Biosynthesis, Characterization, and Efficacy in Retinal Degenerative Diseases of Lens Epithelium-derived Growth Factor Fragment (LEDGF<sub>1–326</sub>), a Novel Therapeutic Protein*  

Received for publication, December 2, 2012, and in revised form, May 1, 2013  
Published, JBC Papers in Press, May 2, 2013, DOI 10.1074/jbc.M112.441618  

Rinku Baid<sup>1</sup>, Arun K. Upadhyay<sup>1</sup>, Toshimichi Shinohara<sup>2</sup>, and Uday B. Kompella<sup>†¶1</sup>  

From the <sup>‡</sup>Skaggs School of Pharmacy and Pharmaceutical Sciences and the <sup>¶</sup>Department of Ophthalmology, University of Colorado Anschutz Medical Campus, Aurora, Colorado 80045 and the <sup>§</sup>Department of Ophthalmology, University of Nebraska Medical Center, Omaha, Nebraska 68198

**Background:** There is no FDA-approved drug therapy for blinding retinal degenerative diseases such as retinitis pigmentosa and dry age-related macular degeneration.

**Results:** LEDGF<sub>1–326</sub> increased viability of retinal cells subjected to mutant rhodopsin aggregation stress and reduced loss of photoreceptors, thereby improving retinal function.

**Conclusion:** LEDGF<sub>1–326</sub> reduced retinal degeneration.

**Significance:** LEDGF<sub>1–326</sub> is a new protein therapeutic to treat vision loss.

For vision-threatening retinitis pigmentosa and dry age-related macular degeneration, there are no United States Food and Drug Administration (FDA)-approved treatments. We identified, biosynthesized, purified, and characterized lens epithelium-derived growth factor fragment (LEDGF<sub>1–326</sub>) as a novel protein therapeutic. LEDGF<sub>1–326</sub> was produced at about 20 mg/liter of culture when expressed in the *Escherichia coli* system, with about 95% purity and aggregate-free homogeneous population with a mean hydrodynamic diameter of 9 ± 1 nm. The free energy of unfolding of LEDGF<sub>1–326</sub> was 3.3 /H9262 for scotopic electroretinogram and from 10.9 /H9262 um-derived growth factor fragment (LEDGF<sub>1–326</sub>) as a novel biosynthesized, purified, and characterized lens epithelium-derived growth factor fragment (LEDGF<sub>1–326</sub>) as a novel therapeutic protein.*  

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Eight weeks after single intravitreal injection in Royal College of Surgeons (RCS) rats, LEDGF<sub>1–326</sub> increased the b-wave amplitude significantly from 9.4 ± 4.6 to 57.6 ± 8.8 µV for scotopic electroretinogram and from 10.9 ± 5.6 to 45.8 ± 15.2 µV for photopic electroretinogram. LEDGF<sub>1–326</sub> significantly increased the retinal outer nuclear layer thickness from 6.34 ± 1.6 to 11.7 ± 0.7 µm. LEDGF<sub>1–326</sub> is a potential new therapeutic agent for treating retinal degenerative diseases.

Retinal degenerations including retinitis pigmentosa (RP)<sup>2</sup> and dry age-related macular degeneration (dry AMD) are a group of heterogeneous, chronic, progressive, and blinding diseases (1). RP is a genetically inherited disease (2) caused by more than 50 different gene mutations (3), including P23H mutant rhodopsin (4), which result in endoplasmic stress and protein aggregation (5). Approximately 1.5 million people worldwide currently suffer from RP (6). For dry AMD, although the cellular mechanisms are not fully understood, it has been associated with chronic oxidative stress and inflammation (7), leading to deposition of tiny protein and fat-containing toxic “drusen” and loss of photoreceptors and ultimately irreversible blindness (8–10). Currently, about 1.75 million individuals are suffering from AMD in the United States, and by 2020, this number is expected to reach 3 million (11). Dry AMD accounts for 90% of AMD cases (12).

Despite the widespread prevalence of RP and dry AMD, there is no treatment approved by the United States Food and Drug Administration (FDA) (13, 14). The primary preventive measure to slow the progression of RP remains daily ingestion of up to 15,000 IU of vitamin A palmitate (15–18). Other treatment options include a recently approved Angus<sup>®</sup> II retinal prostheses system for RP (19). For dry AMD, a nutrition supplement (Ocu-vite PreserVision<sup>®</sup>) containing β-carotene and zinc is recommended (20). However, high doses of β-carotene can increase the risk of cancer in smokers (21). Currently, there is no FDA-approved drug therapy for dry AMD or RP.

Lens epithelium-derived growth factor (LEDGF) is a transcription factor in human lens epithelial cells (22). Glutathione S-transferase-tagged LEDGF (GST–LEDGF) protein promotes retinal cell growth under serum starvation and thermal stress and protects the photoreceptors in rats receiving light damage and in Royal College of Surgeons (RCS) rats (23–25). However, full-length LEDGF has two major drawbacks. First, full-length LEDGF has been associated with human immunodeficiency virus (HIV-1)-mediated diseases (26). The N terminus (amino
acids 1–324) of LEDGF acts as a chromatin tether and binds LEDGF to DNA, whereas the C terminus (amino acids 325–530) binds to the HIV-1 integrase and promotes transcription and replication of HIV (27, 28). In vivo administration of C-terminal LEDGF325–530 potently inhibited HIV replication by competing with endogenous LEDGF for binding to HIV integrase (26). Second, purification of full-length LEDGF in bulk quantities has never been reported, possibly due to inherent instability. Because GST-LEDGF protein degrades to smaller fragments during its biosynthesis and purification (29), attempts were made to stabilize GST-LEDGF with heparin. In the presence of 71 mg/ml heparin in the culture medium, the full-size GST-LEDGF in purified protein fraction increased to 56% from 32% in controls. In a previous study, we identified that gene delivery of LEDGF1–326, an N-terminal fragment of LEDGF, can reduce P23H rhodopsin aggregation and promote cell survival (30). Due to the absence of the C-terminal domain of LEDGF in LEDGF1–326, the possibility of HIV-1 integration is expected to be minimized with LEDGF1–326.

In this study, we cloned, synthesized, and purified LEDGF1–326 protein for the first time in stable and non-degraded form and scaled up its production to large quantities. Further, we established the biophysical properties of LEDGF1–326 and assessed its ability to reduce in vitro P23H rhodopsin aggregation-mediated retinal cell damage. Finally, the ability of LEDGF1–326 to reduce retinal degeneration in the RCS rat model for dry AMD and RP was determined.

**EXPERIMENTAL PROCEDURES**

**Preparations of LEDGF1–326 DNA Construct**

Gene encoding LEDGF1–326 protein was designed to clone into pET-28a (+) vector (Novagen, Madison, WI). Briefly, LEDGF1–326 gene was amplified from the pEGFP-LEDGF plasmid using the forward primer 5'-AGTAGTGATCCATGACTCGCGATTTCAAAC-3' and reverse primer 5'-AAT-AATAAGCTTCACTGCTAGTTTCCATTGTC-3'. Thereafter, the purified LEDGF1–326 gene and pET-28a (+) vector were digested using HindIII and BamHI restriction enzymes and ligated overnight at 4 °C using T4 DNA ligase. Competent Escherichia coli DH5α cells were transformed with the ligation product as per the manufacturer's protocol. Insertion of LEDGF1–326 in pET-28a (+) vector (named as pLEDGF1–326) was confirmed by PCR screening, restriction digest, and finally by DNA sequencing. Purity and the size of the recombinant DNA were analyzed using 2% agarose gel. The colony showing positive PCR signal and correct sequence was cultured further, and the bacterial glycerol stock was made and stored at −80 °C for all future use.

**Cloning and Expression of LEDGF1–326**

For protein biosynthesis, pLEDGF1–326 plasmid was isolated from E. coli DH5α colony and transformed in E. coli BL21 (DE3) as per the manufacturer's protocol. LEDGF1–326 was expressed and purified in shake flask culture under the controlled addition of isopropyl-β-D-thiogalactoside. Cells were harvested and lysed, and crude LEDGF1–326 was collected as soluble cell lysate.

**Purification of LEDGF1–326 Using Fast Protein Liquid Chromatography (FPLC)**

LEDGF1–326 was solely expressed in soluble fraction as determined by SDS-PAGE. LEDGF1–326 was purified using two-step fast protein liquid chromatography (FPLC), first based on charge (cation exchange) and then based on size (gel filtration). Briefly, cation exchange SP Sepharose beads were packed in XK 16/20 column, and the soluble cell lysate was loaded. The column was washed with buffer A (25 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM PMSF, and 5% sucrose). The nonspecifically and loosely bound impurities were eluted using a gradient of NaCl. Fractions containing high amount of protein were pooled together, dialyzed using dialysis buffer (25 mM Tris, pH 7.0, and 0.1% sucrose), and lyophilized for 48 h. The lyophilized protein was resolubilized in 2 ml of deionized water and further purified using prepackaged S-200 gel filtration column in buffer B (25 mM Tris-HCl, pH 7.0, and 100 mM NaCl). Fractions containing the pure LEDGF1–326 were pooled together and dialyzed in the dialysis buffer (25 mM Tris-HCl, pH 7.0, and 0.1% sucrose) for 48 h at 4 °C with three buffer exchanges. The dialyzed LEDGF1–326 was lyophilized, aliquoted, and stored at −80 °C for all future purposes.

**Bioinformatics Analysis**

LEDGF1–326 amino acid sequence was submitted to ExPASy bioinformatics resource portal, and the molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, and estimated half-life of LEDGF1–326 were computed. Post-translational modification of LEDGF1–326 was predicted using NetNGlyc 1.0, NetOGlyc 3.1, and NetCGlyc 1.0 server.

**Physical Characterization**

*Size Exclusion Chromatography (SEC) HPLC—* The lyophilized protein was dissolved in deionized water to final concentration of 500 µg/ml and filtered through 0.22-µm polyvinylidene difluoride (PVDF) filters. The protein was size-separated in an Agilent Bio SEC-3 column using 25 mM Tris buffer containing 1 mM CaCl2, pH 7.0, at 25 °C with a flow rate of 1 ml/min. Retention time was averaged from four chromatograms.

*Matrix-assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry—* Protein homogeneity and molecular weight were confirmed by 4800 Plus MALDI TOF/TOF™ (AB Sciex, Framingham, MA) mass spectrometry. The protein sample was dissolved into a solution of standard MALDI matrix sinapinic acid, spotted, and dried onto the metal target plate. Data were collected as total ion current from 1000 laser shots of 5900 intensity.

*Dynamic Light Scattering (DLS)—* The homogeneity and size of the LEDGF1–326 protein was analyzed using Nano ZS (Malvern, Westborough, MA). Briefly, lyophilized protein sample was dissolved in deionized water to get final protein concentration of 1 mg/ml. The hydrodynamic diameter of LEDGF1–326 using the dynamic light scattering technique with data collection at 173° backscatter angle was obtained. Measurement was an average of 13 scans. Data represents mean of n = 8.
**Biophysical Characterization**—For biophysical characterization, the protein was extensively dialyzed in 25 mM phosphate buffer, pH 7, to remove Tris-HCl and sucrose and filtered through a 0.22-μm PVDF syringe filter. Spectra obtained were analyzed using either Origin® 8.5 (OriginLab Corp., Northampton, MA) or SigmaPlot 11.0 (SYSTAT Software, Inc., Chicago, IL). The data were fitted using Equations 1 and 2, defined by Scholtz et al. (31) as below to determine the ΔG, m-value, and [urea]$_b$.

\[
y = \frac{\left( y_f + m_f [\text{urea}] \right) + \left( y_i + m_i [\text{urea}] \right) \times e^{-\frac{\Delta G(H_2O) - m[\text{urea}]}{RT}}}{1 + e^{-\frac{\Delta G(H_2O) - m[\text{urea}]}{RT}}}
\]

\[
\Delta G = \Delta G(H_2O) - m[\text{urea}]
\]

where $y_f$ and $y_i$ are the intercepts, $m_f$ and $m_i$ are the slopes of the pre- and post-transition phase baselines, and the m-value is the slope of the transition phase. ΔG is the free energy change at any particular urea concentration, and it varies linearly with urea concentration and is used to estimate ΔG(H$_2$O). ΔG(H$_2$O) is defined as the Gibbs free energy of a protein in the absence of urea at 25 °C. R is the universal gas constant, and T is the temperature of the sample. [urea]$_b$ is the concentration of urea at which LEDGF$_{1–326}$ is 50% unfolded. Data represent mean of duplicate studies.

**Circular Dichroism (CD)**—To determine the secondary structures of LEDGF$_{1–326}$ and its conformational stability parameters, far-ultraviolet CD spectra were recorded. Briefly, a 500 μg/ml protein sample was placed in 1-mm quartz cuvette, and spectra were recorded at a scan speed of 0.5 s/time point, step size of 1 nm, and a bandwidth of 4 nm from 200 to 280 nm using a Chirascan® CD instrument (Applied Photophysics Ltd.). All scans were done in triplicate. The native LEDGF$_{1–326}$ spectrum thus obtained was deconvoluted using CDNN 2.1 software (Dr. Gerald Bohm, Martin-Luther-University at Halle-Wittenberg, Germany) to get the percentage of secondary structures present in native LEDGF$_{1–326}$ protein.

LEDGF$_{1–326}$ chemical denaturation was performed at various urea concentrations. Briefly, 300 μg/ml protein was incubated with 0–6 M urea in 25 mM phosphate buffer, pH 7.0, for 24 h. CD signal was recorded as mentioned above. The conformational stability parameters of LEDGF$_{1–326}$ were determined by plotting the CD signal at 230 nm as a function of urea concentration as we obtained the maximum CD signal difference by plotting the CD signal at 230 nm as a function of urea concentration and is used to estimate [urea]$_{1/2}$ (Eq. 2).

\[
[\text{urea}]_{1/2} = \sqrt{\frac{-mG}{y_U}}
\]

where $y_F$ and $y_I$ are the intercepts, $m_F$ and $m_I$ are the slopes of the pre- and post-transition phase baselines, and the m-value is the slope of the transition phase. ΔG is the free energy change at any particular urea concentration, and it varies linearly with urea concentration and is used to estimate ΔG(H$_2$O). ΔG(H$_2$O) is defined as the Gibbs free energy of a protein in the absence of urea at 25 °C. R is the universal gas constant, and T is the temperature of the sample. [urea]$_b$ is the concentration of urea at which LEDGF$_{1–326}$ is 50% unfolded. Data represent mean of duplicate studies.

**Functional Characterization**

**In Vitro Assay**—ARPE-19 cells were maintained as described earlier (30). For cell viability assay, 10,000 cells were plated in 96-well plate and incubated for 24 h. After 24 h, the serum-containing medium was aspirated out. The test groups (pP23H-Rho + LEDGF$_{1–326}$) were transiently transfected with pP23H-Rho plasmid (1 μg/ml) using a 1:3 ratio of Lipofectamine 2000 (LP-2000) in serum-free medium as per the manufacturer’s protocol. After 6 h of transfection, the medium was aspirated out, and cells were treated with increasing amount of LEDGF$_{1–326}$. No cells (just the medium), cells with no LP-2000, and cells with LP-2000 were also maintained as control.

**MTT Assay**—After 48 h, the medium was aspirated out, and 200 μl of fresh serum-free medium was added. 20 μl of MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 5 mg/ml in PBS, pH 7.4) was added to each well, and further incubation was done for 3 h at 37 °C. The MTT-containing medium was aspirated out, and the formazan crystals formed were dissolved in 200 μl of dimethyl sulfoxide (DMSO). The absorbance of the color developed was measured at 570 nm using a SpectraMax M5. The percentage of viability of groups was calculated with reference to the control group containing cells with no LP-2000. All groups were repeated with n = 4.

**Live/Dead Cell Count Assay**—ARPE-19 cells were treated similar to MTT assay as stated above. At the end of LEDGF$_{1–326}$ treatment period, cells were washed with PBS. The cells were labeled with a combination of plasma membrane permeant (Hoechst 33342), a plasma membrane-impermeable molecule (BOBO™ 3), and a nuclear dye (4′,6-diamidino-2-phenylindole, dihydrochloride; DAPI). Hoechst 33342 was used to label cell nuclei, whereas BOBO™ 3 was used to label dying or dead cells. The cells were visualized using the Operetta® high content imaging system. Cell count was obtained using the automated software tool in the Operetta® instrument. The number and percentage of live cells were calculated by subtracting the dead cell count from the “all cell” count.

**Phagocytic Assay**—ARPE-19 cells were seeded in 24-well plates and transfected with 20 pm/ml MERTK siRNA (Santa Cruz Biotechnology Inc., Dallas, TX), using siRNA transfecting agent (Santa Cruz Biotechnology) for 6 h. The transfecting medium was removed, and cells were further incubated in serum-free medium for 24 h. Cells transfected only with the transfecting medium and no MERTK siRNA were maintained as control. Cells were washed once and treated with 0.05, 0.5, or 5 μg/ml LEDGF$_{1–326}$ for 24 h, and then phagocytosis of 2 μm particles was monitored. Briefly, 100 μg/ml 2-μm blue Fluospheres (Life Technologies) was incubated with cells for 3 h.
Thereafter, cells were washed twice with cold PBS, pH 7.4, followed by two washes of cold PBS, pH 5.0, to remove adherent FluoSpheres. Cells were lysed using 1% Triton X, and the fluorescence of the particles in the cell lysate was measured using 350 nm excitation and 430 nm emission. Cells transfected with only transfecting agent without siRNA were taken as control for particle uptake. Cells with no particle treatment were used for background fluorescence measurements.

**In Vivo Efficacy Assay**

**Animal Maintenance**—Homozygous RCS rat breeders were generously provided by Dr. Jeffrey Olson (University of Colorado Anschutz Medical Campus, Aurora, CO). Thereafter, the RCS rat colony was maintained in the animal facility of the University of Colorado Anschutz Medical Campus and with the approval of the Institutional Animal Care and Use Committee (IACUC). The experiments were carried as per the Association for Research in Vision and Ophthalmology (ARVO) statement for the Use of the Animals in Ophthalmic and Vision Research.

**Electroretinography**—At 4 weeks of age, rats were dark-adapted for at least 30 min. Thereafter, the animal was prepared for electroretinogram (ERG) under dim red light. Briefly, rat was anesthetized with intraperitoneal injection of a mixture of 80 mg/kg of ketamine and 12 mg/kg of xylazine. The pupil was dilated with 0.5% tropicamide (Akorn, Lake Forest, IL) and was kept moist using 2.5% hypromellose (Akorn). Thereafter, the animal was placed on a heated water jacket stabilized at 37 °C. A reference electrode (LK Technologies Inc., Gaithersburg, MD) was inserted into the tail and cheek of the animal. A DTL Plus electrode (LK Technologies Inc.) was placed across the cornea of each eye. The animal was exposed to brief flashes of 0.4 log cd-s/m² with an interval of 10 s between each flash, and scotopic ERGs were recorded. Thereafter, the animal was light-adapted for 3 min with a background light of 30 cd/m². Photopic ERG was recorded at the same intensity of flash but with background light on. At least three ERGs were averaged to get a single ERG for each animal. 2 μl of 0.25, 0.5, or 2.5 mg/ml sterile-filtered LEDGF₁–326 was given intravitreally in one eye, and vehicle was given in the contralateral eye. ERGs were recorded every 2 weeks for 8 weeks after intravitreal injection, and vehicle was given in the contralateral eye. ERGs were recorded every 2 weeks for 8 weeks after intravitreal injection, and vehicle was given in the contralateral eye.

**Histology**—At the end of the study, i.e., on the 12th week, eyes were enucleated after ERG measurements and fixed in David-son’s fixative (2% of 37–40% formaldehyde, 35% ethanol, 10% glacial acetic acid, and 53% distilled water) for 24 h at room temperature. The eyes were then stored in 70% ethanol for subse-quent serial dehydration and embedment in paraffin. Three vertical sections of 6 μm thick were cut from the nasal to the inferior edge in 30 h. The thickness of outer nuclear layer (ONL) and inner nuclear layer (INL) was measured methodically using the Ape-rio ImageScope software version 11.1.2.760. Because the photoreceptor cell protection may be uneven across the retina, every 500 μm from the superior edge to the inferior edge in each section was analyzed, and the average of three sections was done for each point. Data represent the average of three eyes.

**Immunofluorescence**—For immunofluorescence, after re-moval of paraffin, eye sections were processed through the fol-lowing sequential steps at room temperature, unless otherwise indicated. Antigen was retrieved by boiling the sections at 80 °C for 15 min. After blocking the nonspecific binding, sections were incubated with mouse anti-rhodopsin (1D4) primary antibody at 4 °C overnight followed by 30-min incubation with Alexa Fluor® 594-conjugated donkey anti-mouse IgG and DAPI. Finally, eye sections were washed and mounted by SuperMount H (Biogenex, San Ramon, CA) mounting medium to prevent rapid loss of fluorescence. The fluorescence was visualized using a confocal microscope (Nikon Eclipse C1) at 20X optical zoom. The excitation-emission wavelengths used for DAPI and Alexa Fluor were 408–450/35 and 637–605/75 nm, respectively. Images were captured using the Nikon EZ-C1 software version 3.40.

**Statistical Analysis**

All data are represented as the mean ± S.D. An independent samples Student’s t test or one-way analysis of variance followed by Tukey’s post hoc analysis (SPSS, version 11.5; SPSS, Chicago, IL) was performed for comparisons between the two or multiple experimental groups, respectively. The differences were considered statistically significant at p ≤ 0.05.

**RESULTS**

**Cloning, Expression, and Purification of LEDGF₁–326**—DNA gel electrophoresis of the PCR amplification product during cloning indicated a positive band of 1000 bp of LEDGF₁–326 (Fig. 1A). A positive band, at about 40 kDa, in the supernatant fraction, indicated expression of LEDGF₁–326 as a soluble protein in E. coli BL21 (DE3) (Fig. 1B). Cation exchange (Fig. 1C) of LEDGF₁–326 preparation removed all negatively charged proteins and eluted LEDGF₁–326 along with other lower molecular weight positively charged proteins (Fig. 1B, lane 4). On further purification using gel filtration (Fig. 1D), LEDGF₁–326 was eluted as a strong positive band of ~40 kDa (Fig. 1B, lane 5). Protein estimation indicated that about 20 mg of protein was obtained per liter of the shake flask culture.

**Bioinformatics Analysis of LEDGF₁–326**—Bioinformatics analysis of LEDGF₁–326 sequence using SIB ExPAsy portal (32) indicated its theoretical molecular mass to be 36.9 kDa. The computed isoelectric point (pI) of LEDGF₁–326 was 9.23, with 73 positively charged (arginine and lysine) and 63 negatively charged (aspartic acid and glutamic acid) amino acid residues. The theoretical molar extinction coefficient was 15,470M⁻¹cm⁻¹ at 280 nm in water. Based on its N-terminal amino acid methionine, its half-life in mammalian cells was predicted to be 30 h. The N-, O-, and C-glycosylation of LEDGF₁–326 was predicted using NetNGlyc 1.0, NetO Glyc 3.1, and NetCGlyc 1.0 servers, respectively. According to the output of NetO Glyc, LEDGF₁–326 has only one potential site for N-glycosylation at 103 amino acids. However, the probability of this site to be glycosylated is 0.6, indicating low likelihood of N-glycosylation. According to NetO Glyc 3.1 and NetCGlyc 1.0 server outputs, LEDGF₁–326 is unlikely to be O- or C-glycosylated. Thus, bac-
**LEDGF**<sub>1–326</sub> Protein for Treating Retinal Degenerations

The purity of LEDGF<sub>1–326</sub> protein, determined by SEC-HPLC (Fig. 2A), indicated 95% pure LEDGF<sub>1–326</sub> with a retention time of 10.63 ± 0.06 min. To further investigate whether LEDGF<sub>1–326</sub> self-associates to form any higher molecular weight oligomers, DLS was performed (Fig. 2B). DLS indicated a homogeneous population of LEDGF<sub>1–326</sub> with a mean hydrodynamic diameter of 9 ± 1 nm.

The molecular weight of LEDGF<sub>1–326</sub> was confirmed by MALDI-TOF mass spectrometry. The major peak obtained in MALDI-TOF spectrum was at 40,314.32 and 80,663.19 m/z (mass to charge) ratio (Fig. 2C). MALDI-TOF indicated that LEDGF<sub>1–326</sub> has a molecular mass of 40.314 kDa, which was equivalent to its theoretical molecular mass, indicating that the protein is not glycosylated. However, a second peak at 80,663 m/z was also seen, which indicated that LEDGF<sub>1–326</sub> may exist as a dimer. To investigate the existence of the dimers, SDS-PAGE (Fig. 2D) and CD (Fig. 2E) were performed. The presence of the strong negative signal at 200 nm indicated that LEDGF<sub>1–326</sub> is primarily a random coiled protein. The CD spectrum was deconvoluted using CDNN 2.1 software to determine the percentage of secondary structures present in LEDGF<sub>1–326</sub>. The native LEDGF<sub>1–326</sub> spectrum was deconvoluted using CDNN 2.1 software to determine the percentage of secondary structures present in LEDGF<sub>1–326</sub>.

**TABLE 1**

| Secondary Structure | % 200–260 nm | % 205–260 nm | % 210–260 nm | Average |
|---------------------|--------------|--------------|--------------|---------|
| C-Helix             | 14.00        | 20.00        | 16.30        | 16.77   |
| β-Sheets (antiparallel) | 20.80    | 12.20        | 15.90        | 16.30   |
| β-Sheets (parallel)  | 15.30        | 14.60        | 15.10        | 15.00   |
| β-Turn              | 22.90        | 19.60        | 21.10        | 21.20   |
| Random coil         | 42.40        | 44.60        | 48.30        | 45.10   |
| Total               | 115.4        | 111.0        | 116.7        | 114.37  |

The native LEDGF<sub>1–326</sub> spectrum was deconvoluted using CDNN 2.1 software to determine the percentage of secondary structures present in LEDGF<sub>1–326</sub>. The native LEDGF<sub>1–326</sub> spectrum was deconvoluted using CDNN 2.1 software to determine the percentage of secondary structures present in LEDGF<sub>1–326</sub>. The native LEDGF<sub>1–326</sub> spectrum was deconvoluted using CDNN 2.1 software to determine the percentage of secondary structures present in LEDGF<sub>1–326</sub>. The native LEDGF<sub>1–326</sub> spectrum was deconvoluted using CDNN 2.1 software to determine the percentage of secondary structures present in LEDGF<sub>1–326</sub>.
Assembly Refinement) protein modeling server (Fig. 2F). The LEDGF\textsubscript{1–326} predicted model had the confidence score of \(-3.18\) and template modeling score of 0.36 \pm 0.12, and root mean square deviation was equal to 14.1 \pm 8 Å.

**LEDGF\textsubscript{1–326} Is Conformationally Stable**—To investigate the conformational stability of LEDGF\textsubscript{1–326} in water, the perturbation in the tertiary structure due to chemical denaturation was measured by the intrinsic fluorescence of tryptophan molecules present in LEDGF\textsubscript{1–326} (Fig. 3A). Emission spectrum of native LEDGF\textsubscript{1–326} protein, in absence of urea, had a \(\lambda_{\text{max}}\) at 340 nm and \(\Delta\lambda_{\text{h}}\) (half-width of \(\lambda_{\text{A}}\)) of 56 nm (Fig. 3A, panel i). As the concentration of urea increased from 0 to 5 M, quenching in the fluorescence signal was observed. The signal decreased slowly until 0.9 M urea concentration was reached. Thereafter, there was a sharp decrease in the fluorescence signal until 2.3 M urea concentration was reached. Beyond this concentration, the decrease in the fluorescence signal was minimal. The \(\lambda_{\text{max}}\) of LEDGF\textsubscript{1–326} shifted to 356 nm, and \(\Delta\lambda_{\text{h}}\) was 71 nm at 5 M urea. When the ratio of LEDGF\textsubscript{1–326} fluorescence signal at 340–356 nm was plotted as a function of urea concentration, a sigmoidal curve was obtained (Fig. 3A, panel ii). Using Equations 1 and 2 (described under “Experimental Procedures”), \(\Delta G(H_2O)\) of LEDGF\textsubscript{1–326} was estimated to be 3.24 \pm 0.48 kcal mol\(^{-1}\), the \(m\)-value was estimated to be 1.70 \pm 0.22 kcal mol\(^{-1}\) M\(^{-1}\), and \([\text{urea}]_{1/2}\) was estimated to be 1.81 \pm 0.02 M, indicating that LEDGF\textsubscript{1–326} might be a stable protein.

Far-ultraviolet CD spectroscopy was performed to investigate the perturbation in the secondary structures of LEDGF\textsubscript{1–326} in the presence of urea (Fig. 3B). The CD signal of the LEDGF\textsubscript{1–326} was traced against the wavelength at each urea concentration (Fig. 3B, panel i). The CD signal continuously became more negative as the concentration of urea was increased. When CD signal at 230 nm was plotted as a function of urea concentration (Fig. 3B, panel ii), a sigmoidal curve was obtained. Fitting the LEDGF\textsubscript{1–326} data to Equations 1 and 2 indicated a \(\Delta G(H_2O)\) of 3.3 \pm 0.4 kcal mol\(^{-1}\), an \(m\)-value of 1.9 \pm 0.2 kcal mol\(^{-1}\) M\(^{-1}\), and \([\text{urea}]_{1/2}\) of 1.6 \pm 0.1 M.

Thermal stability of LEDGF\textsubscript{1–326} was determined using far-ultraviolet CD spectroscopy (Fig. 3C). The CD signal in the presence of heat as a denaturant was measured from 215 to 250 nm (Fig. 3C, panel i). As the temperature of the LEDGF\textsubscript{1–326} solution was increased, the negative dip at about 235 nm increased. The CD signal followed the same pattern as chemical denaturation, a pre-transition phase between \(\sim 30\) and 35 °C, followed by a transition phase between \(\sim 35\) and 55 °C, followed by a post-transition phase from \(\sim 55\) to 70 °C (Fig. 3C, panel ii). When these data were fitted using a global fit analysis equation, the \(T_m\) (the melting temperature) of LEDGF\textsubscript{1–326} obtained was 44.8 \pm 0.2 °C, indicating that LEDGF\textsubscript{1–326} will possibly be stable at 25 °C (room temperature).

**LEDGF\textsubscript{1–326} Rescues ARPE-19 Cells from Aggregation-mediated Stress**—LEDGF\textsubscript{1–326} efficacy to rescue ARPE-19 cells from protein aggregation-mediated stress was measured by MTT assay (Fig. 4A). Initially, the ability of LEDGF\textsubscript{1–326} to increase the viability of ARPE-19 cells in the absence of any stress was investigated (Fig. 4A, No Stress). There was no significant difference in the cell viability in untreated and 0.001–50 \(\mu\)g/ml LEDGF\textsubscript{1–326}-treated cells following 48 h of treatment. At the highest dose of LEDGF\textsubscript{1–326} (50 \(\mu\)g/ml), the cell viability was 108.14 \pm 5.63% (right-most bar) as compared with 100 \pm 13.19% for untreated cells (left-most bar), which was not significantly different. However, in pP23H-Rho-transfected ARPE-19 cells having aggregation stress, LEDGF\textsubscript{1–326} behaved differently (Fig. 4A, Aggregation Stress). Cells expressing P23H mutant rhodopsin showed a decline in cell viability to 48.25 \pm 5.62% (bar 2). This loss in cell viability could be attributed to the toxic effect of expression and accumulation of aggregation-prone P23H mutant rhodopsin protein within the cells. When cells expressing P23H mutant rhodopsin (bars 3–9) were treated with increasing amounts of LEDGF\textsubscript{1–326} an increase in the cell viability was seen. Even at a concentration as low as 0.001 \(\mu\)g/ml, LEDGF\textsubscript{1–326} increased the cell viability of ARPE-19 cells from 48.3 \pm 5.6 to 77.0 \pm 10.2%. At and beyond this point, the cell viability remained significantly higher as compared with the pP23H-Rho-transfected group.

To confirm the results of MTT assay, fluorescence imaging (Fig. 4B) and cell counting (Fig. 4C) were also performed. Based on fluorescence images, the cells were counted automatically by.
high throughput analysis software. Groups treated with LEDGF$_{1-326}$ in the presence of P23H-Rho aggregation stress indicated a greater number of cells per frame as compared with the untreated group, indicating the ability of LEDGF$_{1-326}$ to prevent the loss of cells due to aggregation stress (Fig. 4B). Under P23H-Rho aggregation stress, the live cell count decreased to 38 ± 6% (Fig. 4C). LEDGF$_{1-326}$ increased the live cell count significantly from 38 ± 6 to 118 ± 16% in a dose-dependent manner in 24 h. Further, a single treatment of LEDGF$_{1-326}$ was effective until day 7 for all doses ranging from 0.005 to 5 μg. P23H-Rho-expressing cells treated with LEDGF$_{1-326}$ (blue lines) indicated a significantly higher number of live cells than the untreated group (red line) on day 7.

**LEDGF$_{1-326}$ Increases Phagocytic Activity of ARPE-19 Cells**—ARPE-19 cells were transfected with MERTK siRNA and then treated with LEDGF$_{1-326}$. Uptake of 2-μm FluoroSpheres was measured to assess the phagocytic activity of ARPE-19 cells (Fig. 5). In the absence of MERTK siRNA transfection, the addi-

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**FIGURE 4. LEDGF$_{1-326}$ rescues ARPE-19 cells from aggregation-mediated stress.** A, MTT assay. LEDGF$_{1-326}$ had no effect on the viability of ARPE-19 cells in the absence of aggregation stress at given concentrations. In the presence of aggregation stress, the cell viability of ARPE-19 cells was increased significantly by LEDGF$_{1-326}$ at concentrations as low as 0.01 μg/ml. B, fluorescence imaging. Shown is a representative image of ARPE-19 cells treated with LEDGF$_{1-326}$. The number of live cell nuclei increased with increasing dose of LEDGF$_{1-326}$. C, cell counting. ARPE-19 cells treated with LEDGF$_{1-326}$ in the presence of pP23H-Rho-mediated aggregation stress indicated significant increase in the number of live cell nuclei in a dose- and time-dependent manner. Data are represented as mean ± S.D. for n = 4. *, p < 0.05 as compared with pP23H-Rho-transfected group.
LEDGF<sub>1–326</sub> Protein for Treating Retinal Degenerations

The objective of this study was to develop a protein therapy potential candidate for rescuing retinal pigment epithelial cells from death due to aggregation-mediated cellular stress, and determined its in vivo efficacy in rescuing photoreceptors from degeneration. As elaborated below, our in vitro studies indicated that LEDGF<sub>1–326</sub> improves phagocytic activity and is effective in rescuing retinal pigment epithelial cells from death due to aggregation-mediated stress. Further, our in vivo studies indicated that intravitreally injected LEDGF<sub>1–326</sub> can reduce retinal degeneration for at least 8 weeks in RCS rats.

In our previous study, we identified LEDGF<sub>1–326</sub> gene as a potential candidate for rescuing retinal pigment epithelial cells from P23H mutant rhodopsin aggregation-induced stress (30). The objective of this study was to develop a protein therapy based on LEDGF<sub>1–326</sub>. To develop LEDGF<sub>1–326</sub> as a therapeutic agent, it should be produced in tens of milligrams, which in itself is a daunting task. We designed a cloning strategy to express LEDGF<sub>1–326</sub> as a cytoplasmic protein in pET-28a (+), a bacterial cell-based system, and confirmed the successful cloning by sequence analysis for the correct reading frame, right orientation of the gene, and no mutagenesis.

In light-adapted (photopic) ERG, the base b-wave amplitude, the b-wave amplitude ranged from 65.80 ± 15.44 to 91.13 ± 13.94 μV. There was no significant difference in the untreated and LEDGF<sub>1–326</sub>-treated groups. However, beyond 2 weeks, the b-wave amplitude reduction was less in the LEDGF<sub>1–326</sub>-treated groups. With increasing doses of LEDGF<sub>1–326</sub>, the loss of b-wave amplitude was reduced.

In this study, we successfully cloned and expressed LEDGF<sub>1–326</sub> in large quantities and characterized its biochemical properties, determined its in vitro ability to enhance phagocytic activity and ameliorate protein aggregation-mediated cellular stress, and determined its in vivo efficacy in rescuing photoreceptors from degeneration. As elaborated below, our in vitro studies indicated that LEDGF<sub>1–326</sub> improves phagocytic activity and is effective in rescuing retinal pigment epithelial cells from death due to aggregation-mediated stress. Further, our in vivo studies indicated that intravitreally injected LEDGF<sub>1–326</sub> can reduce retinal degeneration for at least 8 weeks in RCS rats.
during the cloning procedure (Fig. 1A). Utilizing two-step purification, we were able to produce ~95% pure protein (Fig. 1B). Because LEDGF1–326 is not highly likely to undergo glycosylation based on molecular modeling, and based on our observed efficacy, bacterial biosynthesis of LEDGF1–326 is adequate for therapeutic activity. However, the role of other post-translational modifications and an alternation in activity of our LEDGF1–326 as compared with native protein cannot be ruled out. SEC-HPLC of purified LEDGF1–326 (Fig. 2A) indicated 5% higher molecular weight species of LEDGF1–326. Because it has been known that the presence of large aggregates can trigger immunogenic reactions in vivo (34), we also investigated the possible formation of high molecular weight aggregates during purification of LEDGF1–326. Protein, using DLS (Fig. 2B). Interestingly, the number of mean size measurements gave a single narrow size distribution of LEDGF1–326, indicating the absence of higher molecular weight aggregates. Because MALDI-TOF analysis indicated that LEDGF1–326 may exist as a monomer and/or dimer (Fig. 2C), nonreducing SDS-PAGE was utilized to confirm the existence of dimers (Fig. 2D). Higher molecular weight bands were evident in the gel, indicating the possible existence of a dimeric form of LEDGF1–326. Possibly a dynamic equilibrium exists between the monomeric and the dimeric forms of LEDGF1–326. However, the ratio of dimer to monomer is currently not established. The far-ultraviolet CD of LEDGF1–326 (Fig. 2E) indicated that LEDGF1–326 is predominantly a random coiled structure. Deconvolution of the CD signal further strengthened this view.

To establish a protein’s therapeutic value, it is very important to understand its biophysical nature, which includes the conformational stability of the protein. Protein conformational stability is contributed by various environmental factors including pH, ionic strength, and temperature. Such information is useful in developing a stable formulation of the protein (35). In this
study, fluorescence and CD were used to determine conformational stability of LEDGF<sub>1–326</sub> (Fig. 3). The results obtained from fluorescence spectroscopy for LEDGF<sub>1–326</sub> (Fig. 3A) indicated that tryptophan residues of native LEDGF<sub>1–326</sub> are partially exposed to the aqueous environment (36). LEDGF<sub>1–326</sub> free energy of unfolding, ΔG(H<sub>2</sub>O), was positive, indicating that the unfolding of LEDGF<sub>1–326</sub> is unfavorable in the absence of any denaturant. The melting temperature of LEDGF<sub>1–326</sub> was predicted to be about 44 °C, indicating that LEDGF<sub>1–326</sub> is stable at room temperature. ΔG(H<sub>2</sub>O), m-value, [urea]<sup>1/2</sup>, and T<sub>m</sub>, together defined the conformational and thermal stability of LEDGF<sub>1–326</sub>.

RPE cells are known to accumulate rod outer segments containing rhodopsin with aging in human eyes (37). P23H rhodopsin-containing rod outer segment accumulation has also been implicated in vivo in neovascularization of RPE (38). Thus, our study utilizing mutant rhodopsin in ARPE-19 cells is pertinent to the in vitro simulation of retinal degeneration models. The in vitro efficacy data indicated significant decrease in the cell viability of ARPE-19 cells in the presence of P23H mutant rhodopsin protein (Fig. 4). This decrease in the cell viability was previously related to aggregation-mediated stress caused by the expression of P23H mutant rhodopsin and the associated aggregates in the cellular environment (39). Interestingly, upon treatment with LEDGF<sub>1–326</sub>, ARPE-19 cell viability increased significantly. LEDGF<sub>1–326</sub> protein did not alter the viability of ARPE-19 cells in the absence of stress, indicating that LEDGF<sub>1–326</sub> is more active in stressed conditions. Because MTT assay is indicative of mitochondrial respiratory state, but not a direct measure of cell viability, we performed high throughput cell counting using the Operetta High Content Analysis System. We did not see many dead cells in the P23H-Rho group. However, the cell count in the P23H-Rho group was significantly lower than the cells not expressing P23H-Rho, suggesting that rhodopsin aggregation arrested cell proliferation. In the presence of LEDGF<sub>1–326</sub>, the number of cells per frame increased as compared with controls, indicating that LEDGF<sub>1–326</sub> acts as a proliferating agent in the presence of protein aggregation stress. We previously reported that LEDGF<sub>1–326</sub> reduces oligomers of P23H rhodopsin as well as wild type rhodopsin, whereas increasing their monomers in a dose-dependent manner (30). LEDGF<sub>1–326</sub> in this earlier study did not affect the total rhodopsin protein expression at low doses. However, at high doses, there was a decrease in the total rhodopsin protein content with no changes in mRNA levels, suggesting that LEDGF<sub>1–326</sub> may be targeting rhodopsin aggregates to degradation pathways, in addition to preventing/disrupting rhodopsin aggregates.

Because RPE cells are specialized to phagocytose photoreceptor outer segments and other cellular debris (40), the phagocytic activity of ARPE-19 cells was determined in the presence of LEDGF<sub>1–326</sub>. Depletion of MERTK receptors in the retinal pigment epithelium is known to inhibit phagocytosis in RPE (40). However, MERTK siRNA transfection did not reduce the phagocytosis or uptake of FluoSpheres by ARPE-19 cell in our experimental conditions. It could be possible that under the conditions of the experiment, MERTK was not sufficiently depleted in ARPE-19 cells. Interestingly, LEDGF<sub>1–326</sub> significantly increased ARPE-19 phagocytic activity irrespective of MERTK siRNA transfection. This result is of high interest as this is the first time that LEDGF<sub>1–326</sub> has been shown to enhance phagocytosis.

In the absence of any exact replicate animal model for dry AMD, RCS rat is a useful model in understanding retinal degenerations. RCS rats exhibit primary genetic defect in the RPE due to deletion of MERTK gene, leading to loss of phagocytic activity and toxic accumulation of cell debris. The ultimate pathology in these animals is photoreceptor degeneration (41). Histopathology and ERG are established methods to monitor photoreceptor degeneration and retinal functional loss, respectively. B-waves of ERG specifically indicate photoreceptor health. Therefore, we used histopathology and ERG to monitor the ability of LEDGF<sub>1–326</sub> to reduce retinal degeneration. The decline in the b-wave amplitude in scotopic ERG represents loss or degeneration of rod and cone photoreceptors. In photopic ERG, the rods are bleached, so the response is mostly from the cone photoreceptors. The amplitude of the b-wave reflects the number of functionally active photoreceptors. Single intravitreal injection of LEDGF<sub>1–326</sub> significantly reduced the loss of b-wave amplitude, indicating reduction in the functional loss of photoreceptors for a period of at least 2 months from the day of treatment (Fig. 6A). Further, the functional protection of photoreceptors indicated by ERG correlated with the morphological protection of photoreceptors indicated by histology (Fig. 6B) and immunofluorescence (Fig. 7). LEDGF<sub>1–326</sub> rescued photoreceptors, as evidenced by the thicker ONL and thicker band of rhodopsin-stained photoreceptors. It also protected INL, which is made up of amacrine and bipolar cells, from degeneration, as evident in histological analysis. N-terminal LEDGF<sub>1–326</sub> has a stress-related element binding domain (30), which is capable of activating stress-related proteins (42).
Despite some efforts in the current study, the mechanism as to how exactly LEDGF1–326 works has yet to be investigated at the molecular level and is beyond the scope of this study. However, based on existing knowledge, the putative mechanisms of action of LEDGF1–326 are summarized in Fig. 8. We speculate that LEDGF1–326 may reduce both oxidative as well as endoplasmic reticulum stress (generated by protein aggregation) by up-regulating stress response proteins. It may also improve the phagocytic activity of RPE, thereby improving the survival of photoreceptors. Because a close association has been suggested (43) between protein aggregation stress (RP) and oxidative stress (dry AMD), a molecule like LEDGF1–326 can be a universal therapeutic protein to treat multiple retinal degenerative diseases. Further, because many neurodegenerative diseases including Alzheimer, Parkinson, and Huntington diseases have been linked to protein aggregation, a possibility exists where LEDGF1–326 could also be useful in some of these diseases as a therapeutic intervention.

CONCLUSIONS

In conclusion, in this study, we were able to biosynthesize and purify large quantities of LEDGF1–326 in aggregate-free, highly pure form. LEDGF1–326 was found to be a conformationally and thermally stable protein at 25 °C. LEDGF1–326 was able to prevent the loss of cell viability due to aggregation-mediated stress at concentrations ranging from 0.001 to 50 µg/ml. A single intravitreal dose of LEDGF1–326 was effective in reducing retinal degeneration in RCS rats for over 2 months. Thus, LEDGF1–326 is a potential therapeutic agent for retinal degenerative disorders.

Acknowledgment—We are thankful to Dr. Joseph Brzezinski of University of Colorado for assistance in performing immunofluorescence studies.

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