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Advances in Brief

Phosphorylation of Serine 1387 in Brca1 Is Specifically Required for the Atm-mediated S-Phase Checkpoint after Ionizing Irradiation[1]

Bo Xu, Anne H. O'Donnell, Seong-Tae Kim,[2] and Michael B. Kastan[3]

Department of Hematology-Oncology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105

Abstract

Although it is well established that inheritance of mutations in the *Brca1* gene significantly increases the chances of developing breast or ovarian cancers, the mechanisms underlying this specific tumor susceptibility remain to be clarified. It is clear that one of the roles of the Brca1 protein is to facilitate cellular responses to DNA damage. We recently reported that Brca1 function is required for appropriate cell cycle arrests after ionizing irradiation in both the S-phase and the G_2 phase of the cell cycle. We also found that mutation of serine 1423 in Brca1, a target of Atm phosphorylation, abrogates the G_2-M checkpoint but not the ionizing irradiation-induced S-phase checkpoint. Here we demonstrate that mutation of serine 1387 in Brca1, another target of Atm phosphorylation, conversely abrogates the radiation-induced S-phase arrest but does not affect the G_2-M checkpoint. Thus, these two posttranslational modifications of Brca1 have two distinct functional roles in the protein. In addition, although mutation of this site abrogates the ionizing irradiation-induced S-phase arrest, it does not adversely affect cell survival after irradiation. This demonstrates that loss of this checkpoint function by itself does not affect cell survival and suggests that some other function of Brca1 alters cell survival after DNA damage.

Introduction

Progression through the cell cycle is inhibited when cellular DNA is damaged. It has been suggested that transient arrests in the G_1, S, and G_2-M phases of the cell cycle after DNA damage enhance cell survival and/or minimize genetic alterations (1–4). A number of different gene products have been demonstrated to be critical for inducing these cell cycle perturbations in mammalian cells after IR.[4] Arrests in all three of these cell cycle phases after IR require the Atm protein kinase (5–8). A variety of other gene products have been implicated in specific cell cycle arrests after IR, including p53, Chk2, and $p21^{Waf1/Cip1}$ in the G_1 checkpoint (9–11); Nbs1, Brca1, Smc1, Chk2, and Cdc25A in the S-phase checkpoint (8, 12–15); and Brca1 and hRad17 in G_2-M arrest (8, 16, 17).

Many of the proteins involved in these cell cycle perturbations are direct targets of the Atm protein kinase, including p53, Nbs1, Chk2, Smc1, and Brca1, and the sites of Atm phosphorylation are known for all of these substrates (12–15, 18–24). Brca1 has an "SQ" cluster in the 244-amino acid region between amino acids 1280 and 1524, and mass spectroscopic analysis has suggested that several of the serines in this region are phosphorylated after DNA damage (20). In particular, serines 1387, 1423, and 1524 appear to be phosphorylated by ATM in response to IR (20, 25). Phosphorylation of serine 1423 in Brca1 appears to be important for the IR-induced G_2-M checkpoint but is not required for the IR-induced S-phase arrest and is not a determinant of radiosensitivity (8, 17). However, although phosphorylation of serine 1423 in Brca1 is not required for the IR-induced S-phase arrest, a functional Brca1 protein is required for this checkpoint (8). Thus, it remained unclear how Brca1 was involved in this Atm-dependent arrest.

Because serine 1387 in Brca1 also appeared to be phosphorylated after IR (25), we investigated the potential role of this phosphorylation event in the IR-induced S-phase arrest. Acting as a dominant-negative activity, overexpression of a Brca1 protein with serine 1387 mutated to alanine specifically abrogated the IR-induced S-phase arrest. Interestingly, overexpression of this mutant had a specific effect on Brca1 function and did not affect the IR-induced G_2 arrest. Similarly, expression of this Brca1 mutant protein in a cell line containing dysfunctional Brca1 protein restored the defective G_2 checkpoint but did not complement the S-phase checkpoint. Interestingly, this mutant was as effective as wild-type Brca1 in being able to reverse the decreased cell survival of this cell line after irradiation. This result supports prior demonstrations (17) that lack of the S-phase checkpoint by itself does not cause radiosensitivity. Furthermore, this result suggests that some function of Brca1 protein that is not affected by phosphorylation of either serine 1387 or serine 1423 is an important determinant of cell survival after IR.

Materials and Methods

Cell Culture and Irradiation. Human 293T cells that have normal IR-induced S-phase and G_2-M checkpoints were grown as monolayers in DMEM supplemented with 10% fetal bovine serum. The human breast cancer cell line, HCC1937, which has a truncated, nonfunctional Brca1 and which has defective IR-induced S-phase and G_2-M checkpoints, was cultured in RPMI 1640 supplemented with 15% fetal bovine serum. All cell lines were grown at 37°C in a humidified atmosphere containing 5% CO_2. Radiation from a ^{137}Cs source was delivered at a dose rate of ~120 cGy/min.

Expression of *BRCA1* Constructs in Brca1 Mutant Cells. Transfections of HA-tagged wild-type BRCA1 (generously provided by David Livingston, Dana-Farber Cancer Institute, Boston, MA) or mutant Brca1 constructs were performed transiently using Lipofectamine (Life Technologies, Inc., Rockville, MD). Expression of transfected Brca1 was detected by Western blot analysis with an anti-HA monoclonal antibody (Roche Molecular Biochemicals, Indianapolis, IN). Transfection efficiencies were assessed by flow cytometric evaluation of GFP expression. For clonogenic survival assays in HCC 1937 cells transfected with Brca1 constructs, 1 mg/ml of Geneticin (G-418; Life Technologies, Inc.) was added to the medium 36 h after transfection.

G_2-M Checkpoint Assay. Cells were harvested at the indicated time points after IR and fixed in 70% ethanol at −20°C. The cells were suspended in 100 μl of PBS containing 1% BSA and 0.75 μg of a polyclonal antibody that specifically recognizes the phosphorylated form of histone H3 (Upstate Biotechnology, Lake Placid, NY) and incubated for 3 h at room temperature. The

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[2] Present address: Sungkyunkwan University School of Medicine, Samsung Biomedical Research Institute, 300 Chunchun-Dong, Changan-Ku, Suwon, Kyunggi-Do 440-746, Korea.

[3] To whom requests for reprints should be addressed, at Department of Hematology-Oncology, St. Jude Children's Research Hospital, 332 North Lauderdale St., Memphis, TN 38105. Phone: (901) 495-3968; Fax: (901) 495-3966; E-mail: Michael.Kastan@stjude.org.

[4] The abbreviations used are: IR, ionizing irradiation; GFP, green fluorescent protein; HA, hemagglutinin.

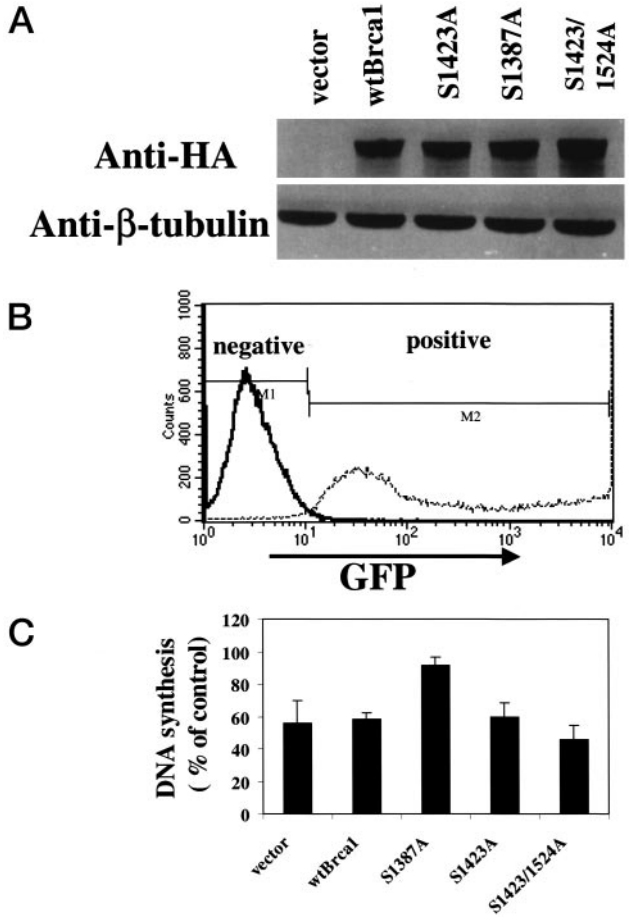

Fig. 1. Transfection of the serine 1387 mutant Brca1 abrogates the IR induced S-phase checkpoint. *A,* expression of HA-tagged Brca1 proteins in 293T cells was measured by immunoblot analysis with an anti-HA antibody. Levels of β-tubulin protein are shown as a loading control. *B,* transfection efficiency of the various Brca1 transgenes in 293T cells was assessed by flow cytometric analysis of expression of a cotransfected GFP vector. *C,* 30 min after exposure to 10 Gy of ionizing radiation, replicative DNA synthesis was measured in the 293T cells that had been transfected with either vector alone (*vector*), wild type (*wtBrca1*), or a series of serine-to-alanine mutants at the indicated sites (*S1378A, S1423A,* or *S1423/1524A*). *Columns,* averages of at least triplicate samples; *bars,* SE.

cells were then rinsed with PBS containing 1% BSA and incubated with fluorescein isothiocyanate-conjugated goat antirabbit IgG antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted at a ratio of 1:30 in PBS containing 1% BSA. After a 30-min incubation at room temperature in the dark, the cells were stained with propidium iodide (Sigma), and cellular fluorescence was measured by a FACScalibur flow cytometer.

S-Phase Checkpoint Assay. Inhibition of DNA synthesis after irradiation was assessed as described previously (8, 12). Cells were prelabeled with 10 nCi of [^{14}C]thymidine (NEN Life Science Products, Inc., Boston, MA) for 24 h. Cells were irradiated and incubated for 30 min and then pulse-labeled with 2.5 μCi/ml [^{3}H]thymidine for 15 minutes (NEN Life Science Products). After harvesting, the amount of radioactivity was assayed in a liquid scintillation counter. The measure of DNA synthesis was derived from the resulting ratios of ^{3}H cpm to ^{14}C cpm, corrected for those cpm that resulted from channel crossover.

Clonogenic Assays. Cell lines were plated in triplicate into 6-well plates, incubated for 24 h, and then exposed to a range of doses of IR (0–6 Gy) followed by incubation for 2 weeks. Before counting the colonies, cells were fixed in 95% methanol and stained with crystal violet. A population of >50 cells were counted as one survived colony. The mean colony counts ± SE appear in the figures.

Results

Brca1 Mutated at Serine 1387 Specifically Affects IR-induced S-Phase Arrest. Brca1 is phosphorylated at several serine sites within its SQ cluster region in response to DNA damage. Among those serine sites, ionizing irradiation appears to induce phosphorylation of serines 1387, 1423, and 1524 in an Atm-dependent manner (20, 25). We had observed previously that overexpression of a Brca1 protein with serine 1423 converted to alanine, a mutant unable to be phosphorylated on this site, and specifically inhibited the IR-induced G_2 arrest without adversely affecting the S-phase arrest and radiosensitivity that are also dependent on Brca1 function (8, 17). To begin to explore whether one of the other phosphorylation sites was an important determinant of the IR-induced S-phase checkpoint or radioensitivity, a *Brca1* construct with serine 1387 mutated to alanine was constructed and transfected into 293T cells. In multiple experiments, the efficiency of transfection of this and other Brca1 constructs into 293 T cells was typically >90% in these cells (Fig. 1, *A* and *B*). Overexpression of this construct blocked the IR-induced S-phase checkpoint, thus inducing the phenomenon termed "radioresistant DNA synthesis" (Fig. 1*C*). In contrast, overexpression of *Brca1* constructs mutated at serine 1423 or both serines 1423 and 1524, although expressed at similar levels to the S1387A mutant (Fig. 1*A*), did not affect the IR-induced S-phase delay (Fig. 1*C*).

Similar conclusions were reached in complementation experiments using these mutants. HCC1937 cells that lack functional Brca1 protein are defective in the S-phase and G_2 arrests after IR and are radiosensitive (8, 17). Efficiency of introduction of wild-type or mutant

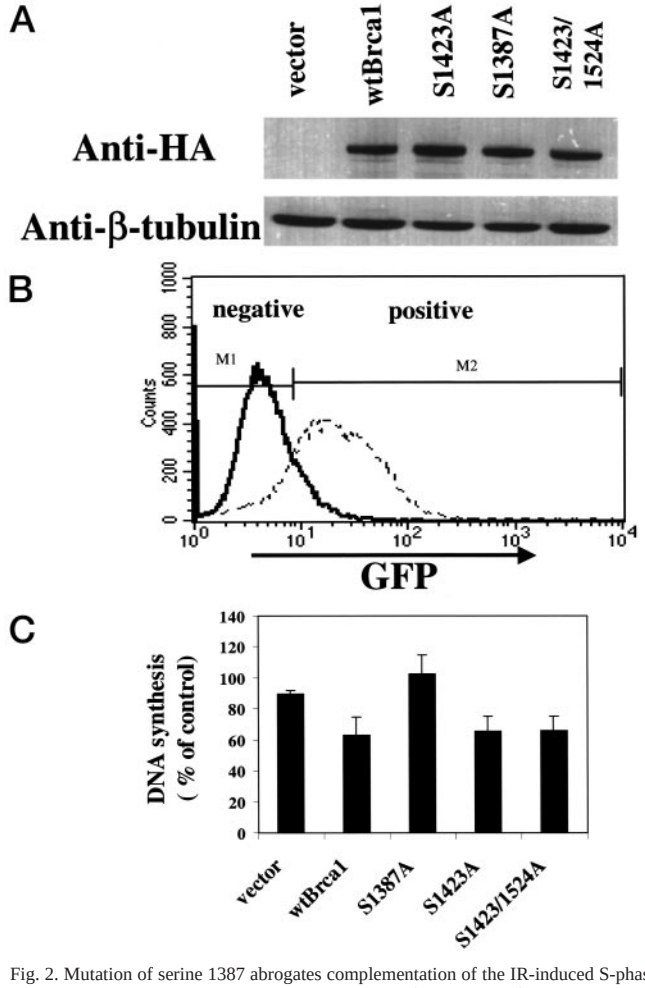

Fig. 2. Mutation of serine 1387 abrogates complementation of the IR-induced S-phase checkpoint in Brca1-null cells. *A,* immunoblot analysis of expression of HA-tagged Brca1 proteins in the HCC1937 cells. Levels of β-tubulin protein are shown as a loading control. *B,* transfection efficiency in HCC1937 cells assessed by GFP expression as described in Fig. 1. *C,* 30 min after exposure to 10 Gy of ionizing radiation. Replicative DNA synthesis was measured in HCC1937 cells that had been complemented with the various HA-tagged Brca1 proteins.

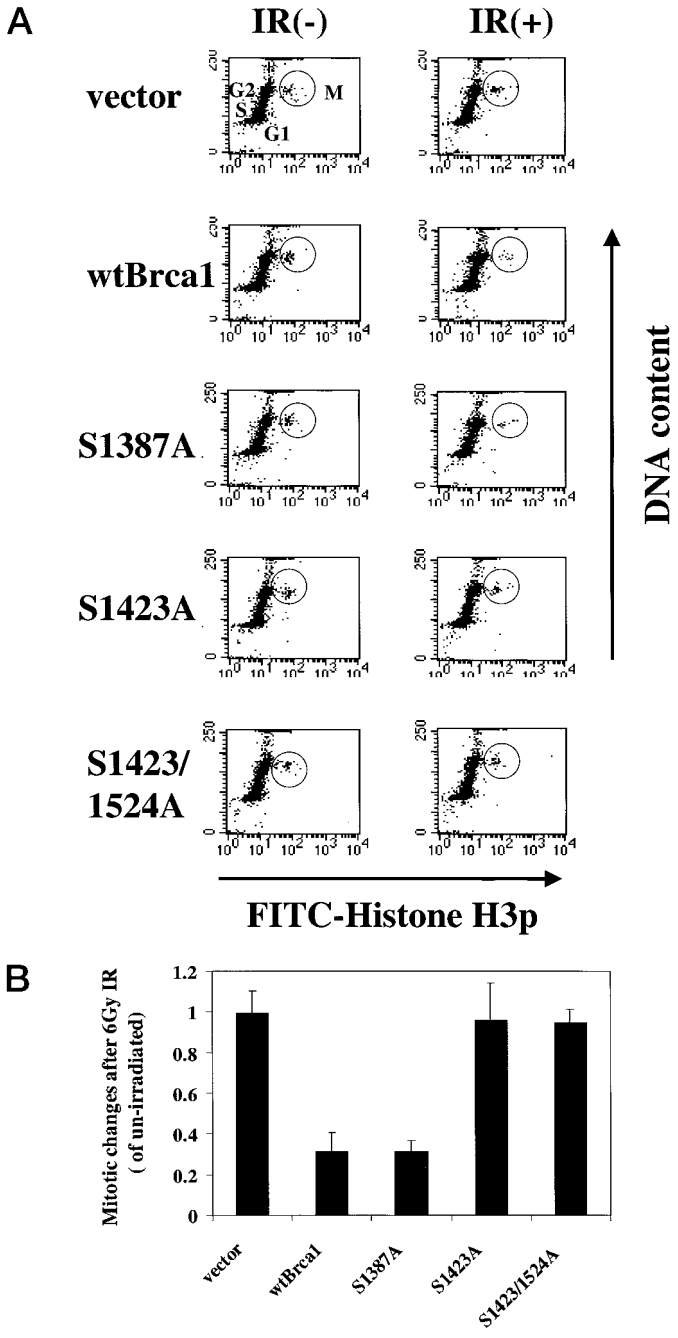

Fig. 3. Phosphorylation of serine 1423, but not serine 1387, is required for the IR-induced G_2-M checkpoint. *A*, flow cytometric profiles of cell cycle distribution before [*IR* (−)] or 1 h after 6 Gy ionizing irradiation [*IR* (+)]. Shown are HCC1937 cells expressing either vector alone (vector), wild type *Brca1* (*wtBrca1*), or a series of serine to alanine mutants at the indicated sites (*S1387A*, *S1423A*, or *S1423/1524A*). Staining for DNA content (propidium iodide; *Y-axis*) and for histone H3 phosphorylation (*FITC-Histone H3p*; *X-axis*) is presented. The mitotic cell population is encircled and labeled *M*. *B*, quantitative analysis of the flow cytometric data in *A*. Shown is the ratio (irradiated: unirradiated) of the percentage of cells in mitosis. *Columns*, average of at least triplicate samples; *bars*, SE.

Brca1 constructs into HCC1937 cells was reproducibly >80% in multiple experiments (Fig. 2, *A* and *B*). It is also noted that the cell cycle distributions without irradiation of neither 293T nor HCC1937 cells was affected by the transient overexpression of any of these *Brca1* constructs (Fig. 3*A* and data not shown). Wild-type Brca1 and Brca1 constructs mutated at either serine 1423 or both serines 1423 and 1524 all complemented the defective IR-induced S-phase delay in HCC1937 cells (Fig. 2*C*). In contrast, transfection of the serine 1387 Brca1 mutant failed to complement this defect. However, the S1387A mutant was functional because it was able to complement the IR-induced G_2 checkpoint defect in these cells (Fig. 3). In confirmation of previous reports (8), although the S1423A and S1423A/S1524A mutants were capable of restoring the IR-induced S-phase delay, these constructs failed to restore the IR-induced G2 arrest (Fig. 3).

Dissociation of Radiation Sensitivity from IR-induced Cell Cycle Checkpoint Defects. Disruption of the IR-induced G_2 checkpoint in yeast appears to reduce cell survival (1). To help clarify the relationships between IR-induced cell cycle arrests and cell survival in human cells, clonogenic assays were performed in HCC1937 cells expressing the various Brca1 constructs. The parental HCC1937 cells are relatively radiosensitive, and re-introduction of wild-type *Brca1* decreases the radiation sensitivity (Fig. 4). However, introduction of either the *S1387A* construct (a situation in which the S-phase checkpoint is defective) or the *S1423A* or *S1423A/1524A* constructs (a situation in which the G_2-M checkpoint is defective) all decreased radiosensitivity as effectively as the wild-type Brca1 construct (Fig. 4). Thus, the radiosensitivity of these Brca1-defective cells is not attributable to the defects in IR-induced cell cycle arrests.

Discussion

Among the mechanisms developed by eukaryotic cells to optimally respond to DNA damage are cell cycle arrests or checkpoints. These perturbations of cell cycle progression presumably enhance genetic stability and limit tumorigenesis in the organism. The fact that so many familial cancer syndromes result from dysfunction of genes involved in checkpoint responses (*e.g.*, p53, Chk2, Atm, Brca1, Nbs1; Ref. 26) supports this concept. The Atm protein kinase is activated by ionizing irradiation and through phosphorylation of a number of different protein substrates appears to initiate multiple cell cycle arrests (26, 27). The Brca1 protein is one of these substrates, and cells defective in Brca1 functions lack both the transient S-phase and G_2 arrests that should be initiated by ionizing irradiation. In the present study, we extend these insights by demonstrating that phosphorylation of Brca1 on serine 1387 is required for the transient S-phase arrest, but not the G_2 arrest, after IR. Conversely, phosphorylation of serine 1423 is required for the G_2 arrest, but not the S-phase arrest, after IR (8).

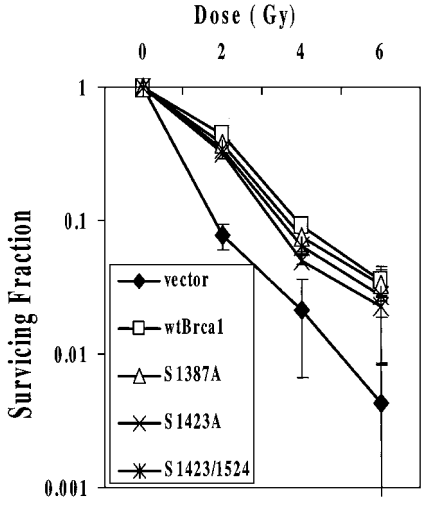

Fig. 4. Abrogation of either the S-phase or G_2-M cell cycle checkpoints is not sufficient to increase radiation sensitivity. Colony formation assays were performed in HCC1937 cells expressing either vector alone (*vector*), wild type (*Brca1*), or a series of serine to alanine mutants at the indicated sites (*S1387A*, *S1423A*, or *S1423/1524A*). Surviving fractions are plotted on a log scale (*Y-axis*) *versus* dose of IR (*X-axis*). Data are the averages of at least triplicate samples; *bars*, SE.

Thus, two different IR-induced phosphorylation events in the same molecule have two different functional consequences. It is not known whether these phosphorylation events are cell cycle stage specific or whether they occur on the same protein molecule. These are questions appropriate for future studies.

Identification of specific posttranslational modifications that affect specific cell cycle checkpoints also allow us to investigate the impact that specific checkpoint defects have on cellular outcome after irradiation. In this case, selective mutations of Brca1 allow us to specifically abrogate either the IR-induced S-phase or G_2 checkpoints. The data presented here demonstrate that neither selective abrogation of the S-phase checkpoint nor the G_2 checkpoint enhances radiosensitivity. Because the *p53* gene is mutated in HCC1937 cells, the G_1 cell cycle checkpoint is also defective in these cells. Thus, our data suggest that even disruption of two cell cycle checkpoints (G_1 plus S, or G_1 plus G_2) is not sufficient to enhance radiation sensitivity. Previous experiments have demonstrated that cells with mutant p53 appear to demonstrate enhanced radiosensitivity when treated with the chemicals caffeine or UCN-01 (28–30). Because these compounds block the G_2 checkpoint, it was suggested that this enhanced radiosensitivity was caused by abrogating the G_2 checkpoint in cells that already had G_1 checkpoint abnormalities. The data shown here demonstrate that radiosensitivity caused by UCN-01 and caffeine must result from some cellular effect of these compounds other than their effect on the G_2 checkpoint itself. A similar line of reasoning leads to the conclusion that some function of Brca1 protein other than S-phase or G_2 cell cycle control affects cell survival after ionizing irradiation. What this function is remains to be determined. Finally, it must be pointed out that these studies do not address the Brca1 function that is critical for preventing breast or ovarian cancer. The Brca1 activity that is relevant to its tissue-specific tumor suppressor function must still be clarified. However, these insights do have potential relevance for understanding or modulating the responses of tumors with Brca1 dysfunction to therapeutic intervention and should allow us to further dissect the various activities of this complex multifunctional protein.

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