Serine 18 Phosphorylation of RAX, the PKR Activator, Is Required for PKR Activation and Consequent Translation Inhibition*

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The mouse protein RAX and its human ortholog PACT are the only known cellular activators for the double-stranded RNA-dependent protein kinase, PKR1 (1–3). RAX and PACT are 98% identical in amino acid sequence and contain three conserved dsRNA binding motifs. The N-terminal first and second motifs bind to dsRNA and associate with PKR, fails to activate PKR following stress. Furthermore, stable expression of RAX(S18A) results in a dominant-negative effect characterized by deficiency of eukaryotic initiation factor 2α subunit phosphorylation, delay of translation inhibition, and failure to undergo rapid apoptosis following removal of interleukin-3. We propose that the ability of RAX to activate PKR is regulated by a sequential mechanism featuring RAX association with PKR, RAX phosphorylation at serine 18, and activation of PKR.

The mechanism(s) by which the withdrawal of IL-3 can mediate RAX function and subsequent PKR activation is undefined. Nevertheless, it is clear that an additional signal is required for RAX to mediate PKR activation, because overexpression of RAX alone is not sufficient to activate PKR (1, 4). The functional significance of our recent discovery that RAX is a phospho-protein, and the mechanism by which RAX/PACT phosphorylation to trigger this cellular shutdown cascade.

Traditionally, PKR has been studied in the context of the host anti-viral response (6, 7). The serine-threonine kinase activity of PKR is activated by viral dsRNA, resulting in the phosphorylation of a physiological substrate of PKR, eIF2α, and consequent translation inhibition. However, it has become clear recently that PKR is also an essential mediator of signaling by both cytokines and growth factors and may even function as a tumor suppressor (9–10). In addition to its role in translation, PKR has been observed to participate in several transcription pathways (11–15). Under such circumstances, where there may be no apparent source of dsRNA necessary to activate PKR, it is believed that RAX/PACT may be responsible for PKR activation (1, 2).

Our laboratory has studied the mechanism of apoptosis following withdrawal of growth factor IL-3 from factor-dependent hematopoietic cells. Multipotential hematopoietic growth factors such as IL-3 are responsible for regulating growth of bone marrow progenitor cells (16–19). The binding of IL-3 to its receptor initiates a signal transduction cascade that results in cell proliferation, differentiation, and suppression of apoptosis. The removal of IL-3 from factor-dependent cells induces cell cycle arrest and apoptosis (20–22). Previous work in our laboratory has established a link between IL-3 withdrawal and RAX/PACT-mediated PKR activation in vivo (16). In the presence of IL-3, PKR remains inactivated, and cells grow normally. However, the stress of IL-3 deprivation results in RAX phosphorylation, promotion of RAX/PKT association, PKR activation, consequent inhibition of translation, and ultimately programmed cell death (1, 4, 16). The correlation between the stress-mediated stimulation of RAX phosphorylation and PKR activation in a mechanism that leads to cell death suggests that stress applications such as IL-3 withdrawal may regulate RAX by phosphorylation to trigger this cellular shutdown cascade.

It is now apparent that the double-stranded (ds)RNA-dependent protein kinase, PKR, is a regulator of diverse cellular responses to stress. Recently, the murine dsRNA-binding protein RAX and its human ortholog PACT were identified as cellular activators of PKR. Previous reports demonstrate that following stress, RAX/PACT associates with and activates PKR resulting in eIF2α phosphorylation, consequent translation inhibition, and cell death via apoptosis. Although RAX/PACT is phosphorylated during stress, any regulatory role for this post-translational modification has been uncertain. Now we have discovered that RAX is phosphorylated on serine 18 in both human and mouse cells. The non-phosphorylatable form of RAX, RAX(S18A), although still able to bind dsRNA and associate with PKR, fails to activate PKR following stress. Furthermore, stable expression of RAX(S18A) results in a dominant-negative effect characterized by deficiency of eukaryotic initiation factor 2α subunit phosphorylation, delay of translation inhibition, and failure to undergo rapid apoptosis following removal of interleukin-3. We propose that the ability of RAX to activate PKR is regulated by a sequential mechanism featuring RAX association with PKR, RAX phosphorylation at serine 18, and activation of PKR.

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MATERIALS AND METHODS

Plasmids and Cell Lines—NSF/N1.H7 and BaF3 cells were maintained in RPMI medium supplemented with 5% fetal bovine serum, 5% calf serum, and 20% WEHI-3B conditioned media as a source of IL-3. REH cells were maintained in RPMI with 10% fetal bovine serum. COS7 cells and PKR null mouse embryonic fibroblasts were maintained

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§ Supported by NSF/N1.H7 and BaF3 cells were maintained in RPMI medium supplemented with 5% fetal bovine serum, 5% calf serum, and 20% WEHI-3B conditioned media as a source of IL-3. REH cells were maintained in RPMI with 10% fetal bovine serum. COS7 cells and PKR null mouse embryonic fibroblasts were maintained in RPMI medium supplemented with 5% fetal bovine serum, 5% calf serum, and 20% WEHI-3B conditioned media as a source of IL-3. REH cells were maintained in RPMI with 10% fetal bovine serum. COS7 cells and PKR null mouse embryonic fibroblasts were maintained

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in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. All cells were cultured at 37 °C and 5% CO2 in a humidified incubator.

FLAG-tagged and HA-tagged RAX constructs were cloned into vector pcDEF3, which utilizes the elongation factor-1α promoter (23). This vector is more effective for RAX expression than any CMV promoter vector tried to date. Suspension cells were transfected by electroporation while LipofectAMINE (Invitrogen) was used to transfect adherent cells according to manufacturer’s protocol. Stable clones were selected in G418 (0.6 mg/ml) and screened by Western blotting with anti-FLAG M2 antibody (Sigma) or anti-HA antibody (Covance), horseradish peroxidase-labeled secondary antibody (Santa Cruz), and ECL detection kit (Amersham Biosciences).

**Cell Viability Assays and Protein Half-life Determination**—Cells stained with 7-AAD and propidium iodide were counted by flow cytometry to determine cell viability. The 7-AAD intensity was measured using the BD FACSCalibur and FloJo flow cytometry software (Tree Star). To determine the rate of protein half-life, cells were transfected with RAX constructs and treated with cycloheximide for 24 h. After treatment, the cells were washed twice with GLB and once with phosphate-buffered saline. The total cellular protein was isolated from 2.5 × 106 cells with 200 μl of RIPA lysis buffer and protein concentration was measured using the BCA assay (Pierce). Protein half-life was determined by loading 50 μg of lysate into each lane of SDS-PAGE, and transferring the protein to nitrocellulose. The total protein was determined by densitometry analysis of the Western blot with a monoclonal antibody to PKR (Santa Cruz B-10).

**PKR Association and dsRNA Binding**—For determination of RAX-PKR association, HA-tagged RAX or RAX(S18A) was immunoprecipitated from 200 μg of lysate from H7 cells that had been starved of IL-3 for 4 h using anti-HA-agarose conjugate. Lysates were quantitated by BCA assay (Pierce). Immunoprecipitates were resolved by 12% SDS-PAGE and transferred to nitrocellulose for Western blotting with antibody to RAX and PKR (Santa Cruz D-20). The percent of protein immunoprecipitated was determined as described.

To determine whether RAX mutants could bind dsRNA, expression vectors were transfected into COS7 cells, and 2 days later 500 μg of each lysate was incubated with 50 μl of poly(I-C)-agarose (Amersham Biosciences) for 2 h at 4 °C. The poly(I-C)-agarose-bound protein was washed three times in GLB and once in phosphate-buffered saline. Bound protein was disassociated by boiling in SDS-PAGE loading buffer and measured by Western blotting.

**Measurement of eIF2α Phosphorylation and Protein Synthesis**—At the indicated times of IL-3 deprivation, 1 × 107 cells were lysed in GLB, and 100 μg of lysate was separated by 12% SDS-PAGE. Western blots using antibody specific for eIF2α and phosphorylated eIF2α (Cell Signaling) were used to quantify the change in phosphorylation versus the change in total protein.

Protein synthesis was measured by incorporation of 3H-labeled amino acids (Amersham Biosciences CFB104). After IL-3 removal for the indicated time, 2.5 μCi of 3H-labeled amino acid mix was added to 5 × 105 cells and incubated at 37 °C for 15 min. Reactions were stopped by the addition of 1 volume of cold 10% trichloroacetic acid. The acid-insoluble fraction was collected on Whatman glass fiber filters (47 mm GF/A), and radioactivity was measured by liquid scintillation counting.

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**RESULTS**

**RAX Is Phosphorylated on Serine 18 following Stress**—We discovered previously that RAX is phosphorylated in vivo during serum starvation to the serine 18 site. To identify which serine(s) is phosphorylated, we created site-specific alanine substitution mutants for 15 of the 28 serine residues of RAX that are predicted to be phosphorylation targets based on three algorithms (24–26). Only 1 of the 15 sites chosen, serine 250, is not conserved between RAX and PACT, whereas the other 14 and their surrounding sequences are identical (Fig. 1A). Phosphorylation of the 15 epitope-tagged mutants was determined by transient expression in COS7 cells. Two days after transfection, the cells were stressed by serum withdrawal for 2 h in the presence of [32P]orthophosphate, and [32P]-labeled RAX was immunoprecipitated. Analysis indicated that all mutants except RAX(S18A) could be phosphorylated (Fig. 1B). The mutation of serine 18 to alanine results in a greater than 90% loss of phosphorylation, whereas the mutation of the other 14 serines did not significantly affect the overall level of RAX phosphorylation (Fig. 1C). Therefore, we conclude that RAX is phosphorylated in vivo exclusively on serine 18.

**Expression of RAX(S18A) in Factor-dependent Hematopoietic Cells Is Associated with a Reduced Rate of Apoptosis following IL-3 Deprivation**—To test the functional significance of RAX phosphorylation, clones of cells stably expressing RAX or RAX(S18A) were established in both IL-3 dependent NFS/1.H7 (H7) and IL-3 responsive BaF3 cells. Consistent with our previous observations with RAX, stable expression of RAX(S18A) has no effect on doubling time or cell cycle distribution in the cell types examined (1). However, unlike RAX, the RAX(S18A) mutant fails to be phosphorylated upon deprivation of IL-3 from these cells, confirming the physiological relevance of the serine 18 site (Fig. 2A). The RAX(S18A) mutant is also unable to be phosphorylated following serum deprivation when stably expressed in human lymphoblastic leukemia REH cells. Collectively, these results indicate that RAX is phosphorylated on serine 18 in both mouse and human cells. Because the region containing serine 18 is identical for both RAX and...
PACT, we predict that PACT will also be phosphorylated on serine 18.

H7 cells stably overexpressing RAX exhibit a greater rate of cell death upon IL-3 withdrawal when compared with vector-only transformed parental cells (1). In contrast, when the RAX(S18A) mutant is expressed at similar levels to RAX, H7 cells exhibit a delayed rate of apoptosis as assessed by both trypan blue and annexin V staining (Fig. 2, B and D). After 12 h of IL-3 deprivation, 27% of vector-only transformed H7 cells and 40% of cells expressing exogenous RAX exhibit annexin V positivity. By contrast, only 7% of cells expressing RAX(S18A) stain positive. These data indicate that an intact serine 18 site is required for rapid cell death following growth factor deprivation. Importantly, all of the RAX(S18A) expressing H7 clones we have tested, as well as a polyclonal population, exhibit nearly the same resistant phenotype and yield similar results in the following experiments. Therefore, we present data only from a representative clone hereafter.

Similar results are also observed for BaF3 cells (Fig. 2C). However, because BaF3 cells are comparatively more resistant to rapid apoptosis following IL-3 deprivation, the expression of exogenous RAX or RAX(S18A) does not appear to have the same magnitude of effect as in H7 cells. Thus, after 48 h of IL-3 deprivation, the expression of RAX in BaF3 cells causes a 15% increase in cell death as compared with parental cells, while RAX(S18A) expression effects a 5% decrease in cell death as

**Fig. 1.** RAX is phosphorylated on serine at amino acid position 18. A, comparison of RAX and PACT amino acid sequences. Only the amino acids that differ from RAX are indicated in the PACT sequence. The dsRNA binding domains are in bold. Serines targeted for site-specific mutagenesis are indicated by an asterisk. Serine 18 is boxed. B, transient transfections of RAX HA-and FLAG-tagged serine to alanine mutants into COS7 cells were used to identify the site of RAX phosphorylation. After metabolic labeling with [32P]orthophosphate, RAX was immunoprecipitated using affinity-tag antibody. The labeled protein and the corresponding Western blot (WB) using anti-RAX antibody are shown. C, the blots in B were quantified by densitometry analysis, and the change in the level of phosphorylation relative to total protein for each point mutant was graphed. wt, wild type.
compared with parental cells and a 20% decrease in death when compared with RAX-expressing cells (Fig. 2C). The difference noted in the rate of apoptosis between H7 and BaF3 cells following IL-3 deprivation may potentially be attributed to inherent cell type differences.

Serine 18 Phosphorylation of RAX Is Required for PKR Activation—Because cells expressing RAX(S18A) fail to undergo rapid apoptosis following IL-3 withdrawal compared with either parental cells or cells expressing equivalent amounts of RAX, we next tested the effect of RAX(S18A) on PKR activation as measured by PKR autophosphorylation (27). The average amount of PKR autophosphorylation relative to total PKR expressed was determined in three separate experiments with each cell line used. Upon the withdrawal of growth factor, PKR is maximally activated at 6 h in vector-transformed H7 cells and earlier, at 2 h, when exogenous RAX is expressed (Fig. 3). By contrast, cells expressing the RAX(S18A) mutant fail to activate PKR to any measurable degree. Therefore, we conclude that RAX phosphorylation is required for the rapid activation of PKR following stress.

RAX Phosphorylation Does Not Affect Protein Stability and Is Not Required for Either RAX-PKR Association or RAX Association with dsRNA—To show that the differences in response to IL-3 withdrawal observed in H7 cells are caused by RAX or RAX(S18A) and are not because of an inherent difference in protein stability, we measured the half-life of exogenously expressed RAX and RAX(S18A) and compared it to that of endogenous RAX. Cells were treated with cycloheximide to inhibit new protein synthesis, and aliquots were taken at the indicated times to assess the level of RAX by Western blot. Endogenous RAX has a half-life of about 16 h as does exogenous RAX and RAX(S18A) (Fig. 4A). Furthermore, exogenous expression of RAX or RAX(S18A) has no significant affect on the stability of PKR as compared with vector control cells (Fig. 4A). Thus, we concluded that RAX phosphorylation has no effect on either RAX or PKR protein stability and that the difference in sensitivity to stress observed in cells expressing RAX or RAX(S18A) is not because of a difference in protein stability.

Because RAX-PKR association is one potential mechanism by which RAX may activate PKR (5), we examined the role of RAX phosphorylation on this interaction. Epitope-tagged RAX or RAX(S18A) was immunoprecipitated from the lysates of H7 cells that were deprived of IL-3 for 4 h, and the associated PKR was evaluated by Western blotting. Results indicate that both RAX(S18A) and RAX associate with PKR to a similar degree following stress (Fig. 4B). In both instances, PKR coimmunoprecipitated at about one-third the level of RAX immunoprecipitation (10–12% versus 30–40%, respectively). This indicates that RAX phosphorylation is not necessary for PKR association but is required for PKR activation. Thus, the RAX(S18A) mutant may function in a “dominant-negative” manner to block PKR activation suggesting that RAX-PKR association may precede RAX phosphorylation and “activation.”

The ability of RAX(S18A) to bind dsRNA was also tested. Affinity-tagged RAX and RAX(S18A) were transiently expressed in COS7 cells, and tested for binding to poly(I-C)-agarose. Results indicated that RAX and RAX(S18A) comparably bind poly(I-C) (Fig. 4C). Two RAX mutants containing

At the indicated times, cell viability was determined using trypan blue dye exclusion. C, IL-3 was withdrawn from stable clones of BaF3 cells expressing either RAX or RAX(S18A). Cell viability was determined at the indicated times as above. ○, parental BaF3; ▲, RAX cl. 9; ●, RAX(S18A) cl. 16. D, annexin V staining of NFS/N1.H7 cell lines after IL-3 withdrawal for 12 and 24 h. Vector control H7, RAX cl. 13, and RAX(S18A) cl. 8 are compared. The percent annexin V positive cells is specified in the lower right corner of each graph.
deletions of the dsRNA binding domains were used as controls. As reported previously, the deletion of the first two dsRNA binding domains but not the second alone results in the failure to bind poly(I-C)-agarose (28). These findings indicate that RAX phosphorylation does not affect dsRNA binding and, importantly, that the potential binding to dsRNA is not sufficient for RAX activation of PKR.

Expression of RAX(S18A) Results in Decreased eIF2α Phosphorylation and Delayed Translation Inhibition following IL-3 Deprivation—To assess the downstream effect of RAX phosphorylation on PKR signaling, the physiological role of PKR as an eIF2α kinase was examined (29). Using antibodies specific for eIF2α and phosphoserine-51 eIF2α, the phosphorylation status of eIF2α was followed by Western blotting (Fig. 5A). Densitometry analysis of the blots from three separate experiments was used to measure the change in eIF2α phosphorylation relative to the change in overall protein level during stress (Fig. 5B). Control vector-transformed parental H7 cells exhibit a 6–6.5-fold increase in eIF2α phosphorylation following the removal of IL-3 for 10 h, whereas the expression of exogenous RAX results in a more rapid phosphorylation of eIF2α with maximal phosphorylation observed after only 8 h (Fig. 5B). In contrast, when RAX(S18A) is overexpressed, eIF2α phosphorylation is strikingly diminished such that even at 12 h only about a 2-fold decrease in phosphorylation is observed (Fig. 5B).

Fig. 3. PKR activation is suppressed by RAX(S18A) expression in NFS/N1.H7 cells. A, PKR was immunoprecipitated at the indicated times from NFS/N1.H7 cells expressing RAX or RAX(S18A) after IL-3 withdrawal and assayed for autophosphorylation activity by the incorporation of 32p. Autoradiography was used to detect 32p-labeled PKR, and the total PKR immunoprecipitated was visualized by Western blot (WB) with an anti-PKR antibody. B, three sets of blots like those shown in A were quantified by densitometry analysis, and the change in the fraction of active PKR/total PKR at each time point was graphed. ●, H7 vector control; ▲, RAX cl. 13; ●, RAX(S18A) cl. 8.

Fig. 4. RAX phosphorylation does not affect protein stability and is not required for either PKR association or dsRNA binding. A, protein stability of PKR and RAX. New protein synthesis was stopped by the addition of cycloheximide. At the indicated times, lysates were analyzed by Western blot (WB) for the presence of PKR and RAX. Endogenous RAX and PKR were detected with antibodies to those proteins while exogenous HA-tagged RAX and RAX(S18A) were monitored with antibody to the affinity tag. B, RAX-PKR association. Lysates were made from clones of H7 cells either expressing HA-tagged RAX (cl. 13) or RAX(S18A) (cl. 8) 4 h after removal of IL-3. Immunoprecipitation was performed with anti-HA antibody and Western blots with anti-PKR and anti-RAX antibody were used to determine whether PKR coimmunoprecipitated with RAX. L, 200 μg of total cell lysate; IP, immunoprecipitation. The percent immunoprecipitated protein as compared with the input is indicated below each lane. C, RAX(S18A) binds to dsRNA. RAX and RAX(S18A) were transiently expressed in COS7 cells for 48 h. As experimental controls, two RAX mutants containing dsRNA binding domain deletions were also tested. RAX(ΔI/ΔII) has both the first and second dsRNA binding domain deletions, whereas RAX(ΔI) has only the second domain removed. Lysates were precipitated with poly(I-C)-agarose. RAX was detected by Western blot. L, 100 μg of total cell lysate; P, precipitated with poly(I-C)-agarose.
H7 vector; 

incorporation of 14C-labeled amino acids following the removal of IL-3.

H7 RAX(S18A) cl. 8.

H7 RAX(S18A)

Protein synthesis was measured by the ability of cells to incorporate 14C-labeled amino acids at various times. Results indicated that expression of RAX enhances, while expression of RAX(S18A) delays, protein synthesis inhibition following stress (Fig. 5C). For example, after removal of IL-3 for 4 h, cells expressing RAX exhibited a 65% decrease in translation, whereas cells expressing RAX(S18A) display only a 20% reduction (Fig. 5C). Compared with either parental or vector-transfected control cells, where protein synthesis is reduced by ~40% at this time, the significantly decreased rate of steady-state translation inhibition observed in RAX(S18A) cells signifies a dominant-negative effect. Furthermore, after 12 h, parental, vector-control, and RAX-expressing cells all display ~80% translation inhibition, whereas cells expressing RAX(S18A) display only 50% inhibition (Fig. 5C). These results clearly indicated that serine 18 phosphorylation of RAX is required for its full and potent ability to inhibit steady-state protein synthesis following the withdrawal of IL-3 from factor-dependent cells.

### DISCUSSION

Findings here established that the PKR-binding and -activating protein, RAX, is phosphorylated exclusively on serine 18. Furthermore, RAX phosphorylation is required for PKR activation, eIF2α phosphorylation, and consequent translation inhibition as well as rapid apoptosis following the removal of IL-3 from factor-dependent hematopoietic cells. Because PACT, the human RAX ortholog, is virtually identical at this phosphorylation locus, we predict that PACT will also be phosphorylated on serine 18. RAX phosphorylation is neither required for RAX-PKR association nor binding to dsRNA, indicating that the RAX-PKR association likely precedes both RAX phosphorylation and PKR activation. Therefore, we propose a sequential model for RAX/PACT-mediated PKR activation featuring RAX/PACT association with PKR upon stress application to cells, followed by RAX/PACT phosphorylation and activation, which results in activation of PKR kinase activity (Fig. 6). Once activated, PKR then phosphorylates its downstream targets, including eIF2α, thereby resulting in translation inhibition and subsequent apoptosis. These data extend recent reports with PACT deletion mutants indicating that the first two dsRNA binding motifs are responsible for both dsRNA and PKR binding but dispensable for PKR activation, whereas the third motif is required for PKR activation (5, 30). Thus, we propose that the phosphorylation of serine 18 may lead to a conformational change in RAX/PACT that facilitates the interaction of its requisite C-terminal dsRNA binding motif with PKR thereby leading to PKR activation (Fig. 6). In addition, the phosphorylation and activation of RAX could explain, at least in part, how PKR is differentially regulated, because it apparently functions in either its kinase-inactive or active states to promote cell survival or apoptosis, respectively (10).

The sequence (REDGSGTF) surrounding serine 18 in RAX/PACT corresponds to a minimal putative kinase recognition sequence for both PKC ((K/R)(X/S/T)) and CaMK II (RXX(X/S/T)) (31). However, we have not observed an inhibition of RAX phosphorylation in vivo when metabolic labeling was performed in the presence of kinase inhibitors specific for PKC or CaMK II (data not shown). Furthermore, PKR does not appear to be responsible for RAX phosphorylation, because we found that RAX can be phosphorylated when stably expressed in PKR null mouse embryonic fibroblasts (data not shown). Although recent evidence indicates that these fibroblasts may retain “some” PKR kinase activity (32), these data are consistent with our previous observation that RAX phosphorylation is not blocked by the often used PKR inhibitor, 2-aminopurine (1).

Thus, additional studies will be required to identify the RAX/PACT kinase(s).

It is uncertain whether RAX/PACT has another physiologic
function(s) in addition to PKR activation. Recent evidence suggests that RAX/PACT may play a role in stimulating transcription and translation under certain circumstances. For example, it has been reported that RAX synergizes with the SV40 large T-antigen to stimulate SV40-dependent DNA replication and gene expression independent of PKR (28). In addition, PACT has been reported to promote the translation of mRNA from exogenous genes (33). However, whether or not RAX phosphorylation affects these activities or other cellular functions independent of PKR is not known.

In conclusion, the evidence presented here reveals that phosphorylation of RAX at serine 18 is necessary for full and potent stress-induced activation of PKR. Understanding the mechanism by which RAX/PACT is activated through phosphorylation will be important to address in future studies especially in the context of the role of PKR in both host anti-viral and potential anti-neoplastic responses. A more complete molecular understanding of how RAX is regulated and its role in PKR activation following stress may prove relevant for the development of novel anti-tumor/viral strategies that could target this important signaling pathway.

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