Endoplasmic Reticulum Stress and Unfolded Protein Response in Atm-Deficient Thymocytes and Thymic Lymphoma Cells Are Attributable to Oxidative Stress

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Abstract

Both oxidative stress and endoplasmic reticulum (ER) stress have been implicated in carcinogenesis. It is well documented that cells deficient in the ataxia–telangiectasia mutated (ATM) gene undergo oxidative stress, which is critically involved in thymic lymphomagenesis in Atm−/− mice. Here we demonstrate that undifferentiated Atm−/− thymocytes show signs of ER stress and of the unfolded protein response (UPR). Using two-dimensional (2-D) gel electrophoresis and mass spectrometry (MS) analysis, we identified 22 differentially expressed proteins, including the ER stress marker glucose-regulated protein 78 (GRP78), in Atm−/− thymocytes and in Atm−/− thymic lymphoma cells relative to Atm+/+ thymocytes. The phosphorylated α subunit of eukaryotic translation initiation factor 2 (p-eIF2α), a UPR marker, was also increased in Atm−/− thymocytes. Cells of the ATL-1 line, which were derived from an Atm−/− mouse thymic lymphoma, were more sensitive to the ER stress inducer tunicamycin than were Atm+/+ thymic leukemia ASL-1 cells. Notably, treatment with hydrogen peroxide duplicated the effects of ATM deficiency in cultured thymocytes, and treatment with the novel cell-permeable thiol antioxidant N-acetylcysteine amide (AD4) reduced elevated p-eIF2α levels in thymocytes of Atm−/− mice. Thus, we propose that ER stress and the UPR are secondary to oxidative stress in Atm−/− thymocytes.


Introduction

Ataxia–telangiectasia mutated (ATM) is a multifunctional protein kinase that regulates cell cycling and cellular responses to events that damage DNA. When activated, ATM undergoes autophosphorylation [1]. Phosphorylated ATM then initiates the phosphorylation of multiple substrates. Many of these substrates are cell cycle regulators, such as p53. Patients with ataxia–telangiectasia have motor problems and develop lymphoid malignancies at an early age. In Atm−/− mice, abnormal T cell development with thymic lymphomagenesis inevitably occurs [2]; thus, ATM may play an essential role in preventing thymic lymphomagenesis during thymocyte development.

In cells, ATM participates in oxidative defense by upregulating major antioxidants, including superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase [3]. In hematopoietic stem cells from Atm−/− mice, reactive oxygen species (ROS) levels

Abbreviations: ATF6, activating transcription factor 6; ATM, ataxia–telangiectasia mutated; AD4, N-acetylcysteine amide; 6AN, 6-aminonicotinamide; 2-D, two-dimensional; eIF2α, α subunit of eukaryotic translation initiation factor 2; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; IRE1, inositol requiring 1; MS, mass spectrometry; NAC, N-acetylcysteine; ROS, reactive oxygen species; PERK, double-stranded RNA-activated protein kinase–like ER kinase; UPR, unfolded protein response; XBP1, x-box–binding protein 1

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are markedly increased, and the resultant progressive bone marrow failure is prevented by treatment with the antioxidant N-acetylcysteine (NAC) [4]. Similarly, in thymocytes from Atm−/− mice, increased spontaneous DNA synthesis, elevation of ROS, upregulation of the oxidative stress indicator Nrf2, and increased oncoprotein c-Myc can be attenuated by NAC treatment [5]. ROS accumulation in Atm−/− thymocytes is associated with increased DNA synthesis and oncoprotein c-Myc expression, both of which participate in the process of tumorigenesis, and treatment of Atm−/− mice with antioxidants prevents thymic lymphoma development [6].

The term ER stress refers to intracellular events in which newly synthesized unfolded proteins accumulating in the endoplasmic reticulum (ER) when ER function is perturbed by various pathologic conditions [7]. The hallmark marker for ER stress, glucose-regulated protein 78 (GRP78), plays an important role in many cellular processes, including helping newly synthesized proteins translocate to the ER membrane, recognizing and targeting misfolded proteins for proteasome degradation, and regulating calcium homeostasis [7]. In response to ER stress, mammalian cells trigger unfolded protein response (UPR) signaling pathways to cope with the accumulated unfolded proteins accumulating in the ER [6]. Three molecules are critically involved in UPR processes: activating transcription factor 6 (ATF6), double-stranded RNA-activated protein kinase-like ER kinase (PERK), and inositol requiring 1 (IRE1) [8].

Under normal conditions, GRP78 interacts with ATF6, PERK, and IRE1, and keeps these UPR signaling molecules in an inactive state [6]. Under ER stress, however, GRP78 preferentially associates with unfolded proteins and releases the three signaling molecules, which then become active [6]. Activated ATF6 (p90) is transported to the Golgi complex and is cleaved by proteases S1P and S2P [9,10]. The cleaved form of ATF6 (p50) translocates to the nucleus and binds to the ER stress response element and subsequently activates target genes, including GRP78 and GRP94 [11]. Activated PERK phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α) [12]. The phosphorylated eIF2α (p-eIF2α) then inhibits protein synthesis to prevent further accumulation of unfolded protein in the ER lumen. In addition, p-eIF2α phosphorylation induces translation of the transcription factor ATF4, resulting in the expression of ATF4 target genes GADD34 and GADD153 [13]. Activated IRE1 modifies x-box–binding protein 1 (XBP1) (α) mRNA by cutting out its 26 nucleotides to generate spliced XBP1 (s) mRNA. This mRNA encodes the more stable XBP1 (s) protein [14]. XBP1 (s) binds to the UPR element and activates expression of the corresponding target genes [15].

ER stress and UPR activation have been shown to occur in various tumors [16]. In human tumor biopsy samples, for example, GRP78 levels are highly elevated [17]. In studies from a variety of tumor cell models, the UPR-related molecules XBP1 and ATF6 were found to be activated, and the molecules CHOP, GRP78, GRP94, and GRP170 were upregulated [18–20]. Quickly growing tumors experience nutrient starvation, acidosis, and hypoxia, all of which promote the formation of new blood vessels to the tumor, to protect the tumor cells from death [16]. The fact that GRP78 expression increases in hypoxic tumors links ER stress and hypoxia.

ER stress and UPR activation are generally viewed as the cytoprotective responses in tumor cells. However, long-term ER stress induces apoptosis in tumor cells through both mitochondria-dependent and mitochondria-independent pathways [21]. Interestingly, ER stress can alter the sensitivity of tumors to chemotherapeutic agents, for example, diminishing the antitumor effects of the topoisomerase II inhibitor etoposide, and synergizing the antitumor effects of cisplatin [16].

In this study, we demonstrated for the first time that ER stress and UPR occur in Atm-deficient thymocytes and thymic lymphoma cells. ER stress and UPR activation in Atm−/− thymocytes are attributable to oxidative stress, because treatment with hydrogen peroxide increases GRP78 and p-eIF2α in normal thymocytes in vitro, and treatment with antioxidant N-acetylcysteine amide (AD4) attenuates elevated p-eIF2α in Atm−/− thymocytes in vivo. These results shed light on our understanding of the potential connection between oxidative stress and ER stress, and the roles of the two events in tumorigenesis.

Materials and methods

Mice

The Atm−/− mice used in this study were created by Barlow et al. [2]. Heterozygous Atm+/− mice of this line were purchased from the Jackson Laboratory (Bar Harbor, ME), mated, and kept in the Animal Center at The University of Texas MD Anderson Cancer Center, Science Park–Research Division. Genotyping was carried out by polymerase chain reaction, as described by Liao et al. [22]. The mice were bred in the institution-accredited specific pathogen-free animal facility under standard conditions with a 14:10-hour light/dark cycle and were given standard diet and water ad libitum. All studies were reviewed and approved by the Institutional Animal Care and Use Committee of MD Anderson Cancer Center.

Cells and Cell Lines

Thymocytes were isolated from Atm+/+ and Atm−/− mice using routine methods [23]. Atm−/− thymic lymphoma cell lines were established as described previously [24]. The phenotype of the ATL-1 thymic lymphoma cell line used in this study was CD4+/CD8+ double positive. The ASL-1 thymic leukemia cell line [24] was used as an Atm+/+ control.

Two-Dimensional Gel Electrophoresis and Mass Spectrometry Analysis

Protein extracts were prepared from the thymocytes of 8-week-old Atm+/+ and Atm−/− mice, and from thymic lymphoma cells of 16-week-old Atm−/− mice, in a modified radioimmunoprecipitation assay buffer [25]. A traditional two-dimensional (2-D) gel electrophoresis was performed, as described previously [26] with some minor modifications. Briefly, samples of each type (Atm−/− thymocytes, Atm+/+ thymocytes, and Atm−/− thymic lymphoma cells) were pooled together to avoid sample-to-sample variation, separated in the first dimension by using immobilized pH gradient strips (pH ranges of 4–7, 58, and 710), and then separated in the second dimension by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis using a precast Criterion 8% to 16% gradient gel (Bio-Rad, Hercules, CA). Two-dimensional gel electrophoresis images were captured on a charge-coupled device camera (Image Station 440CF; Eastman Kodak Company, Rochester, NY).

Two-dimensional gel image analysis of the traditional 2-D gels was performed using the PDQuest software program (Bio-Rad). Differentially expressed spots from the PDQuest analysis were confirmed visually by two independent observers. Selected spots were excised manually and subjected to in-gel digestion [27]. The tryptic digests...
were then analyzed on a Voyager-DE PRO matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA) [26,28].

**Western Blot Analysis**

Western blot analysis was performed as described previously [5]. The antibodies used in the Western blot analysis included anti-GRP78, GRP75, GRP94, CHOP, and XBP1 (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-p-ERK, p-ATM, p-eIF2α, p-ATM, caspase-3, activated caspase-3, caspase-9, and caspase-12 (Cell Signaling Technology, Danvers, MA). A monoclonal anti-β-actin antibody (Sigma, St. Louis, MO) was used as a control for protein loading.

**Cell Viability Assay**

ASL-1 (*Atm*+/+) and ATL-1 (*Atm*−/−) cells were treated with various concentrations of tunicamycin (Sigma) at 37°C for 16 hours. Cell viability was assessed using the One Solution Cell Proliferation Assay kit (Promega, Madison, WI) according to the manufacturer’s instructions.

**[^3]H]Thymidine Incorporation into DNA**

Single-cell suspensions of ASL-1 and ATL-1 cells (4 × 10⁴ cells/well) were cultured in 96-well plastic tissue culture dishes at 37°C for 6 hours with or without 6-aminonicotinamide (6AN) (Sigma). The cells were then cultured with or without etoposide (Sigma) for an additional 8 hours. For the last 4 hours of culture, 0.5 μCi of [3H]-ThdR (Amersham Pharmacia Biotech, Piscataway, NJ) was added to each well. The cells were harvested, and [3H]thymidine incorporation into DNA was measured in a scintillation counter (Packard, Meriden, CT).

**Statistical Analysis**

Results for cell viability and [3H]thymidine incorporation are expressed as means ± SD. The statistical significance of the results was determined by analysis of Student’s two-tailed, unpaired t test. P values of < .05 were considered statistically significant.

**Results**

**Differentially Expressed Proteins in *Atm*+/+ and *Atm*−/− Thymocytes and in *Atm*−/− Thymic Lymphoma Cells**

The thymic atrophy, impaired thymocyte maturation, and thymic lymphomagenesis in *Atm*−/− mice indicate the critical role that ATM plays during thymocyte development. To discover the global functional proteins that are altered in *Atm* gene-deficient cells, we used the proteomic approach of 2-D gel electrophoresis and mass spectrometry (MS) analysis to identify proteins differentially expressed in *Atm*−/− thymocytes and *Atm*−/− thymic lymphoma cells compared with *Atm*+/+ thymocytes (control). Among the 45 protein spots we collected, 22 were either increased or decreased about 2- to 20-fold in their expression levels (Figure 1 and Table 1). These results provided us with a spectrum of proteins that may functionally link ATM in thymocytes. In this study, we focused on the elevated GRP78 expression in *Atm*−/− thymocytes to understand whether and how ER stress and the UPR occur during postnatal thymocyte development in *Atm*−/− mice.

**Endoplasmic Reticulum Stress Markers, UPR Markers, and Activated Caspase-3 in *Atm*−/− Thymocytes and in *Atm*−/− Thymic Lymphoma Cells**

To assess ER stress markers and UPR markers in *Atm*−/− lymphoid cells, proteins were extracted from freshly isolated *Atm*+/+ and *Atm*−/− thymocytes and cultured *Atm*−/− thymic lymphoma cells (ATL-1), and Western blot analysis was performed.

We found that the protein levels of the ER stress markers GRP78 and GRP75, but not GRP94, were increased in *Atm*−/− thymocytes and *Atm*−/− thymic lymphoma cells, compared with those in *Atm*+/+ thymocytes (Figure 2A). Although amounts of the UPR marker p-eIF2α were increased in both *Atm*−/− thymocytes and *Atm*−/− thymic lymphoma cells (Figure 2B), levels of the eIF2α upstream molecule p-ERK were normal (data not shown), whereas the downstream molecule CHOP was decreased (Figure 2B), suggesting that elevated p-eIF2α in *Atm*−/− thymocytes is independent of PERK. The level of another UPR-related molecule XBP1 was increased only in *Atm*−/− thymic lymphoma cells (Figure 2B).

It has been reported that ER stress, UPR, or both induce the activation of caspase-12, caspase-9, and caspase-3 and eventually cause...
apoptosis [29,30]. In this study, we found that the caspase-12 and caspase-9 levels were normal (data not shown) and that the activated caspase-3 levels were increased both in Atm−/− thymocytes and in Atm−/− thymic lymphoma cells (Figure 2C).

These results indicate that Atm−/− thymocytes and Atm−/− thymic lymphoma cells undergo ER stress and UPR, and that chronic ER stress induces mitochondria-dependent apoptosis in these cells, which is consistent with earlier observations that Atm−/− thymocytes undergo spontaneous apoptosis [31,32].

Atm−/− Thymic Lymphoma Cells are Sensitive to Tunicamycin and Resistant to Etoposide

To understand the sensitivity of ER stressed Atm−/− thymic lymphoma cells to the ER stress inducer tunicamycin, we further examined GRP78 and p-eIF2α levels in Atm−/− ATL-1 cells treated with tunicamycin, and compared the results with those in ASL-1 cells and Atm+/+ thymic leukemia cells, in which the Atm gene is intact and ATM autophosphorylation at S1981 occurs in response to ionizing radiation (Figure 3A). The results show that the basic levels of GRP78 and p-eIF2α were higher in ATL-1 cells than in ASL-1 cells (Figure 3B, lanes 1 and 4). When stimulated with tunicamycin, however, both GRP78 and p-eIF2α increased in ASL-1 cells (Figure 3B, lanes 2 and 3) but decreased in ATL-1 cells (Figure 3B, lanes 5 and 6). The cell viability results showed that the ATL-1 cells were more sensitive to tunicamycin than were the ASL-1 cells (Figure 3C). These results suggest that ER-stressed ATL-1 cells are vulnerable to further ER stress challenges.

It has been shown that GRP78 upregulation by treatment with 6AN causes cells to become resistant to the topoisomerase IIα–targeted anticancer drug etoposide [33]. In our study, we found that 6AN itself suppressed DNA synthesis in both Atm+/+ ASL-1 cells and Atm−/− ATL-1 cells in a dose-dependent manner (Figure 3D). Indeed, ASL-1 cells were more sensitive to etoposide than were ATL-1 cells, and

![Figure 2](image-url)
both ASL-1 and ATL-1 cells became slightly resistant to etoposide when pretreated with 6AN.

**Hydrogen Peroxide Upregulates GRP78 and p-eIF2α in Primary Cultured Atm+/+ Thymocytes**

To determine whether ROS accumulation in Atm−/− thymocytes causes ER stress and the UPR in these cells, we treated normal thymocytes with hydrogen peroxide, and then examined their GRP78 and p-eIF2α levels by Western blot analysis. Figure 4A shows that p-eIF2α upregulation was initiated at 30 minutes, and Figure 4B shows that GRP78 upregulation was initiated at 4 to 6 hours after hydrogen peroxide treatment, indicating that ROS initiates both ER stress and he UPR in normal thymocytes. If hydrogen peroxide was washed out after 30 minutes of treatment, p-eIF2α levels were initially elevated and then gradually decreased with time, whereas the elevated GRP78 levels persisted for at least 20 hours (Figure 4C).

**AD4 Treatment of Atm−/− Mice Diminishes p-eIF2α Elevation in Their Thymocytes**

To confirm that ROS causally increases ER stress and the UPR in Atm−/− thymocytes, we administrated AD4, a novel low–molecular weight thiol antioxidant [34,35], to 6-week-old Atm−/− mice for 7 days and then examined GRP78 and p-eIF2α expression levels in their thymocytes by Western blot analysis. The increased p-eIF2α level in Atm−/− thymocytes was attenuated by AD4 treatment, but levels of the ER stress marker GRP78 were sustained high, as they were in untreated Atm−/− thymocytes (Figure 5). We assume that high levels of ROS are required for eIF2α phosphorylation in thymocytes, whereas...
Proteins were extracted at each time point to examine p-eIF2α expression in cultured thymocytes. (A) Hydrogen peroxide increases GRP78 and p-eIF2α expression in cultured thymocytes. Cells were treated with 100 μM hydrogen peroxide at 37°C for 30 minutes, at which time their proteins were extracted to examine p-eIF2α levels. (B) Hydrogen peroxide increases GRP78 expression in cultured Atm+/+ thymocytes. Cells were treated with different concentrations of hydrogen peroxide at 37°C for 3 or 6 hours, and proteins were extracted at each time point. (C) Dynamics of GRP78 and p-eIF2α after hydrogen peroxide treatment. Primary cultured Atm+/+ thymocytes were treated with 100 μM of hydrogen peroxide at 37°C for 30 minutes, washed twice with phosphate-buffered saline, and cultured for an additional 1, 4, 8, or 20 hours. Proteins were extracted at each time point to examine p-eIF2α and GRP78 levels in these cells.

GRP78 upregulation by ROS, once initiated, is stable for a longer period. Together these results suggest that ROS accumulation in Atm−/− thymocytes is responsible for ER stress and UPR development.

Discussion
A recent study has identified more than 700 proteins and 900 phosphorylation sites recognized by ATM and ataxia telangiectasia and Rad3-related (ATR) by a large-scale proteomic analysis of proteins phosphorylation in response to DNA damage [36]. In the current study, using proteomic and MS analyses, we identified 22 differentially expressed proteins in Atm−/− thymocytes and Atm−/− thymic lymphoma cells. The levels of these unphosphorylated proteins were changed by about 2- to 20-fold compared with those of Atm+/+ thymocytes. To our knowledge, few of these differentially expressed proteins have been reported previously to be associated with ATM. These data thus provide us with new clues to our understanding of ATM function.

ER stress and the UPR have been implicated in carcinogenesis [16,17,37]. The ER stress marker GRP78 is elevated in a wide variety of cancer cells and cancer biopsy tissues [17]. Because most studies on the correlation of ER stress and cancer have been executed in cell cultures in which ER stress was induced with pharmacologic agents, it is unclear whether and how ER stress promotes tumorigenesis and how tumor cells adapt to chronic ER stress in vivo. In the current study, all data from proteomic and Western blot analyses relating to ER stress and UPR markers were derived from freshly isolated Atm−/− thymocytes, which closely reflected the in vivo situations of the mice. We showed that the ER stress marker GRP78 and the UPR marker p-eIF2α increased during thymocyte development as early as 4 weeks of age in Atm−/− mice. This finding suggests that ER stress and UPR are likely to participate in initiation of the thymic lymphoma development in the animals.

Atm−/− thymic lymphoma cells appear to be overloaded with ER stress, and they are vulnerable to further such challenges by the addition of the ER stress inducer tunicamycin. It has been reported that the nicotinamide analogue 6AN sensitizes human tumor cell lines to the DNA cross-linking agent cisplatin [38]. When the cell lines were pretreated with 6AN for 18 hours, their sensitivity to cisplatin was increased. Chatterjee et al. [39] have shown that the V79 cell lines become resistant to topoisomerase IIα-targeted anticancer drug etoposide when GRP78 is upregulated by 6AN treatment. Notably, 6AN treatment downregulates topoisomerase IIα and its activity [33]. In the work presented here, we provide new evidence showing that 6AN treatment suppresses the DNA synthesis, both in ASL-1 and in ATL-1 T cell tumor lines in a dose-dependent fashion. Etoposide treatment along also suppresses the DNA synthesis in these cells, but ASL-1 cells are more sensitive than ATL-1 cells to etoposide. When pretreated with 6AN to upregulate GRP 78, both the cells are slightly resistant to later etoposide treatment.

Oxidative stress has also been implicated in carcinogenesis [40–42]. Accumulation of ROS causes oxidative stress, which leads to DNA damage, genomic instability, and cell death. Increasing evidence indicates that ROS play an important role in cell proliferation and participates in cell signaling regulation [43,44]. Mitogen-activated protein kinase family members extracellular-regulated kinase, Jun N-terminal kinase, and p38 respond to ROS for their activation (i.e., phosphorylation) [45]. Transcription factors nuclear factor–kappa B and activating protein 1 are also activated by ROS [46]. Interestingly, protein kinase C can be activated by oxidants, and inactivated by antioxidants [47]. Protein oxidation in the ER is connected to the generation of ROS, whereas oxidative stress by ROS accumulation disrupts Ca2+ homeostasis [48], causing ER stress. Finally, Nrf2 activation maintains normal levels of glutathione during UPR, and

**Figure 4.** Hydrogen peroxide upregulates GRP78 and p-eIF2α expression in cultured thymocytes. (A) Hydrogen peroxide increases p-eIF2α expression in cultured Atm+/+ thymocytes. Cells were treated with 100 μM hydrogen peroxide at 37°C for 30 minutes, at which time their proteins were extracted to examine p-eIF2α levels. (B) Hydrogen peroxide increases GRP78 expression in cultured Atm+/+ thymocytes. Cells were treated with different concentrations of hydrogen peroxide at 37°C for 3 or 6 hours, and proteins were extracted at each time point. (C) Dynamics of GRP78 and p-eIF2α after hydrogen peroxide treatment. Primary cultured Atm+/+ thymocytes were treated with 100 μM of hydrogen peroxide at 37°C for 30 minutes, washed twice with phosphate-buffered saline, and cultured for an additional 1, 4, 8, or 20 hours. Proteins were extracted at each time point to examine p-eIF2α and GRP78 levels in these cells.

**Figure 5.** AD4 treatment attenuates the elevated p-eIF2α levels in thymocytes of Atm−/− mice. Six-week-old Atm+/+ and Atm−/− mice were treated with 200 mg/kg AD4 intraperitoneally twice a day for 1 week. Control untreated mice received the same volume of normal saline. Thymocytes were isolated from AD4-treated and untreated Atm+/+ and Atm−/− mice at the end of the treatment period, and proteins were extracted for Western blot analysis.
inhibition of ROS production attenuates apoptotic induction after ER stress [49]. Apparently, a close relationship exists between oxidative stress and ER stress, although the mechanisms that link the two events have not been fully elucidated. In light of hydrogen peroxide upregulates GRP78 and p-eIF2α in cultured thymocytes (Figure 4), we treated Atm−/− mice with a novel thiol antioxidant AD4, a precursor of glutathione [50], that efficiently crosses the cell membrane into the cell and inhibits p38, extracellular-regulated kinase 1/2, and Jun N-terminal kinase phosphorylation [51]. This treatment represses the elevated p-eIF2α level in thymocytes of Atm−/− mice, thus we propose that ROS is the initial factor that triggers ER stress and UPR in Atm−/− thymocytes.

In summary, our proteomic and MS analyses revealed that at least 22 differentially expressed proteins are present in Atm−/− thymocytes and Atm−/− thymic lymphoma cells. Because ATM is a protein kinase, it may act indirectly on these proteins. ER stress and the UPR occur in Atm−/− thymocytes by ROS accumulation. Both oxidative stress and ER stress promote cell proliferation and induce neoplastic transformation. ER stress alters the sensitivity of tumor cells to some chemotherapeutic agents. Therefore, ER stress and oxidative stress are potential targets for tumor chemoprevention and chemotherapeutic strategies.

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