Sphingosine-phosphate Lyase Enhances Stress-induced Ceramide Generation and Apoptosis*

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Sphingosine 1-phosphate (S1P) is a sphingolipid metabolite that regulates cell fate, survival, differentiation, and angiogenesis, and development. S1P has been shown to activate an extracellular signaling pathway mediated through a family of specific G protein-coupled receptors (1, 2). However, evidence indicates that S1P also mediates effects through a receptor-independent mechanism by functioning as a second messenger within cells (3). Intracellular S1P levels are regulated primarily by three highly conserved enzymes: sphingosine kinase (SPHK), which catalyzes the phosphorylation of sphingosine-producing S1P, S1P phosphatase (S1PP), which reverses the former reaction, and S1P lyase (SPL), a PLP-dependent enzyme that catalyzes the essentially irreversible cleavage of S1P at the C2−3 carbon–carbon bond, yielding ethanolamine phosphate and a long-chain aldehyde (4–6).

One approach to uncovering roles of S1P signaling in biology has been to manipulate expression of the recently cloned genes of S1P metabolism. Toward that end, altered SPL expression in a variety of cell lines and mutant model systems has yielded pronounced effects and severe phenotypes. For example, Saccharomyces cerevisiae dpl1 null mutants, which lack SPL activity, are highly resistant to heat stress and nutrient deprivation, whereas overexpression of DPL1 can correct certain defects of endocytosis (7–11). In mouse embryonal carcinoma cells, disruption of the SLP gene enhances cellular differentiation in response to retinoic acid (12). In Drosophila melanogaster, Caenorhabditis elegans, and Dictyostelium discoideum, SPL expression is required for global functions, including embryogenesis, reproduction, survival, and movement (13–15). These observations indicate that SPL has important and, in some cases, essential functions in cells and organisms. The effects of manipulating SPL expression are presumed, and in some cases have been demonstrated, to be secondary to alterations in intracellular pools of S1P and its availability to carry on downstream signaling functions. However, the specific intracellular mechanisms by which SPL exerts its effects are not well understood.

In this study, a recombinant human SPL-GFP fusion protein was overexpressed in HEK293 cells to elevate SPL activity and diminish cellular S1P. Somewhat unexpectedly, stable expression also enhanced stress-induced increases in ceramide. Overexpression of catalytically active SPL also increased apoptosis, which was reversed by the addition of exogenous S1P or the ceramide synthase inhibitor fumonisin B1. In contrast, the products of the reaction catalyzed by SPL, ethanolamine phosphate, and long-chain aldehydes, did not promote apoptosis. These studies show that SPL can affect cell fate and influence sphingolipid metabolism beyond the regulation of intracellular S1P.
**EXPERIMENTAL PROCEDURES**

**Materials**—ε-erythro-Sphingosine, fumonisin B₁, ethanolamine phosphate, and MTT reagent were obtained from Sigma Chemical Co. (St. Louis, MO), [4,5-³H]ε-erythro-dihydroxy-6-phosphinosine 1-phosphate (DHIS1P) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO), S1P was from Avanti Polar Lipids (Alabaster, AL), ISP-1 was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA), Sequenase 2.0 and 5'-32P]dATP were from Amerham Pharmacia Biotech (Piscataway, NJ), and Taq polymerase and PCR reagents were from Promega Corp. (Madison, WI). Hexadecanal and hexadecenal were synthesized essentially as described previously (16).

- **cDNA Cloning**—BLAST search using the mouse SPL cDNA sequence against GenBank® dbEST identified two homologous human EST sequences, GenBank® accession numbers T86263 and A3383781 (17). T86263 was homologous to the 3'-end and A3383781 to the 5' region of mouse SPL. To clone the overlapping region of these two ESTs, primers H070F2 (Table I) and H967R2 were used for PCR amplification of the target cDNA from a human cDNA library constructed in the yeast shuttle vector pADNS (18). This PCR product was then cloned into pBluescript, and DNA sequencing was performed using the Sanger method (19). To clone the remainder of the 5'-end of the hSPL cDNA, a generic primer, ADHP2 (based on the vector's promoter sequence), and a gene-specific primer, H967R3 (based on EST sequence T86263), were used for PCR amplification using the same cDNA library. The coding region of the hSPL cDNA was then re-cloned by RT-PCR amplification of total RNA from normal human diploid fibroblasts using N-terminal primer 5'-H967 and C-terminal primer 3'-H967. This RT-PCR product was cloned into pBluescript SK(+) at KpnI/XhoI sites to give plasmid, pBS/hSPL.

**DNA Vector Constructs**—The hSPL coding sequence was subcloned into pcDNA3.0 vector (Invitrogen, Carlsbad, CA) at KpnI/NotI sites with an engineered ribosome binding site corresponding to the Kozak sequence, GCCACCATGG, for efficient expression in mammalian cells (20). This construct is referred to as pc-hSPL. A change of the coding sequence from +4 C to +4 G after the start codon ATG changes the codon for a proline to that of an alanine residue. The pc-hSPL-GFP fusion construct was generated by cloning of the GFP coding region (a codon for a proline to That of an alanine residue. The pc-hSPL-GFP sequence from (20). This construct is referred to as pc-hSPL. A change of the coding sequence, GCCACCATGG after the start codon ATG changes the codon for a proline to That of an alanine residue. The pc-hSPL-GFP sequence from (20). This construct is referred to as pc-hSPL. 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tions were obtained by ultracentrifugation at 100,000 \( \times g \) for 60 min. Pellets were resuspended by sonication for 5-s intervals with repeats at low voltage. Protein content was determined by the Bradford method using Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). SPL activity was determined using \( [4,5-\text{H}] \text{D-erythro-DHS1P} \) as substrate. 50 \( \mu \text{g} \) of protein was assayed, and reactions were carried out at 37 °C for 60 min.

SPL activity of cells transiently or stably expressing three human SPL constructs. SPL enzyme activity was measured in the membrane fractions of HEK293 cells either transiently or stably transfected with pc-hSPL, pc-GFP, pc-hSPL-GFP, or pc-hSPL(K353L)-GFP. SPL activity in 50 \( \mu \text{g} \) of protein extract was determined using \( [4,5-\text{H}] \text{DHS1P} \) substrate. Data shown represent three separate experiments.

SPL-GFP Localization and Microscopy—Co-localization of recombinant SPL to the endoplasmic reticulum (ER) was performed by comparing the pattern of fluorescence from the hSPL-GFP fusion protein versus that of a red fluorescent protein containing the ER targeting sequence of calreticulin and the ER retention sequence KDEL in HEK293 cells stably transfected with pc-hSPL-GFP or pc-hSPL(K353L)-GFP. SPL activity in 50 \( \mu \text{g} \) of protein extract was determined using \( [4,5-\text{H}] \text{DHS1P} \) substrate. Data shown represent three separate experiments.

Sphingolipid Measurements—For sphingolipid measurements in stably transfected cell lines, sphingolipids were analyzed by liquid chromatography, electrospray ionization, and tandem mass spectrometry as described previously (22, 23). The internal standards for quantitation of the sphingolipids were obtained from Avanti Polar Lipids (Alabaster, AL).

Labeling and Isolation of Cellular Sphingolipids and Phospholipids—Biosynthesis of sphingolipids was determined by following incorporation of \( L-[^{14}\text{C}]\)serine into newly synthesized sphingolipids as previously described (24). Briefly, cells were seeded at \( 5 \times 10^6 \) cells per dish in 100-mm dishes and grown under standard conditions in medium supplemented with 10% fetal calf serum for 24 h. Medium was then replaced with serum-free medium containing 1 \( \mu \text{Cl/mmol} L-[^{14}\text{C}]\)-serine (specific activity, 57 mCi/nmol). After 24 h, cells were harvested and lipids extracted from cell pellets by incubating with chloroform/metha-
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**RESULTS**

Cloning and Characterization of Recombinant Human SPL—We previously reported the identification of SPL cDNA from *Mus musculus* (17). Using the BLAST search program we compared the mouse SPL cDNA sequence against the GenbankTM EST data base and identified two homologous human EST sequences. Using this EST sequence information and a PCR cloning strategy as described under “Experimental Procedures,” we cloned human SPL (hSPL) cDNA from a human cDNA library. The coding region of hSPL was then re-cloned by RT-PCR from normal human diploid fibroblast RNA, and resequenced on both strands. This full-length open reading frame of hSPL cDNA contains 1707 nucleotides (GenBankTM accession number AF144638) encoding 568 amino acids with a predicted molecular mass of 63,482 Da. This sequence is consistent with that recently published by others (27). To confirm that the cloned sequence encoded human SPL, it was expressed in the *S. cerevisiae* Δdpl1 mutant strain, which is devoid of SPL enzyme activity and is especially sensitive to the toxic effects of exogenous D-erythro-sphingosine. Expression of hSPL in this background using the pYES2 yeast expression vector (Invitrogen) resulted in correction of sphingosine hypersensitivity and restoration of SPL enzyme activity (data not shown).

Overexpression of Recombinant hSPL in HEK293 Cells—HEK293 cells were transiently transfected with hSPL using the pcDNA3.0 expression system. Western blotting with an antibody recognizing mouse and human SPL demonstrated a large increase in hSPL protein in transiently transfected HEK293 cells. The endogenous SPL protein, however, was not detectable under these immunoblotting conditions (Fig. 1). As shown in Table II, the majority of both the endogenous and recombinant hSPL activity resides in the membrane fraction. Furthermore, SPL activity in the membrane fraction of extracts from hSPL-transfected HEK293 cells was more than 100-fold higher than vector transfected cells, indicating that the recombinant protein is active. A fusion protein constructed to contain hSPL and GFP, pc-hSPL-GFP, localized to the ER in HEK293 cells (Fig. 2) and demonstrated similar activity to recombinant hSPL, as shown in Fig. 3.

Stable Overexpression of hSPL-GFP Leads to Diminished Viability under Stress Conditions—SPL has been shown to affect metazoan organ development and mammalian cell differentiation. To determine whether human SPL expression directly affects cell fate decisions, cell lines stably expressing the hSPL-GFP fusion protein were generated. In all experiments, pooled stable transfecants were employed, to avoid potential mutations or artifacts associated with the selection and propagation of individual clones from single transfected cells. The hSPL-GFP-overexpressing cells demonstrated a 19-fold increase in SPL activity compared with cells stably transfected with a GFP control construct (Fig. 3). Under normal growth conditions, cells overexpressing hSPL-GFP appeared morphologically similar to the control cells; growth characteristics, as determined by cell number and MTT assay, were indistinguishable (Fig. 4). However, when deprived of serum, the two groups behaved differently. Whereas the control cells exhibited a leveling off of the growth curve, the hSPL-GFP-expressing cells demonstrated a marked reduction in cell number.
conditions. As shown in Fig. 6, mass spectrometry in HEK293 cells grown under standard myelin, and higher order sphingolipids were quantified using and unphosphorylated long-chain bases, ceramides, sphingosine overexpression. Toward that end, phosphorylated whether the cellular sphingolipid profile is affected by contin-

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activity is required to promote apoptosis (Fig. 5). However, transient and stable expression of pc-hSPL(K353L)-GFP expressing site was generated by site-specific mutagenesis of the—a predicted protein kinase C phospho-

acid stretch (human SPL amino acids 343–366). Contained within this region are a predicted protein kinase C phosphor-
ylation site (amino acids 351–353) and a predicted PLP binding site (Ly853), based on comparison to the sequences of other PLP-dependent enzymes. A mutant hSPL-GFP with a single (K353L) amino acid substitution at the predicted cofactor-bind-
ing site was generated by site-specific mutagenesis of the hSPL-GFP protein. Like the wild type hSPL-GFP, mutant hSPL-GFP is highly expressed and localizes properly to the ER, as determined by fluorescence microscopy (data not shown). However, transient and stable expression of pc-hSPL(K353L)-GFP indicated that the mutant protein is devoid of significant SPL activity (Fig. 3). When stable hSPL(K353L)-GFP expressing cells were evaluated under serum deprivation conditions, they responded similarly to control cells, indicating that SPL activity is required to promote apoptosis (Fig. 5).

Baseline S1P and Sphingosine Levels Are Diminished in SPL-overexpressing Cells—We were interested in determining whether the cellular sphingolipid profile is affected by contin-

uous SPL overexpression. Toward that end, phosphorylated and unphosphorylated long-chain bases, ceramides, sphingo-
myelin, and higher order sphingolipids were quantified using mass spectrometry in HEK293 cells grown under standard conditions. As shown in Fig. 6 (A and B), C16 ceramide, S1P, and sphingosine levels were substantially diminished under baseline conditions in cells overexpressing wild type but not mutant SPL, whereas the content of most other sphingolipids was not appreciably changed.

Accumulation of Long- and Very Long-chain Ceramides Occurs in SPL-overexpressing Cells under Stress Conditions—To determine the effects of SPL overexpression on sphingolipid mass and subspecies, cellular sphingolipids were analyzed by mass spectrometry. Because serum contains sphingolipids (including S1P), these analyses were conducted in serum-free medium. Under these conditions, the amounts of S1P and sphingosine were not diminished in the cells stably overexpressing SPL (Fig. 7A), and some subspecies of ceramide (C16:0, C18:0, C24:0, and C26:0 ceramides) were significantly elevated. In most cases, this difference was evident early and was sustained throughout the time course. In contrast, the amounts of sphingomyelin and other (glyco)sphingolipids (data not shown) were not significantly affected by SPL overexpression. The finding of increased amounts of ceramide in SPL-overexpressing cells compared with control cells under these conditions was unexpected and suggested that a direct substrate availability relationship between sphingosine and ceramide does not exist when SPL is overexpressed.

Changes in Ceramide Composition Occur in SPL-overexpressing Cells—Backbone composition changes were evident when the subspecies distributions at 48 h are compared as “donut” diagrams (Fig. 7B) (similar trends were seen at other time points, data not shown). These show that hSPL-overexpressing cells have proportionately less C16:0-dihydroceramide (16:0DH) (as well as less C16:0-ceramide overall) and a larger fraction of the 24:0 fatty acid species versus 24:1. The latter appears not to be due to SPL activity, because it is also seen in the cells with mutant (K→L) hSPL, suggesting that SPL may exert some effects on cells through properties that are independent of its catalytic function. The ceramide backbones of glucosylceramide also displayed the shift in 24-carbon ceramides in wild type and mutant hSPL-overexpressing cells (Fig. 7B), and the hSPL-transfected cells showed a somewhat lower fraction of the backbones as C16 species. These results show that overexpression of SPL leads to shifts in ceramide composition that include changes in backbone composition of the ceramides, as well as some shifts in fatty acid profile. Although these changes are subtle, they demonstrate that transfection with hSPL alters both the total amount and structure of ceramides and that, in some cases such the C24 species, these changes may occur independent of catalytic function.
FIG. 7. Sphingolipid profile under stress conditions in control cells and cells overexpressing wild type or mutant human SPL. In A (includes panels A–H): HEK293 cells stably expressing either empty vector (hatched bars), pc-hSPL-GFP (solid bars), or the mutant pc-hSPL(K353L)-GFP (dotted bars) were serum-deprived as described in Fig. 5 and analyzed for sphingolipid content by mass spectrometry as described under "Experimental Procedures." Data are presented as averages of triplicate measurements. All measurements are in picomoles per 1 million cells. Sph, sphingosine; S1P, sphingosine 1-phosphate; SM, sphingomyelin; C16 Cer, C16 ceramide; Total Cer, total ceramide; C18 Cer, C18 ceramide; C24 Cer, C24 ceramide; C26 Cer, C26 ceramide. Shown are unsaturated C24 ceramide and C26 ceramide levels; comparison of the combined C24:0 plus C24:1 (and similarly C26:0 plus C26:1) ceramides yielded similar results (data not shown). In B: fatty acid and dihydro-
The de Novo Synthesis of Sphingolipids Is Increased in Cells Overexpressing hSPL—We were interested in the reason for ceramide elevation in cells overexpressing SPL. We considered that long-term S1P depletion might not be well tolerated by the cell and could induce a cellular response to increase sphingolipid synthesis. In fact, when sphingolipid biosynthesis was measured by the incorporation of L-[14C]serine into new sphingolipids, a 32\% increase in incorporation into sphingomyelin and a 47\% increase in incorporation into total sphingolipids were observed in cells overexpressing wild type but not mutant SPL. These findings suggest that sphingolipid biosynthesis may be enhanced in response to expression of an enzymatically active SPL. To investigate whether sphingolipid biosynthesis was required for SPL-induced apoptosis, de novo biosynthesis was blocked by incubating cells with ISP-1, which inhibits serine palmitoyltransferase. Although treatment of SPL-overexpressing cells with 5 nM ISP-1 led to a 10–20\% reduction in stress-induced apoptosis (data not shown), ISP-1-induced apoptosis in control cells at doses greater than 5 nM.

SPL-induced Apoptosis Is Reversed by Addition of Exogenous S1P—S1P has been shown to afford protection against apoptosis induced by ceramide and other noxious stimuli (31–34). To assess whether SPL-induced apoptosis could be abrogated by S1P, we incubated cells with increasing amounts of exogenous S1P after introduction into serum-free medium. As shown in Fig. 8 (A and B), SPL-induced apoptosis was reversed by addition of S1P in a dose-dependent manner.

Products of the SPL Reaction Do Not Contribute to SPL-induced Apoptosis in HEK293 Cells—To determine whether the effects of SPL on promoting apoptosis might be due to the increased formation of the products of the reaction catalyzed by this enzyme, cells stably expressing a vector control plasmid were incubated with ethanolamine phosphate, hexadecenal, or hexadecanal, the products of the lyase-catalyzed cleavage of S1P or DHSLP, respectively. As shown in Fig. 8A, incubation of cells with doses of up to 50 \% of these three molecules for 48 h did not elicit an apoptotic response, as measured by caspase-3 activity.

Enhanced Apoptosis in Cells Overexpressing SPL Requires the Generation of Ceramide—To determine whether the elevation in long- and very long-chain ceramides contributed to the enhanced apoptosis exhibited by cells overexpressing SPL, cells were treated with the ceramide synthase inhibitor fumonisins.

**Fig. 7—continued**

![Graphs showing ceramide distribution](image)
Importantly, fumonisin B1 (Fig. 9) treatment of cells overexpressing SPL reduced stress-induced apoptosis to the level of stressed vector-transfected control cells. This suggests that the increase in apoptosis observed in this cell line requires the generation of ceramide.

**Cytochrome c Release in Cells Overexpressing Wild Type or Mutant SPL Proteins**—To correlate ceramide elevation with earlier events in the apoptotic pathway, we evaluated the release of cytochrome c into the cytosol of cells expressing wild type or mutant SPL during serum deprivation. As shown in Fig. 10, early (by 12 h) and sustained (through 48 h) cytochrome c release into the cytosol was observed in cells overexpressing wild type or mutant human SPL. In contrast, wild type cells did not release appreciable amounts of cytochrome c until 48 h. Interestingly, cytochrome c was observed transiently in the cytosol of cells overexpressing mutant SPL. Because expression of mutant SPL did not induce either caspase activation or ceramide elevation, these results suggest that ceramide elevation may contribute to the increased apoptosis in SPL-overexpressing cells, but that cytochrome c release alone is either not sufficient or must be sustained to promote apoptosis.

**DISCUSSION**

The fundamental role of S1P in the regulation of cell growth, survival, differentiation, and movement and its contribution to the process of angiogenesis are becoming increasingly evident. The production of S1P by SPHK, which is activated by numer-
ous agonists and through different molecular mechanisms, has garnered considerable attention, even placing it in the limelight as a potential therapeutic target in the treatment of cancer (35, 36). Some attention has also been given to phosphatases that dephosphorylate S1P (S1PP) (5, 37); however, SPL has been generally regarded as having a “housekeeper” function despite being the only enzyme that terminally degrades S1P. This view is changing, because recent studies in genetically tractable metazoan models and in mammalian cells suggest that SPL may play a more substantial role in biology than previously recognized, as a mediator of differentiation and animal development (12–15). Thus, it is not surprising to find that, in addition to SPHK and S1PP, SPL can regulate human cell fate decisions.

SPL expression not only influences the metabolism of both S1P and ceramide but also induces cell death under certain conditions. Our observations that caspase activation in SPL-overexpressing cells is inhibited by supplying cells with exogenous S1P or by blocking ceramide generation suggest that both these bioactive lipids affect apoptosis induced by SPL. The depletion of S1P by SPL and, conversely, the addition of exogenous S1P to cells depleted of this lipid might affect cell survival through either extracellular or intracellular mechanisms. To gain insight into how SPL might enhance apoptosis in serum-deprived cells, we explored the release of cytochrome c from the outer mitochondrial membrane into the cytosol, an important molecular event upstream of caspase-3 activation in the apoptotic pathway. The long- and very long-chain ceramide species that accumulate in stressed SPL-overexpressing cells have been shown recently to contribute coordinately to both induction and amplification of the apoptotic response (38). Therefore, ceramide accumulation and/or S1P depletion in cells overexpressing catalytically active SPL might contribute to the sustained cytochrome c release necessary for apoptosis to proceed. Our observation that overexpression of a catalytically inactive SPL mutant leads to a transient release of cytochrome c (although caspase 3 activation is not enhanced in this cell line) raises the intriguing possibility that non-catalytic functions of SPL might exist.

It was somewhat surprising that ceramide amounts were not reduced by SPL overexpression, because elevated lyase activity might be predicted to divert cellular sphingoid bases toward degradation rather than acylation. This might be due to an increase in de novo sphingolipid synthesis, an explanation consistent with our labeling study and with studies by van Echten-Deckert et al. (24, 39) that have suggested that phosphorylated long-chain bases may reduce serine palmitoyltransferase activity and de novo sphingolipid biosynthesis. It was interesting also that cells overexpressing SPL differ in ceramide composition. They contain lower proportions of dihydroceramides, which might indicate that additional enzymes of this pathway (e.g. the desaturases that convert dihydroceramides to ceramides) are also induced. The other change in ceramide composition (a shift in the portions of C24:0 versus C24:1 fatty acids) was not due to SPL activity, because the shift was seen in cells overexpressing the mutant hSPL, raising the possibility that the lyase polypeptide may influence the types of ceramides that are made or turned over. Although we do not fully understand why these changes in ceramide composition have occurred, they may indicate a higher order regulation of this pathway (for example, perhaps through interaction of some of the proteins in a macromolecular complex).

These findings are important on several levels. First, SPL expression regulates not only intracellular S1P and sphingosine but also appears to increase ceramide generation under stress conditions. This observation underscores the fact that sphingolipid metabolism is a highly complex network and that manipulation of a single enzyme in this pathway can give rise to distant and sometimes paradoxical effects, through feedback inhibition and other mechanisms of regulation. Furthermore, recent studies have shown that SPL is involved in regulation of sterol regulatory element binding protein processing through its influence on phospholipid metabolism (40). Thus, SPL exerts global effects on lipid metabolism in eukaryotic cells. Second, the ability of SPL to regulate cell fate decisions may also indicate its role in metazoan development and is consistent with observations of dysregulated developmental apoptosis in Drosophila SPL mutants (13). Third, the ability of SPL to affect cell fate leads to the prediction that loss of SPL expression could contribute to cancer cell survival. In fact, deletions of the 10q21 chromosomal region where the human SPL locus resides have been demonstrated in a variety of cancers, including leukemia and solid tumors of thyroid, colon, and brain (41–53). Thus, mutations in the SPL locus of cancer cells could potentially have diagnostic and predictive value. Lastly, our findings indicate that SPL may be a unique target for pharmacological agonists in the treatment of cancer. It has been suggested that the effective deployment of ceramide-induced apoptosis as a cancer therapy must take into account the fact that increased ceramide production eventually also gives rise to increased S1P production, limiting the desired effect on the cancer cell (54). The generation of ceramide analogs that do not give rise to S1P or the simultaneous generation of ceramide and inhibition of SPHK are schemes proposed to circumvent this problem. However, SPL activation causes both ceramide accumulation and S1P depletion, efficiently accomplishing this goal in one step. The manipulation of SPL for therapeutic purposes may not be far-fetched, because temporal, spatial and differentiation-induced regulation of SPL expression has recently been demonstrated in metazoan models and mammalian cells. The identification of specific factors involved in SPL gene regulation should facilitate our ability to manipulate SPL for therapeutic purposes in the future.

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