on 7% SDS-polyacrylamide gels. Gels were Western-blotted (WB) and probed with a monoclonal antibody specific for the phospho-Thr-68 (P-T68) residue of Chk2. Blots were stripped and reprobed with a monoclonal anti-Chk2 antibody. Quantitation of Thr-68 phosphorylation in individual cell lines relative to total Chk2 protein was performed by densitometry and is shown below each lane.

Fig. 5. Autophosphorylation of Chk2 following irradiation in NBS cells expressing nibrin mutants. NBS-ILB1 cells stably transduced with the pLXIN vector alone, wild type nibrin (NBS1), or the nibrin mutants Nb652, NbFR5, and S278A/S343A were unirradiated or exposed to 10 Gy of ionizing radiation, and total cellular lysates were prepared after 60 min. Controls included GM637 normal fibroblasts (control), AT3B1 A-T fibroblasts (A-T), and parental NBS-ILB1 cells. 20 μg of protein/lane was separated on 7% SDS-polyacrylamide gels and Western-blotted (WB). Immunoblots were probed with a polyclonal antibody specific for the phospho-Thr-68 (P-T68) residue of Chk2, and then were stripped and reprobed with a monoclonal anti-Chk2 antibody.

Fig. 6. Phosphorylation of Smc1 following irradiation in NBS cells expressing nibrin mutants. NBS-ILB1 cells stably transduced with the pLXIN vector alone, wild type nibrin (NBS1), or the nibrin mutants Nb652, NbFR5, and S278A/S343A were unirradiated or exposed to 3 Gy of ionizing radiation, and total cellular lysates were prepared after 60 min. As controls, GM637 normal fibroblasts (control), AT3B1 A-T fibroblasts (A-T), and parental NBS-ILB1 cells were included. 20 μg of protein/lane was separated on 3-8% SDS-polyacrylamide gels and Western-blotted (WB). Immunoblots were probed with a polyclonal antibody specific for the phospho-Ser-957 (P-S957) residue of Smc1, and then were stripped and reprobed with a polyclonal anti-Smc1 antibody. Quantitation of Ser-957 phosphorylation in individual cell lines relative to total Smc1 protein was performed by densitometry and is shown below each lane.
DISCUSSION

Studies using NBS and ATLD cells have shown that the MRN complex is required for Atm activation and phosphorylation of downstream targets in vivo (16, 20–26). Although these studies indicate a role for the intact MRN complex in Atm activation and function, they do not delineate the roles of the individual MRN complex proteins, because all of the known mutations in NBS and ATLD have global effects on the MRN complex as a whole. Defining the specific roles of Mre11, Rad50, and nibrin is important both to explain the distinctly different phenotypes of NBS and ATLD, as well as to understand the cellular functions of each protein. In the current study we have generated mutant forms of nibrin that allow us to manipulate the subcellular localization of nibrin and Mre11-Rad50 independently. These constructs were expressed in NBS-IL1B cells, which are homozygous for the common NBS mutation 657del5 and produce no full-length nibrin protein. Maser et al. (36) have reported that reinitiation of translation on the 657del5 allele can produce a 70-kDa C-terminal polypeptide that includes the Mre11 binding domain, making this mutation hypomorphic. The p70 protein is found primarily in lymphoblasts but is undetectable in fibroblasts. We have not observed the p70 protein in NBS-IL1B cells under the conditions used, and thus it is unlikely to contribute significantly to the background in these experiments.

We found that Mre11-Rad50, but not nibrin, stimulated Atm activation at early times after low doses of ionizing radiation. This does not reflect a strict dependence of Atm activation on Mre11-Rad50, because at later time points and higher doses of irradiation Atm activation was observed independent of the MRN complex. Rather, Mre11-Rad50 appear to affect the kinetics of Atm activation. These results place Mre11-Rad50 upstream of Atm in the DNA DSB response and are consistent with Mre11-Rad50 playing a detector role, possibly through their ability to bind and process DNA DSBs (7, 8). Bakkenist and Kastan (1) have reported that chromatin alterations even in the absence of DNA DSBs can activate Atm. It is not clear from our study whether the processing of DNA DSBs by Mre11-Rad50 produces or enhances a chromatin change, which in turn signals Atm, or whether Mre11-Rad50 stimulates Atm activation by another mechanism.

The role of nibrin in stimulating Atm activation appears to be indirect, translocating Mre11-Rad50 to the nucleus. However, nibrin has been reported to stimulate Mre11 nuclease activity and DNA binding by Mre11-Rad50 proteins in vitro (9, 10). In the latter study, a fragment of nibrin encompassing amino acids 221–754 was sufficient to increase the DNA binding and nuclease activity of Mre11-Rad50. It is possible that the C-terminal NbFR5 protein used here may have had a similar stimulatory effect. Nibrin has also been shown to relocalize to the sites of DNA DSBs immediately after damage (12). Whether this relocalization is dependent upon Mre11-Rad50 has not been shown. Our finding that Mre11-Rad50 can stimulate Atm activation in the absence of full-length nibrin raises the possibility that the initial localization of the MRN complex to DNA damage may be dependent on Mre11-Rad50 binding and processing the DNA breaks. Subsequent accumulation of the MRN complex at the sites of DNA damage appears to be mediated via interactions of the nibrin FHA and BRCT domains with γH2AX and presumably other DNA damage response proteins that also accumulate in radiation-induced foci (30, 37–39).

Because Mre11-Rad50 can stimulate Atm activation, deficient phosphorylation of Atm targets in NBS and ATLD cells could simply reflect a failure to fully activate Atm. However, we found the requirement for Mre11-Rad50 or nibrin for the phosphorylation of Atm targets varied in timing after irradiation, suggesting a more complex relationship between the MRN proteins and downstream Atm targets. In the case of nibrin, phosphorylation at Ser345 by Atm was dependent on Mre11-Rad50 at early but not late time points after irradiation. Similarly, Chk2 Thr-68 phosphorylation by Atm was detected even 60 min after irradiation, even though time point activation was readily observed in these cells. Thus, the effect of Mre11-Rad50 on Smc1 phosphorylation cannot be attributed to impaired Atm activation alone. In addition, full activation of Chk2 by autophosphorylation was dependent on Mre11-Rad50, and to a lesser extent on nibrin, at 60 min after irradiation. The timing of these events suggests an additional function for Mre11-Rad50 beyond stimulating Atm activation. We propose that the MRN complex functions as an adaptor for Atm phosphorylation of Smc1 and for Chk2 autophosphorylation. Consistent with this model, Lee and Paull (40) recently reported that in vitro the MRN complex appears to increase the interaction of Atm with its substrates. Atm was shown to interact directly with nibrin or Mre11-Rad50, as well as the intact MRN complex, and this was reflected in the requirement for various components of the MRN complex for phosphorylation of Atm targets.

The differential roles of Mre11-Rad50 versus nibrin in Atm function observed in this study may provide some insight into the unique clinical phenotypes associated with NBS, ATLD, and A-T. Although these radiosensitivity disorders share several features, A-T and ATLD are characterized by progressive cerebellar degeneration resulting in ataxia, whereas NBS is characterized by microcephaly and growth retardation (16–18). The differential role for Mre11-Rad50 in activation of Atm and in the phosphorylation of some downstream Atm targets may be reflected in the similarity between A-T and ATLD. However, given the effects on the MRN complex in ATLD and NBS, this connection is likely to be too simplistic. Additional factors such as developmental and/or cell type expression of Atm, Mre11, Rad50, nibrin, or even the p70 protein in NBS patients may contribute to the phenotypes observed in these disorders.

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