Vasoinhibin Gene Transfer by Adenoassociated Virus Type 2 Protects against VEGF- and Diabetes-Induced Retinal Vasopermeability

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PURPOSE. Specific proteolytic cleavages of the hormone prolactin (PRL) generate vasoinhibins, a family of peptides (including 16kDa PRL) that are able to inhibit the pathologic increase in retinal vasopermeability (RVP) associated with diabetes. Here the authors tested the ability of an adenoassociated virus type 2 (AAV2) vasoinhibin vector to inhibit vascular endothelial growth factor (VEGF)– and diabetes-induced RVP.

METHODS. AAV2 vectors encoding vasoinhibin, PRL, or soluble VEGF receptor 1 (soluble FMS-like tyrosine kinase-1 [sFlt-1]) were injected intravitreally into the eyes of rats. Four weeks later, either VEGF was injected intravitreally or diabetes was induced with streptozotocin. Tracer accumulation was evaluated as an index of RVP using fluorescein angiography or the Evans blue dye method. RT-PCR verified transgene expression in the retina, and the intravitreal injection of an AAV2 vector encoding green fluorescent protein revealed transduced cells in the retinal ganglion cell layer. In addition, Western blot analysis of AAV2-transduced HEK293 cells confirmed the expression and secretion of the vector-encoded proteins.

RESULTS. The AAV2-vasoinhibin vector prevented the increase in tracer accumulation that occurs 24 hours after the intravitreal injection of VEGF. Diabetes induced a significant increase in tracer accumulation compared with nondiabetic controls. This increase was blocked by the AAV2-vasoinhibin vector and reduced by the AAV2-sFlt-1 vector. The AAV2-PRL vector had no effect.

CONCLUSIONS. These results show that an AAV2-vasoinhibin vector prevents pathologic RVP and suggest it could have therapeutic value in patients with diabetic retinopathy. (Invest Ophtalmol Vis Sci. 2011;52:8944–8950) DOI:10.1167/iovs.11-8190

D iabetic retinopathy (DR) is the primary cause of irreversible blindness and visual impairment in working-age adults in developed countries.1 Blood-retinal barrier breakdown and enhanced retinal vasopermeability (RVP) are early complications of DR that contribute to diabetic macular edema (DME) and can lead to the overproliferation of retinal blood vessels in advanced DR.2 The current treatments for DR and DME, which include laser photocoagulation, vitrectomy, and intravitreal pharmacotherapy, are frequently effective, but the first two can treat only the advanced disease and the latter often requires repeated injections, which raise important issues with patient convenience and compliance.2,3 Thus, developing new strategies to prevent both excessive RVP and angiogenic responses remains a major research focus.

Vasoinhibins are natural inhibitors of ocular blood vessels that may play a role in the progression of DR.4–6 They comprise a family of peptides generated by specific proteolytic cleavages of prolactin (PRL) that exert potent antiangiogenic, vasoconstrictive, and antivasopermeability actions.7 Vasoinhibins prevent vascular endothelial growth factor (VEGF)–induced angiogenesis8 and promote apoptosis-mediated vascular regression,9 as observed, for example, in retinopathy of prematurity.10 Moreover, elevation of intraocular vasoinhibins inhibits ischemia-induced retinal angiogenesis11 and prevents excessive RVP in diabetic rats and in rats treated with intravitreal injection of either VEGF or vitreous from patients with DR.5 Notably, patients with DR have reduced levels of vasoinhibins in the circulation,12 and increasing systemic PRL levels in diabetic rats raises the concentration of vasoinhibins in the retina, which in turn reduce RVP.6

Viral vector-mediated delivery of antiangiogenic and antivasopermeability factors offers considerable promise for the treatment of DR.13 The most widely used vectors for ocular gene therapy are based on adenoassociated virus (AAV) because they produce long-term transgene expression in a variety of retinal cell types.14 In this study, we evaluated an AAV type 2 (AAV2)–vasoinhibin vector for the ability to inhibit VEGF- and diabetes-induced vasopermeability in rats after intravitreal injection.

METHODS

Plasmid Constructions and Production of Recombinant AAV2

Human PRL cDNA was amplified by PCR from an existing construct, digested with BamHI and NotI, and cloned into the BamHI and NotI sites of pcDNA3. Human vasoinhibin cDNA (codons 1 to 142 of human PRL) was amplified from a modified construct in which codon 58 had been converted from Cys to Ser. The amplification inserted a stop codon at position 143. The product was cloned into pcDNA3 as

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described for PRL. Full-length cDNAs encoding soluble FMS-like tyrosine kinase-1 (sFlt-1) and green fluorescent protein (GFP) were used. All plasmid constructs were fully sequenced and verified to be free of PCR-induced errors. AAV2 vector genomes were constructed by placing the transgene of interest downstream of a cytomegalovirus (CMV) immediate early promoter and a chimeric CMV/human β-globin intron and upstream of a human β-globin polyadenylation site (Fig. 1A). AAV2 particles were produced by the triple transfection method and purified by polyethylene glycol precipitation followed by cesium chloride density gradient fractionation by a previously described method.15 The purified vectors were formulated in 10 mM Tris-HCl and 180 mM NaCl (pH 7.4) and stored at −80°C before use. Vector preparations were examined by SDS-PAGE for purity and dynamic light scattering for aggregation state. Quantification of vectors was done by real-time PCR using linearized plasmid standards. A 2.1010 vector genomes (vg) of each vector or 2 μL vehicle was injected into the vitreous.

In Vitro Transduction Analysis

HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% penicillin–streptomycin, 1% fungizone, and 10% fetal bovine serum (Gibco, Grand Island, NY). HEK293 cells (9 × 104 in 300 μL medium) were transduced with 1.4 × 1011 vg of AAV2 (multiplicity of infection: 1.5 × 106) encoding each transgene in 24-well plates in the presence of etoposide (Sigma-Aldrich Co., St. Louis, MO), at a final concentration of 2.5 nM. After 16 hours, the cells were washed with PBS and then cultured in DMEM. The cells and conditioned medium were harvested 24 hours later to evaluate the expression and secretion of the transgenes by Western blot.

RT-PCR

Total RNA extracted from retinal lysates was treated with DNase and subjected to RT-PCR and gel electrophoresis as previously described.16 Detection of human PRL and vasoinhibin cDNA was accomplished using primers CTG CCC GAT GCC AGG TGA (sense) and GAA AGT CTT TIT GAT TCA TCT GT (antisense) that generate a 219 basepair (bp) product. These primers correspond to the sequence shared by human PRL and human vasoinhibins and were verified not to amplify rat PRL. The amplification parameters used were 30 seconds at 94°C, 30 seconds at 57°C, and 30 seconds at 72°C for 35 cycles. Oligonucleotide primers used to amplify human sFlt-1 were GAC CTG GAG TTA CGC TCA GGA (sense) and ACT GTG TAC TTT TCA CAC GAC (antisense), with an annealing temperature of 61°C for 30 cycles, and yielded a 159-bp product; for actin, ACA TCA TGA AGT GTG ACG TTG (sense), with an annealing temperature of 61°C for 30 cycles, and generated a 219-bp product.

Western Blot Analysis

HEK293 cells were homogenized in 100 μL of lysis buffer (50 mM Tris, pH 7.4, 0.5% Igepal, 100 mM NaCl, 1 μg/mL aprotinin, 0.5 mM PMSF) and centrifuged to remove cellular debris (12,000 g for 10 minutes at 4°C). Cell lysates containing 20 μg protein or 15 μL conditioned medium were resolved by 15% SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane. Blots were incubated overnight with a 1:500 dilution of either an anti-human PRL antiserum (HC-1)10 or an anti–sFlt-1 monoclonal antibody (ab9540; Abcam Inc., Cambridge, MA). Detection was performed using an alkaline phosphatase–coupled secondary antibody and a colorimetric detection kit (Bio-Rad Laboratories, Hercules, CA).

Deglycosylation Procedure

Deglycosylation of glycoproteins in media conditioned by HEK293 cells was carried out using N-glycosidase F (New England BioLabs Inc., Ipswich, MA) following the manufacturer’s instructions. Briefly, 5 μL medium was incubated with 5 μL denaturing buffer (5% SDS, 0.4 M dithiothreitol) at 100°C for 10 minutes. Subsequently, 4 μL H2O, 2 μL 0.5 M sodium phosphate buffer (pH 7.5), 2 μL 10% NP-40, and 1 μL (500 U) N-glycosidase F were added and the mixture was incubated at 37°C for 1 hour. A 10 μL sample of the final reaction mixture was analyzed by SDS-PAGE/Western blot.

Animals

Male Wistar rats (250 to 300 g) were maintained and treated in accordance with the guidelines in the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The Bioethics Committee of the Institute of Neurobiology of the National University of Mexico (UNAM) approved all animal experiments. Animals were anesthetized intraperitoneally (IP) with 70% ketamine and 30% xylazine (1 μL/g body weight), and AAV2 vector or vehicle was injected into the vitreous as described.19 A month later, some animals were anesthetized in a CO2-saturated inhalation chamber and euthanized by decapitation, and their retinas were dissected to evaluate the expression of the vector-encoded proteins by RT-PCR or histochemistry. Other animals, injected a month earlier with the AAV2-vasoinhibin vector or vehicle, received an intravitreal injection of 300 ng VEGF (rhVEGF165; a gift from Genentech, South San Francisco, CA) in 2 μL PBS. Finally, other rats were injected with the various AAV2 vectors or vehicle and, a month later, were fasted overnight, then treated with a single IP dose of streptozotocin (60 mg/kg) (Sigma-Aldrich Co.). Forty-eight hours later, blood glucose levels were measured, and only the rats with a blood glucose concentration > 250 mg/dL were considered diabetic.17 One month after
inducing diabetes, animals were euthanized to evaluate RVP by the Evans blue dye method.

**Retinal Localization of AAV2-GFP Transduction**

Green fluorescent protein (GFP) fluorescence was directly detected in retinas fixed in 4% paraformaldehyde for 30 minutes, flat-mounted, and coverslipped with mounting media for fluorescence (Vectorshield; Vector Laboratories, Burlingame, CA).18 GFP in retinal cell bodies and processes was also visualized by immunohistochemistry. Retinas fixed in 4% paraformaldehyde for 30 minutes were placed sequentially in PBS containing 2% and 30% sucrose (24 hours each). Cryosections (12 µm) were blocked in PBS containing 1% bovine serum albumin, 1% normal goat serum, and 0.1% Triton X-100 for 1 hour at room temperature and labeled overnight at 4°C with a 1:100 dilution of anti-GFP polyclonal antibody (ab6556; Abcam Inc.). After incubation with the primary antibody, samples were rinsed three times in PBS and labeled for 2 hours with a 1:1000 dilution of a fluorescent dye–conjugated secondary antibody (Alexa Fluor 546; Invitrogen, Carlsbad, CA) goat anti-rabbit IgG (Molecular Probes, Eugene, OR). Retinal flat-mounts and sections were examined with a confocal laser scanning microscope (LSM 510; Carl Zeiss Microimaging GmbH, Göttingen, Germany). Photographs were taken and images prepared using a digital photo editing program (Adobe Photoshop CS3 Extended Version 10.0.1; Adobe, San Jose, CA).

**Fluorescein Angiography**

Twenty-four hours after the intravitreal injection of VEGF, the animals were injected (intrajugularly) with 100 mg/kg fluorescein isothiocyanate–labeled dextran (50 mg/mL, FITC-dextran; MW 2 × 10⁶ Da; Sigma-Aldrich Co.). One hour later, the rats were euthanized and their retinas were flat-mounted, fixed for 4 hours in 4% paraformaldehyde at room temperature, washed with PBS, and mounted on glass slides using 50% glycerol in PBS. Retinal flat-mounts were observed and photographed under a fluorescence microscope (Olympus BX60 with a DP70 Olympus camera), and the fluorescence intensity of each image was analyzed using commercial software (Image Pro-Plus Media; Cybernetics, Silver Spring, MD). The area of retinal hemorrhages in the flat-mounted retinas was quantified using a digital scanner (ScanScope; Aperio Technologies, Inc. Vista, CA) and color-deconvolution software (Aperio Technologies).

**Evans Blue Dye Method**

Retinal albumin accumulation was measured using the Evans blue dye technique.19 Briefly, after being anesthetized, the rats were injected (intrajugularly) with Evans blue dye (45 mg/kg; Sigma-Aldrich Co.). Two hours later, blood (1 mL) was drawn from the heart to measure the Evans blue concentration in plasma, and the rats were perfused via the left ventricle at physiologic pressure and heart to measure the Evans blue concentration in plasma, and the retinas were flat-mounted, fixed for 4 hours in 4% paraformaldehyde at room temperature, washed with PBS, and mounted on glass slides using 50% glycerol in PBS. Retinal flat-mounts were observed and photographed under a fluorescence microscope (Olympus BX60 with a DP70 Olympus camera), and the fluorescence intensity of each image was analyzed using commercial software (Image Pro-Plus Media; Cybernetics, Silver Spring, MD). The area of retinal hemorrhages in the flat-mounted retinas was quantified using a digital scanner (ScanScope; Aperio Technologies, Inc. Vista, CA) and color-deconvolution software (Aperio Technologies).

**Statistical Analysis**

Values are expressed as mean ± SEM. The statistical significance of differences between groups was determined by ANOVA followed by the unpaired two-tailed Student’s t-test. Statistical analysis was performed using commercial analytical software (SigmaStat 7.0, Systat Software Inc., San Jose, CA). Differences in means with \( P < 0.05 \) were considered statistically significant.

**RESULTS**

**Verification of Transgene Expression in HEK293 Cells**

AAV2-PRL, -vasoinhibin, and -sFlt-1 vectors were tested in vitro using HEK293 cells to validate their usefulness for the expression and secretion of the transgenes. Western blot analysis detected the expected bands of 23 and 16 kDa for the AAV2-PRL and AAV2-vasoinhibin vectors, respectively, in both cell lysates and conditioned medium (Fig. 1B). Transduction by both vectors also yielded immunoreactive proteins having a slightly higher molecular weight (25 and 18 kDa for the AAV2-PRL and the AAV2-vasoinhibin vectors, respectively) than that predicted for the translation products (Fig. 1B). These larger proteins resulted from N-linked glycosylation of PRL and vasoinhibin because their mobility increased after incubating the conditioned medium with N-glycosidase F such that only 23- and 16-kDa bands were seen (Fig. 1C). The N-linked glycosylation of Asn31 occurs naturally in some PRL molecules,20 but this posttranslational modification does not appear to affect the biological activity of vasoinhibins.11,21 HEK293 cells transduced with the AAV2-sFlt-1 vector contained and secreted substantial amounts of an immunoreactive protein of 82 kDa, the molecular mass of sFlt-1 (Fig. 1D). These results demonstrated that all vectors were able to deliver their transgenes to cells and establish protein expression and secretion.

**Verification and Localization of Retinal Transgene Expression**

Transgene expression in target retinal cells was examined by RT-PCR analysis. Total retinal RNA from eyes injected with vehicle only (control) or with AAV2 was treated with DNase to eliminate genomic DNA contamination. Amplification of DNase-treated RNA samples without reverse transcriptase generated no products for PRL, vasoinhibin, or sFlt-1 (data not shown). A common human PRL cDNA region was amplified in retinas transduced with the AAV2-PRL and AAV2-vasoinhibin vectors, and the cDNA for human sFlt-1 was detected in retinas injected with the AAV2–sFlt-1 vector (Fig. 2A). No PRL message was detected in control eyes or eyes injected with the AAV2 vector encoding sFlt-1, confirming that the primers do not amplify endogenous rat PRL (Fig. 2A). The intravitreal injection of AAV2-GFP revealed transduced cells and their projections within the retinal ganglion cell layer, as indicated by the fluorescence detected directly on flat-mounted retinas (Fig. 2B) or after immunohistochemical analysis of retinal sections (Fig. 2C). No positive fluorescence signal was detected in the vehicle-injected controls (not shown). These results verified the transgene expression of each of the various AAV2 vectors in retinal cells and confirmed previous observations showing that intravitreal delivery of AAV2 vectors primarily transduces retinal ganglion cells.22

**AAV2-Vasoinhibin Vector Reduces VEGF-Induced Hemorrhages and FITC-Dextran Accumulation**

Retinal hemorrhages and FITC-dextran accumulation were examined in AAV2-vasoinhibin–transduced retinas that were challenged with intravitreally injected VEGF, a major vasopermeability factor in DR.21 In these experiments, rats were injected intravitreally with VEGF or with the VEGF vehicle (PBS) I
month after the intravitreal injection of vector vehicle or the AAV2-vasoinhibin vector, and retinas were evaluated 24 hours after VEGF injection. VEGF treatment caused multiple retinal hemorrhagic areas (Fig. 3B) that were absent in control retinas injected with PBS (Fig. 3A) and nearly absent in VEGF-injected eyes transduced with the AAV2-vasoinhibin vector (Fig. 3C). Image analysis showed that the AAV2-vasoinhibin vector reduced VEGF-induced hemorrhagic areas by 95% (Fig. 3D). The effect of the AAV2-vasoinhibin vector was further evaluated by measuring FITC-dextran accumulation. The strong fluorescence outside the vasculature that accompanied VEGF treatment (Fig. 3F) was markedly reduced in retinas transduced by the AAV2-vasoinhibin vector (Fig. 3G), corresponding to 64% inhibition after quantifying the intensity of retinal fluorescence (Fig. 3H). No extravascular fluorescence was detected in PBS-injected controls (Fig. 3E).

AAV2-Vasoinhibin Vector Prevents Diabetes-Induced, Evans Blue-Stained Albumin Accumulation

In these experiments, 1 month after intravitreal delivery of the vehicle or the various AAV2 vectors, the rats were made diabetic with streptozotocin, and a month later albumin accumulation was quantified by the Evans blue method. Diabetes induced a statistically significant increase in Evans blue-stained albumin accumulation in control, vehicle-injected rats (Fig. 4). Treatment with the AAV2-vasoinhibin vector prevented this effect, whereas using the AAV2-sFlt-1 vector reduced, but did not eliminate, diabetes-induced Evans blue-stained albumin accumulation. The AAV2-PRL vector had no effect and none of the vectors modified Evans blue-stained albumin accumulation in nondiabetic rats.

**FIGURE 2.** Verification and localization of AAV2-mediated transgene expression in the retina. (A) RT-PCR analysis of vasoinhibins (Vi), sFlt-1, and PRL cDNA in retinas obtained from rats 1 month after being injected intravitreally with vehicle (C) or AAV2-Vi, AAV2-sFlt-1, or AAV2-PRL vectors. The sizes of RT-PCR products are given in base pairs (bp). Amplification of β-actin was used as an internal standard. (B, C) Confocal microscope visualization of direct GFP fluorescence in the ganglion cell layer of a flat-mounted retinal preparation (B) or of GFP immunoreactivity in a retinal section (C), obtained from rats 1 month after being injected intravitreally with the AAV2-GFP vector. Bars indicate 50 μm. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, internal nuclear layer; ONL, outer nuclear layer.

**FIGURE 3.** The intravitreal injection of AAV2-vasoinhibin vector inhibits VEGF-induced retinal vasopermeability. Age-matched rats were injected intravitreally with PBS (A, E) or VEGF in PBS (B, C, F, G) 1 month after receiving no injection (PBS) (A, E), vector vehicle (VEGF) (B, F), or the AAV2-vasoinhibin vector (VEGF + AAV2-Vi) (C, G). Animals were perfused with FITC-dextran 24 hours after treatment with PBS or VEGF. Representative flat-mounted retinas under light-field (A–C) and fluorescence (E–G) microscopy illustrate the presence of hemorrhages and the accumulation of FITC-dextran into the retinal parenchyma. Quantification of hemorrhagic area (D) and of FITC-dextran fluorescence intensity (H) from three and five flat-mounted retinas, respectively, is shown. *P < 0.05 vs. PBS- or VEGF + AAV2-Vi-injected rats.
pable of treating a broader segment of the patient population. They are natural inhibitors of vasopermeability in clinical trials as intraocular therapeutic agents in DR rats. Here we show that the intravitreal injection of an AAV2-vasoinhibin vector reduces VEGF-induced retinal hemorrhages (Fig. 3), it is very likely that the inhibition of tracer accumulation observed in retinas transduced by the AAV2-vasoinhibin vector is largely due to a reduction of VEGF- and diabetes-induced RVP.

In this regard, the action of the AAV2-vasoinhibin vector on VEGF-induced RVP is similar to the effect of the injected vasoinhibin protein. This inhibitory effect is likely to be a property of the vasoinhibin molecule independent of the delivery method. Vasoinhibins inhibit VEGF action by blocking VEGF-activated endothelial nitric oxide synthetase (eNOS), which is one of the signaling pathways by which VEGF stimulates vasopermeability. Vasoinhibins act directly on endothelial cells through a still-unidentified saturable high-affinity binding site distinct from the PRL receptor, and they block VEGF-induced eNOS activation by activating protein phosphatase 2A, which dephosphorylates and inactivates eNOS, and by interfering with Ca2+-calmodulin-dependent activation of eNOS.

VEGF is the predominant mediator of elevated RVP in diabetes; its levels increase in the vitreous of patients with DR and DME and clinical trials using anti-VEGF molecules show reduced retinal permeability and leakage. Accordingly, the demonstration that the AAV2-vasoinhibin vector inhibits VEGF-induced RVP makes it an attractive potential therapeutic tool for the treatment of DR and DME. However, the development of DME depends not only on excessive RVP but also on impaired fluid absorption from the retinal tissue. The water content, retinal thickness, and osmotic swelling of Müller cells should be measured to predict the therapeutic value of AAV2-vasoinhibin vectors against DME. Notably, the pathologic cascade leading to DR and DME involves not only VEGF but also other vasoactive substances such as angiopeptin-2, erythropoietin, bradykinin, basic fibroblast growth factor (bFGF), interleukin-1β, interleukin-6, tumor necrosis factor-α, and stromal-cell–derived factor-1, all of which are elevated in the vitreous of patients with DR and DME and are under intensive investigation as therapeutic targets. It has been demonstrated that vasoinhibins inhibit the increase in RVP induced by the intravitreal injection of vitreous from patients with DR, and that they interfere with the vascular effects and signaling pathways of bradykinin, bFGF, and interleukin-1β. Because vasoinhibins block the vascular actions of several factors, they may offer a broader and more comprehensive approach to suppress excessive RVP in diabetes than agents targeting solely VEGF.

To address this possibility, the effect of the AAV2-vasoinhibin vector on diabetes-induced RVP was compared with that of an AAV2 vector encoding sFlt-1, a natural VEGF inhibitor. sFlt-1 corresponds to the secreted extracellular domain of VEGF receptor 1, and blocks VEGF action by either sequestering VEGF or forming inactive heterodimers with membrane VEGF receptors 1 and 2. Previous studies have shown that sFlt-1 vectors inhibit the breakdown of the blood–retinal barrier, improve vision, and decrease the importance of VEGF as the predominant mediator of RVP in diabetes. In our study, both the AAV2-vasoinhibin and –sFlt-1 vectors inhibited diabetes-induced RVP. Interestingly, the AAV2-vasoinhibin vector completely blocked the diabetes-induced increase in RVP, whereas the AAV2–sFlt-1 vector reduced, but did not eliminate, this increase. The greater inhibition by the AAV2-vasoinhibin vector is consistent with vasoinhibins blocking the action of other vasopermeability factors in addition to VEGF.

AAV vectors are attractive tools for therapeutic gene transfer to the retina and are being increasingly used to target retinal disorders in animal studies. Recently, they have been successfully used to treat genetic retinal diseases in the clinic. Our results show that the intravitreal delivery of vasoinhibins via an AAV2 vector can confer protection against excessive
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RVP associated with diabetes, and suggest that these vectors may be effective therapeutic tools in DR, DME, and other vasoproliferative retinopathies. Preclinical studies of the efficacy, safety, expression, and biodistribution of the AAV2-vasoinhibin vector over longer time intervals are required to confirm this therapeutic potential. Moreover, AAV2-vasoinhibin vectors may be of benefit in other vascular-related disorders. Elevated production of vasoinhibins blocks angiogenesis, growth, leukocyte infiltration, and metastasis in experimental tumors.\(^{21,54–56}\) The value of this vector to treat these types of conditions should also be assessed.

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