Disruption of Tsc2 in pancreatic β cells induces β cell mass expansion and improved glucose tolerance in a TORC1-dependent manner

Latif Rachdi^{*†}, Norman Balcazar^{*}, Fernando Osorio-Duque^{*}, Lynda Elghazi^{*}, Aaron Weiss^{*}, Aaron Gould^{*}, Karen J. Chang-Chen^{*}, Michael J. Gambello[‡], and Ernesto Bernal-Mizrachi^{*§}

^{*}Division of Endocrinology, Metabolism, and Lipid Research, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8127, St. Louis, MO 63110; and [‡]Division of Medical Genetics, Department of Pediatrics, University of Texas Health Science Center, Houston, TX 77030

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Regulation of pancreatic β cell mass and function is a major determinant for the development of diabetes. Growth factors and nutrients are important regulators of β cell mass and function. The signaling pathways by which these growth signals modulate these processes have not been completely elucidated. Tsc2 is an attractive candidate to modulate these processes, because it is a converging point for growth factor and nutrient signals. In these experiments, we generated mice with conditional deletion of Tsc2 in β cells (β Tsc2^{-/-}). These mice exhibited decreased glucose levels and hyperinsulinemia in the fasting and fed state. Improved glucose tolerance in these mice was observed as early as 4 weeks of age and was still present in 52-week-old mice. Deletion of Tsc2 in β cells induced expansion of β cell mass by increased proliferation and cell size. Rapamycin treatment reversed the metabolic changes in β Tsc2^{-/-} mice by induction of insulin resistance and reduction of β cell mass. The reduction of β cell mass in β Tsc2^{-/-} mice by inhibition of the mTOR/Raptor (TORC1) complex with rapamycin treatment suggests that TORC1 mediates proliferative and growth signals induced by deletion of Tsc2 in β cells. These studies uncover a critical role for the Tsc2/mTOR pathway in regulation of β cell mass and carbohydrate metabolism *in vivo*.

mTOR | pancreas | islets

The defects that result in diabetes are diverse, but the extent of loss of pancreatic β cell mass is a critical determinant for the development of this disease (1, 2). Overall pancreatic β cell mass is dictated by a balance of neogenesis, proliferation, cell size, and apoptosis (3). Growth factors, insulin, incretins, and nutrients are important modulators of β cell mass (4–9). The molecular mechanisms and downstream signaling events linking growth factor and nutrient signaling to regulation of β cell mass and function are ill defined.

mTOR integrates growth factors and nutrient signals and is essential for cell growth and proliferation (10). One of the mechanisms by which these signals regulate mTOR activity involves the tuberous sclerosis complex (TSC) gene product TSC2 (tuberin). TSC2 forms a complex with TSC1 (hamartin) and the small G protein Rheb. Phosphorylation of TSC2 by AKT, GSK3 β , and AMPK reduces its GTPase-activating protein (GAP) activity toward Rheb, leading to activation of mTOR (11–15). Recent observations suggest that mTOR is associated with two distinct complexes (TORC1 and -2) (16, 17). The mammalian TORC1 contains Raptor and G protein β -subunit-like protein (G β L) and modulates the activity of ribosomal S6 kinase (S6K) and eukaryote initiation factor 4E-binding protein 1 (4EBP1) and eukaryote initiation factor 4E (eIF4E), key regulators of protein translation (18). Decreased β cell mass and hyperglycemia in mice deficient for S6K and mutant of ribosomal protein S6 provide evidence for the importance of this pathway in β cell mass and function (19–21). In contrast, the TORC2 complex is insensitive to rapamycin and includes mTOR and rictor (16, 17). This complex is potentially important for regu-

lation of β cell mass, because it is necessary for activation of Akt by phosphorylation of Ser 473 (22). The importance of the mTOR complexes in β cells is not completely understood.

In the current experiments, the hypothesis that Tsc2/mTOR signaling is a major regulator of β cell mass and carbohydrate metabolism was tested. To activate mTOR signaling in β cells, we generated mice with conditional deletion of Tsc2 in pancreatic β cells (β Tsc2^{-/-}). These mice exhibited hypoglycemia, hyperinsulinemia, and improved glucose tolerance that was maintained up to 52 weeks. The improved glucose tolerance resulted from increased β cell mass due to augmented proliferation and cell size. Moreover, rapamycin treatment reversed the metabolic phenotype observed in β Tsc2^{-/-} mice by induction of insulin resistance and reduction in β cell mass. The decreased β cell mass in β Tsc2^{-/-} mice treated with rapamycin is compatible with the concept that the TORC1 complex is a major component in mediating proliferative and growth signals induced by deletion of Tsc2 in β cells. These studies show a direct role for Tsc2 and activation of mTOR/S6K/4EBP signaling in regulation of β cell mass.

Results

Generation of Mice Deficient for Tsc2 in β Cells. The study of Tsc2 *in vivo* has been limited because of embryonic lethality of mice with disruption of Tsc2 (23). Therefore, we generated mice with conditional deletion of the Tsc2 gene in pancreatic β cells (β Tsc2^{-/-}) by breeding mice carrying a loxP flanked Tsc2 gene with mice expressing cre recombinase driven by the rat insulin promoter (24). Levels of the Tsc2 gene product tuberin in islet lysates from β Tsc2^{-/-} mice were almost nondetectable (Fig. 1A). Immunostaining for Tsc2 in pancreatic sections from 8-week-old WT and β Tsc2^{-/-} mice demonstrated staining only in islets from WT mice (Fig. 1B). Tsc2 levels in other tissues were comparable between WT and β Tsc2^{-/-} mice (data not shown). To determine the activation of mTOR signaling, we assessed the activity of S6 kinase, a downstream target of mTOR. S6K activation was determined by immunostaining for phospho-ribosomal S6 protein, a direct target for S6K. In contrast to WT mice, increase in phospho-ribosomal S6 protein levels was observed in islets from β Tsc2^{-/-} mice [supporting information (SI) Fig. S1]. Increased levels for phospho-ribosomal S6 protein in islet lysates from

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The authors declare no conflict of interest.

[†]Present address: Paris Descartes University, INSERM U845, Necker Faculty of Medicine, Paris, France.

[§]To whom correspondence should be addressed. E-mail: ebernal@wustl.edu.

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$\beta Tsc2^{-/-}$ was also demonstrated by immunoblotting (Fig. 1C). These studies demonstrated that the deletion of *Tsc2* in pancreatic β cells resulted in activation of mTOR signaling.

$\beta Tsc2^{-/-}$ Mice Exhibited Normal Body Weight and Improved Glucose Tolerance. Evaluation of body weight showed that progression of body weight in $\beta Tsc2^{-/-}$ and $\beta Tsc2^{+/-}$ mice was comparable with that of the WT controls (Fig. S2). Assessment of fed glucose concentrations at 4 weeks of age showed lower glucose levels in $\beta Tsc2^{-/-}$ mice than in $\beta Tsc2^{+/-}$ and WT mice. The significant decrease in fed glucose concentrations was maintained in 52-week-old mice (Fig. 2A and data not shown for 52 weeks). In 6-h-fasted mice, $\beta Tsc2^{-/-}$ mice also exhibited lower glucose levels as early as 8 weeks of age, and this difference persisted in 52-week-old mice (Fig. 2B and data not shown for 52 weeks). Compared with WT controls, $\beta Tsc2^{-/-}$ mice exhibited higher fed and 6-h-fasting insulin levels at 4, 8, 12, 16, and 20 weeks of age (Fig. 2C and D). The fed and 6-h-fasting insulin values in $\beta Tsc2^{+/-}$ mice were comparable with those of WT controls (Fig. 2C and D). i.p. glucose tolerance tests at 4 weeks of age showed that glucose levels were lower in $\beta Tsc2^{-/-}$ mice after overnight fasting (Fig. 2E). Blood glucose concentrations in $\beta Tsc2^{-/-}$ mice were reduced at 30, 60, and 120 min after glucose injection (Fig. 2E). The results of the glucose tolerance test in 40-week-old mice demonstrated fasting hypoglycemia and improved glucose tolerance in $\beta Tsc2^{-/-}$ mice (Fig. 2F). The glucose tolerance test changes were maintained at 52 weeks of age (Fig. S3). Insulin secretory response was then assessed after i.p. glucose admin-

istration. Insulin levels after overnight fasting were 3-fold higher in $\beta Tsc2^{-/-}$ than in WT mice (Fig. 2G). Five minutes after glucose challenge, serum insulin levels almost doubled in $\beta Tsc2^{-/-}$ and WT mice (Fig. 2G). The elevation of insulin levels in $\beta Tsc2^{-/-}$ mice persisted at 15 min and returned to basal levels 30 min after glucose injection (Fig. 2G). The insulin secretory response in $\beta Tsc2^{+/-}$ was similar to that of WT mice. These experiments showed that deletion of *Tsc2* in β cells resulted in improved glucose tolerance as a consequence of increased insulin levels and that glucose mediated insulin secretion in $\beta Tsc2^{-/-}$ mice was conserved.

Deletion of *Tsc2* Increases β Cell Mass by Augmented Proliferation and Cell Size. Islet histology showed that islets from $\beta Tsc2^{-/-}$ mice exhibited normal architecture consisting of insulin cells in the core and non β cells in the periphery (Fig. S4A). β cell mass in 8-week-old $\beta Tsc2^{-/-}$ mice was 2.1-fold higher than in WT mice (Fig. 3A and Fig. S4B). The increased β cell mass in $\beta Tsc2^{-/-}$ mice was still observed in 52-week-old mice ($P < 0.05$, data not shown). The size of individual β cells was 1.6-fold higher in $\beta Tsc2^{-/-}$ mice (Fig. 3B and Fig. S4C). Proliferation of β cells measured by Ki67 immunostaining showed a 2-fold increase in frequency of β cell proliferation in β cells from $\beta Tsc2^{-/-}$ mice (Fig. 3C and Fig. S4D, $P < 0.05$). In contrast, the frequency of β cell apoptosis as measured by cleaved caspase-3 staining was similar between $\beta Tsc2^{-/-}$ and WT mice (Fig. 3D and Fig. S4E). The results of these experiments suggest that deletion of *Tsc2* in β cells augments β cell mass by increased proliferation and cell size.

Inhibition of the TORC1 Complex by Rapamycin Reverted the Metabolic Phenotype Observed in $\beta Tsc2^{-/-}$ Mice. To elucidate the molecular mechanisms involved in regulation of β cell mass and function by Tsc2, we inhibited the TORC1 complex in $\beta Tsc2^{-/-}$ and WT mice. Inhibition of the TORC1 complex *in vivo* was obtained by daily i.p. administration of rapamycin for 14 days. Inhibition of the TORC1 complex by rapamycin was assessed by immunoblotting for phospho-S6 protein, a downstream target of TORC1/S6K activation (Fig. 4A). In contrast to WT treated with vehicle or rapamycin for 14 days, β cells from $\beta Tsc2^{-/-}$ mice exhibited increased levels of phospho-S6 protein (Fig. 4A). Phosphorylation of S6 protein disappeared after rapamycin treatment of WT and $\beta Tsc2^{-/-}$ mice (Fig. 4A). Similar findings were obtained by Immunostaining for phospho-S6 protein in sections from WT and $\beta Tsc2^{-/-}$ mice (Fig. S5A). Phosphorylation of Akt on Thr-308 and Ser-473 was decreased in islet lysates from $\beta Tsc2^{-/-}$ mice (0.5 ± 0.02 and 0.3 ± 0.06 , respectively; $P < 0.05$) (Fig. 4B). Rapamycin treatment of islets from WT and $\beta Tsc2^{-/-}$ mice had no effect on the levels of Akt phosphorylation on Thr 308 and Ser 473 (Fig. 4B; $P > 0.05$). Assessment of carbohydrate metabolism showed that, in contrast to WT mice treated with vehicle, fasting glucose concentrations were elevated in WT mice treated with rapamycin (Fig. 4B). Fasting glucose levels in $\beta Tsc2^{-/-}$ and WT mice treated with rapamycin were comparable (Fig. 4B). Fed glucose levels were similar among all of the groups (Fig. S5B). Fasting and fed insulin levels in rapamycin-treated WT and $\beta Tsc2^{-/-}$ mice were similar but higher than those of WT injected with vehicle (Fig. 4C and Fig. S5B). Glucose tolerance testing performed before rapamycin treatment showed improved glucose tolerance in $\beta Tsc2^{-/-}$ mice (Fig. 4C). Assessment of glucose tolerance after 7 days of rapamycin treatment demonstrated that rapamycin-injected $\beta Tsc2^{-/-}$ mice had similar glucose levels to those of WT injected with rapamycin or vehicle (Fig. 4D). After 14 days of treatment, glucose levels in rapamycin injected WT were higher at 60 min after glucose injection and returned to normal after 2 h (Fig. 4D). Rapamycin-treated $\beta Tsc2^{-/-}$ mice exhibited glucose con-

The Increased β Cell Mass and Proliferation Observed in $\beta Tsc2^{-/-}$ Mice Is TORC1 Dependent. Analysis of islet morphometry demonstrated that β cell mass in rapamycin-treated WT and $\beta Tsc2^{-/-}$ mice were no different from that of WT treated with vehicle (Fig. 5A). The frequency of β cell proliferation in WT mice treated with rapamycin was lower than vehicle treated WT mice (Fig. 5B). The frequency of proliferation in rapamycin treated $\beta Tsc2^{-/-}$ and WT mice was comparable (Fig. 5B). No difference in cell size was observed between WT and rapamycin-treated $\beta Tsc2^{-/-}$ and WT mice (Fig. 5C). The frequency of apoptosis between rapamycin-treated WT and $\beta Tsc2^{-/-}$ mice was similar (Fig. 5D).

The current studies were performed to understand the role of Tsc2/mTOR signaling in growth and function of pancreatic β cells. These experiments showed that *in vivo* activation of mTOR signaling by conditional deletion of *Tsc2* in β cells resulted in lower glucose levels, hyperinsulinemia, and improved glucose tolerance. Deletion of *Tsc2* in β cells induced expansion of β cell mass by increased proliferation and cell size. These experiments provide evidence for a critical role of Tsc2 levels in regulation of β cell mass and function. Rapamycin treatment reversed the metabolic changes in $\beta Tsc2^{-/-}$ mice by induction of insulin resistance and reduction of β cell mass. The reduction of the expansion of β cell mass by rapamycin treatment is compatible with the concept that the TORC1 complex plays a crucial role in relating proliferative signals induced by *in vivo* activation of

The IRS2/phosphoinositide 3-kinase (PI3K)/Akt pathway plays a critical role in regulation of β cell mass *in vivo* and *in vitro* (6, 25–30). Tsc2 is one of the important downstream molecules regulated by Akt signaling. Akt phosphorylates Tsc2, and this event results in activation of mTOR signaling. The current work evaluates the importance of the TSC/mTOR arm of Akt signaling. In addition, these experiments address the mechanism by which nutrient signals regulate β cell mass and function. We showed that activation of Tsc2/mTOR signaling in β cells regulates glucose metabolism by increasing insulin levels. These metabolic changes resulted from expansion in β cell mass by means of increased proliferation and cell size. In contrast to models with activation of Akt signaling, the islet organization in $\beta Tsc2^{-/-}$ mice was conserved, suggesting that activation of this pathway does not cause unrestrained proliferation. The mechanisms involved in progression of cell cycle induced by activation of mTOR are not known, but, in preliminary experiments, the levels of the cell cycle inhibitor p27 were not altered in $\beta Tsc2^{-/-}$ mice (data not shown). The metabolic and β cell mass changes were maintained in 52-week-old mice, suggesting that mTOR activation prevents some of the β cell defects associated with aging. It is important to note that follow-up of mice up to 52 weeks of age showed no evidence of tumor or hamartoma formation. The current work suggests that Tsc2/mTOR signaling could be a major downstream pathway relating the effects of IRS2/PI3K/Akt and nutrient signaling in β cells.

Fig. 3. Effect of Tsc2 deficiency on islet morphology. (A) Quantitation of β cell mass in 8-week-old WT and β Tsc2^{-/-} mice. (B) Quantitation of β cell size in WT and β Tsc2^{-/-} mice was performed in sections immunostained for insulin and β -catenin. (C) Frequency of β cell proliferation was assessed by Ki67 staining in insulin-stained sections from WT and β Tsc2^{-/-} mice. (D) Frequency of β cell apoptosis was assessed by cleaved caspase-3 staining in insulin-stained sections from WT and β Tsc2^{-/-} mice. Data are presented as mean + SE ($n > 4$). For all panels: *, $P < 0.05$.

To dissect the molecular mechanisms involved in the metabolic and β cell mass phenotype by Tsc2, we focused on the two mTOR complexes (TORC1 and -2) (16, 17). The TORC2 complex is resistant to rapamycin treatment and has been recently described as the kinase responsible for phosphorylation of Ser-473 in Akt (22). Interestingly, Ser-473 phosphorylation of Akt was reduced in islets from $\beta Tsc2^{-/-}$, suggesting that the effect of TORC2 on Akt phosphorylation was not operational in β cells and that other mechanisms are involved (Fig. 4B). The reduction in phosphorylation of Akt on Thr-308 indicates that the negative feedback inhibition of mTOR on IRS signaling is functional. Taken together, the results of these experiments indicate that activation of mTOR by deletion of Tsc2 inhibits Akt signaling by feedback inhibition of IRS signaling and that the

effects of Tsc2 deletion are mediated predominantly by the TORC1 complex.

Therefore, we focused on the study of the TORC1 complex. TORC1 contains at least two proteins: Raptor and mLst/GbL (18). Rapamycin treatment destabilizes Raptor-mTOR binding and inhibits function of the TORC1 complex (31). Nutrient deprivation also inhibits the activity of the TORC1 complex by stabilizing the Raptor-mTOR complex. The importance of this arm of mTOR signaling in the phenotype was evaluated by the administration of rapamycin to $\beta Tsc2^{-/-}$ mice. The metabolic studies in rapamycin-treated mice suggest that rapamycin induced insulin resistance in WT and $\beta Tsc2^{-/-}$ mice. Interestingly, rapamycin treatment reversed the improved glucose tolerance observed in $\beta Tsc2^{-/-}$ mice. The reversal of the metabolic phenotype in rapamycin-treated $\beta Tsc2^{-/-}$ mice resulted from the development of insulin resistance and inhibition of β cell expansion, proliferation, and cell size. Assessment of cell death showed a similar frequency of apoptosis between WT and $\beta Tsc2^{-/-}$ after 14 days of rapamycin treatment. Additionally, immunoblotting for cleaved caspase-3 levels in islets from WT and $\beta Tsc2^{-/-}$ mice treated or untreated with rapamycin was comparable (data not shown). Therefore, the rapid changes in β cell mass after rapamycin treatment for 14 days suggest that cell size rather than proliferation or apoptosis is a major component. It is possible that the effects of decreased proliferation or apoptosis in β cell mass after rapamycin treatment become evident with longer treatment. The mechanisms involved in inhibition of the TORC1 complex by rapamycin or nutrient deprivation are complex, and it is difficult to determine whether the rapamycin effect is comparable with nutrient deprivation. These results indicate that the TORC1 pathway is a major component relating proliferative and growth signals from mTOR. The individual contribution of 4EBP or S6K to these phenotypes could be assessed further by genetic experiments.

The current work uncovers an important function for Tsc2 in regulation of β cell mass, proliferation, and carbohydrate metabolism. An attractive feature of this signaling pathway is the activation of proliferative responses that lack oncogenic potential. This previously undescribed finding is of importance, because it allows development of alternative approaches to expand β cell mass *in vivo* and *in vitro* without the risk of oncogenic transformation. This information is critical for the development

Fig. 4. Rapamycin treatment reverses the metabolic phenotype observed in $\beta Tsc2^{-/-}$ mice. (A) Immunoblotting for phospho-S6 protein and phospho-Akt at Thr-308 and Ser-473 in islet lysates from WT – RAP, WT + RAP, and $\beta Tsc2^{-/-}$ + RAP. Immunoblotting is representative of three experiments in duplicate. (B) Fasting glucose and insulin concentrations in WT – RAP, WT + RAP, and $\beta Tsc2^{-/-}$ + RAP. (C and D) i.p. glucose tolerance tests performed before treatment (C) and 7 and 14 days after treatment (D) with rapamycin. Data are expressed as mean $\pm$ SE for at least three mice per group.

□ WT ■ WT + RAP ■ $\beta Tsc2^{-/-}$ + RAP

Fig. 5. Effect of rapamycin treatment in β cell mass, proliferation, cell size, and apoptosis. (A) β cell mass in 8-week-old WT treated with vehicle (WT) or rapamycin (5 mg/kg a day for 14 days) (WT + RAP) and $\beta Tsc2^{-/-}$ mice treated with rapamycin ($\beta Tsc2^{-/-}$ + RAP). (B) Frequency of β cell proliferation was assessed by Ki67 staining in insulin-stained sections from WT and $\beta Tsc2^{-/-}$ mice. (C) Size of individual β cells in the same group of mice as described in A. (D) Frequency of β cell apoptosis was determined by cleaved caspase-3 staining. At least 2,000 β cells per group were measured. Data are presented as mean $\pm$ SE for at least three mice per group (*, $P < 0.05$).

of improved therapeutic strategies for the treatment and cure of diabetes and to understand the effects of mTOR inhibitors on β cell function. Finally, the adverse effects of rapamycin on β cells imply that this agent could negatively affect the success of islet transplantation. This information could be used to modify immunosuppressant therapy used for islet transplantation

Methods

Animals. β cell-specific *Tsc2* knockout mice ($\beta Tsc2^{-/-}$) were generated by crossing *Tsc2^{fllox/fllox}* mice with mice that express Cre recombinase gene under the control of the rat insulin promoter (24). The *Tsc2^{fllox/fllox}* mice harbor a modified endogenous *Tsc2* gene with *loxP* sites flanking exon 2 to 4 (32). The mice included for these experiments were on a mixed 129 $\times$ C57BL/6 background. $\beta Tsc2^{-/-}$ and WT littermates were on comparably mixed backgrounds, and experiments were performed in males. For the rapamycin treatments, rapamycin (Santa Cruz Biotechnology) was injected i.p. at 5 mg/kg

every day for 2 weeks. All procedures were performed in accordance with the Washington University Animal Studies Committee.

Western Blotting. Isolated islets were lysed and subjected to immunoblotting as described (33, 34). The following antibodies were used: tuberlin, actin, phospho-S6 ribosomal protein (Ser-235/236), and phospho-Akt (Ser-473 and Thr-308) (all from Cell Signaling Technology) and α -tubulin (from Sigma). Immunoblotting experiments were performed at least three times in duplicate. Scanning densitometry of protein bands was determined by pixel intensity using NIH ImageJ software (v1.38, <http://rsb.info.nih.gov/ij/index.html>) and normalized against that of tubulin.

Immunohistochemistry, Islet Morphometry and Analysis of Proliferation, and Apoptosis. Immunostaining for insulin, glucagon, somatostatin, and pancreatic polypeptide was done as described (35). Sections were stained with antibodies against tuberlin/*Tsc2* (Santa Cruz Biotechnology), β -catenin (Sigma), anti-Ki67 (Novocastra), and cleaved caspase-3 (Cell Signaling Technology). The β cell mass was calculated by point counting morphometry using NIH ImageJ software (v1.3.8x, freely available at <http://rsb.info.nih.gov/ij/index.html>) as described (33, 34). Proliferation and apoptosis were assessed in insulin-stained and Ki67-stained or cleaved caspase-3-stained sections. At least 2,000 insulin-stained cells were counted for each animal. A mean cross-sectional area of individual β cells, a measure of β cell size, was determined on insulin-stained sections. This measurement of cell size was calculated by dividing the β cell area by the number of β cell nuclei in the covered area using NIH ImageJ software (v1.3.8x, <http://rsb.info.nih.gov/ij/index.html>) (35).

Metabolic Studies. Fasting blood samples were obtained from the tail vein from 6-hour-fasted mice using a Freestyle glucometer (TheraSense). Fed glucose measurements were performed early in the morning between 9:00 and 10:00 a.m. Plasma insulin levels were determined by using Mouse Insulin Ultra sensitive ELISA kit (ALPCO Diagnostics). Glucose tolerance tests were performed in overnight-fasted animals by injecting glucose (2 mg/g) i.p. as described (35).

Statistical Analysis. All of the values are presented as mean $\pm$ SE. Statistical analyses were conducted by using two-way analysis of variance for interactions between variables and the unpaired Student *t* test to compare independent means. Differences were considered statistically significant with a *P* value ≤ 0.05 .

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