Evolutionarily Conserved ELOVL4 Gene Expression in the Vertebrate Retina

Pamela S. Lagali,1 Jiafan Liu,2 Rajesh Ambasudhan,2 Laura E. Kakuk,2 Steven L. Bernstein,3 Gail M. Seigel,4 Paul W. Wong,1,5 and Radha Ayyagari2,5

PURPOSE. The gene elongation of very long chain fatty acids-4 (ELOVL4) has been shown to underlie phenotypically heterogeneous forms of autosomal dominant macular degeneration. In this study, the extent of evolutionary conservation and the existence and localization of retinal expression of this gene was investigated across a wide variety of species.

METHODS. Southern blot analysis of genomic DNA and bioinformatic analysis using the human ELOVL4 cDNA and protein sequences, respectively, were performed to identify species in which ELOVL4 orthologues and/or homologues are present. Retinal RNA and protein extracts derived from different species were assessed by Northern hybridization and immunoblot techniques to assess evolutionary conservation of gene expression. Immunohistochemical analysis of tissue sections prepared from various mammalian retinas was performed to determine the distribution of ELOVL4 and homologous proteins within specific retinal cell layers.

RESULTS. The existence of ELOVL4 sequence orthologues and homologues was confirmed by both Southern blot analysis and in silico searches of protein sequence databases. Phylogenetic analysis places ELOVL4 among a large family of known and putative fatty acid elongase proteins. Northern blot analysis revealed the presence of multiple transcripts corresponding to ELOVL4 homologues expressed in the retina of several different mammalian species. Conserved proteins were also detected among retinal extracts of different mammals and were found to localize predominantly to the photoreceptor cell layer within retinal tissue preparations.

CONCLUSIONS. The ELOVL4 gene is highly conserved throughout evolution and is expressed in the photoreceptor cells of the retina in a variety of different species, which suggests that it plays a critical role in retinal cell biology. (Invest Ophthalmol Vis Sci. 2003;44:2841–2850) DOI:10.1167/iovs.02-0991

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Stargardt macular dystrophies are inherited disorders of the retina that are characterized by impaired central vision, cellular atrophy of retinal and retinal pigment epithelial (RPE) cells initiating in the macular region, and the presence of yellow fundus flecks surrounding the atrophic area resulting from the subretinal accumulation of lipofuscin-like material.1–4 Both autosomal recessive and autosomal dominant forms of Stargardt’s disease (STGD) have been described.5,6

Classical or recessive Stargardt’s disease (STGD1; OMIM 248200; http://www.ncbi.nlm.nih.gov/omim, provided by the National Institutes of Health, Bethesda, MD) is the most common form of hereditary macular dystrophy, occurring at an incidence of approximately 1 in 10,000 live births, and accounts for roughly 7% of all retinal dystrophies.7 A distinguishing clinical feature of the disease is the dark or silent choroid observed on fluorescein angiography of the fundus, resulting from the accumulation of lipofuscin. Mutations in the ABCA4 (ABCR) gene on human chromosome 1p32 have been found to underlie STGD1.8,9 This gene encodes a retina-specific adenosine triphosphate–binding cassette transport protein found in photoreceptor cell outer segment disc membranes and functions as an outwardly directed flipase to facilitate the shuttling of N-retinylidene-phosphatidylethanolamine from the interior to the exterior of the outer segments for subsequent retinoid recycling by RPE cells after phototransduction.10,11 ABCR mutations have also been implicated in the etiology of a variety of other retinal diseases, including retinitis pigmentosa, cone–rod dystrophy, and age-related macular degeneration (AMD).12–15 Cases of autosomal dominant Stargardt’s macular dystrophy (adSTGD) are comparatively rare, and a number of genetic loci for diseases with typical adSTGD traits have been mapped, including STGD3 and adMD (OMIM 600110) on 6q14.14,15 and STGD4 (OMIM 605786) on 4p.16 Forms of adSTGD are clinically quite similar to the phenotype characteristic of recessive Stargardt’s disease; however, there are a number of key differentiating features. The most notable differences between STGD1 and STGD3/adMD-associated phenotypes in particular are the later and more variable age of onset and the absence of the dark choroid observed in fluorescein angiograms of patients with dominant STGD.15,17,18

Using a positional candidate approach, we and others recently identified the gene defect corresponding to the STGD3/adMD locus responsible for the disease phenotype in five related pedigrees throughout North America.19 The mutation, a 5-bp deletion, lies in the elongation of very long chain fatty acids-4 gene (ELOVL4), a novel member of a family of genes that encode enzymatic components of the microsomal fatty acid elongation system referred to as fatty acid elongases (ELOs). The ELOVL4 gene consists of six exons and encodes a 2.9-kb full-length mRNA transcript that in turn encodes a 514-amino-acid protein that contains the three typical features that define a fatty acid elongase20,21: five putative transmembrane segments, a single HXXHH histidine box redox center motif, and a carboxyl-terminal dilysine targeting signal thought to be

From the 1Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada; 2Department of Ophthalmology and Visual Sciences, W. K. Kellogg Eye Center, University of Michigan, Ann Arbor, Michigan; the 3Department of Ophthalmology, University of Maryland, Baltimore, Maryland; and the 4Department of Ophthalmology, State University of New York at Buffalo, Buffalo, New York.

1Contributed equally to the work and therefore should be considered equivalent authors.

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Corresponding author: Paul W. Wong, Department of Biological Sciences, G-502 Biological Sciences Center, University of Alberta, Edmonton, Alberta, T6G 2E9, Canada; pwwong@ualberta.ca.
responsible for the retrieval of transmembrane proteins to the endoplasmic reticulum (ER) from the Golgi apparatus. The 5-bp deletion in the gene identified in dominant STGD/adMD-affected pedigrees causes a reading frame shift that results in premature termination of the translation product, leading to substitutions in amino acids 264–271 and the loss of the 51 carboxyl-terminal residues in the mutant protein. This mutation has subsequently been verified in affected members present in other branches of the same family. In addition, a novel complex mutation in the ELOVL4 gene has been described in an unrelated family that manifests diverse macular dystrophy phenotypes.

Thus, it is clearly established that the ELOVL4 gene plays a critical role in the pathogenesis of macular degeneration. However, the precise nature of this role is at present unknown. Clues to the importance of this gene in retinal cell biology have also come from the identification of both mouse and yeast homologues, implying evolutionarily conserved gene expression. This limited assessment of conservation, its implied role in fatty acid metabolism, and its association with retinal degeneration suggests that ELOVL4 is an essential retinal-expressed gene. To further characterize this gene in normal retinal biology, we used nucleic acid hybridization and bioinformatic and immunodetection analyses to examine the sequence conservation and expression of ELOVL4 gene products across a wide range of species. We report that ELOVL4 represents a highly evolutionarily conserved gene that is expressed in the photoreceptor cell layer within the retina of every mammalian species examined.

METHODS

Animal Tissues and Donor Eyes

Human ocular tissue was obtained from eyes donated to the Michigan Eye Bank (Ann Arbor, MI) and through the National Disease Research Interchange (NDRI, Philadelphia, PA). Archival human retinal sections were obtained from the Ocular Pathology Laboratory at the University of Rochester (Rochester, NY). Rhesus monkey eyes were kindly provided by John Cogan (Bureau of Biologics, Bethesda, MD). Ocular dissections were performed as previously described. Mouse, Sprague-Dawley rat, and feline eyes were obtained from normal control animals in the University of Michigan animal facility and dissected immediately after harvesting. Bovine eyes were obtained from a local abattoir and stored on ice after harvesting. Dissections were performed as previously described.25 Mouse, dog, cow, rabbit, chicken, and yeast) was purchased (BD Biosciences-Clontech, Palo Alto, CA). A 376-bp DNA probe corresponding to exon 6 of the human ELOVL4 gene was generated from a genomic DNA template by polymerase chain reaction (PCR), with primers 5’-GAAATGGCAGTGGTGTAAAAAG-3’ and 5’-GTCAACACAGTTAAGGCCC-3’. PCR cycling parameters used were as follows: 1 cycle of denaturation at 94°C for 3 minutes; 30 cycles of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, primer extension at 72°C for 1 minute, primer extension at 72°C for 3 minutes; and 1 cycle of extension at 72°C for 7 minutes. A probe corresponding to the human ELOVL4 and β-actin coding regions. The ELOVL4 probe used was the 743-base pair cDNA fragment described earlier. The β-actin probe was amplified by PCR from a plasmid clone with primers 5’-GTCCACACCCGCCGCACAGTACC-3’ and 5’-ACTGGTCTCGAATGTCAGTGACGTGA-3’ to generate a 1698-bp nearly full-length cDNA product. Radiolabeled probes were prepared and hybridizations were performed as just described at either 42°C or 55°C for blots containing multispecies RNA samples or at 65°C for primate RNA blots. For increased specificity of probe binding, multispecies blots were blocked with 0.5 μg of denatured human Cot-1 DNA (Invitrogen) added to the hybridization solution. ELOVL4-probed blots were exposed to film for 18 to 48 hours.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from primate ocular tissues and retinal tissue derived from various mammalian species using extraction reagent (Trizol; Invitrogen), according to the manufacturer’s protocol. Five micrograms of each total RNA sample was electrophoresed on a 1% agarose-formaldehyde gel and subsequently blotted onto a nylon membrane (Pall Corp., Mississauga, Ontario, Canada) by capillary transfer. Northern blots were consecutively hybridized with probes corresponding to the human ELOVL4 and β-actin coding regions. The ELOVL4 probe used was the 743-base pair cDNA fragment described earlier. The β-actin probe was amplified by PCR from a plasmid clone with primers 5’-GTCCACACCCGCCGCACAGTACC-3’ and 5’-ACTGGTCTCGAATGTCAGTGACGTGA-3’ to generate a 1698-bp nearly full-length cDNA product. Radiolabeled probes were prepared and hybridizations were performed as just described at either 42°C or 55°C for blots containing multispecies RNA samples or at 65°C for primate RNA blots. For increased specificity of probe binding, multispecies blots were blocked with 0.5 μg of denatured human Cot-1 DNA (Invitrogen) added to the hybridization solution. ELOVL4-probed blots were exposed to film for 18 to 48 hours.

Preparation of Retinal Protein Extracts

Volumes of retinal tissue samples were estimated, and an equal volume of 2× lysis buffer (100 mM Tris-HCl [pH 7.4], 300 mM NaCl, 1% Triton X-100, 1 tablet of a protease inhibitor cocktail dissolved in 10 ml of water [Complete; Roche Diagnostics Corp., Indianapolis, IN]) was added. Tissues were homogenized using a sonicator (Sonifier 250; Branson Ultrasonics, Danbury, CT), with samples pulsed for 10 seconds at 2-minute intervals for 20 minutes at 4°C. Homogenates were spun at 5000 rpm for 3 to 5 minutes in a microcentrifuge to precipitate insoluble material, and the supernatants were further analyzed for protein content. Total protein concentration was estimated with the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL).

SDS-PAGE and Immunoblot Analysis

Retinal protein extracts (approximately 200 μg of total protein) were added to an equal volume of 2× sodium dodecyl sulfate–polyacrylam-
ide gel electrophoresis (SDS-PAGE) loading buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol) and electrophoresed on a 12% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (Sigma-Aldrich Corp., St. Louis, MO) by electroblotting (Mini-Blot Protein Blotting Cell; Bio-Rad Laboratories, Hercules, CA). Immunoblots were blocked with 3% skim milk powder for 1 hour at room temperature, followed by incubation with the ELOVL4 polyclonal antiserum diluted 1:1000 in 3% skim milk at room temperature for 1 hour in the presence or absence of 20 μg of competing ELOVL4 peptide. Blots were subsequently labeled with a 1:5000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Pierce Biotechnology) and subjected to immunodetection by enhanced chemiluminescence (ECL Western Blotting Detection Reagents; Amersham Biosciences, Piscataway, NJ).

Bioinformatic Analysis

ELOVL4 homologues and orthologues were identified by performing protein homology searches of sequence databases with the translated ELOVL4 mRNA sequence (GenBank accession no. AF277094; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information [NCBI], Bethesda, MD) and the BLASTP program also provided in the public domain by NCBI (http://www.ncbi.nlm.nih.gov/blastp).26 Proteins identified by the BLASTP search program with greater than 25% identity to the human ELOVL4 amino acid sequence were classified as homologues. Sequence alignments and phylogenetic analysis were performed using the Clustal W (versions 1.8, 1.82)27 and PHYLIP 28,29 programs and Command Line applications for sequence analysis and phylogeny (http://www.cbr.nrc.ca/ all provided in the public domain through Canadian Bioinformatics Resources, National Research Council Canada, Halifax, Nova Scotia, Canada).

Immunohistochemistry

Human Retina Sections. Whole human eyes were paraffin embedded and cut into 4-μm tissue sections. Human tissue sections were permeabilized in 0.25% Triton X-100 for 5 minutes and rinsed in phosphate-buffered saline (PBS). Endogenous peroxidase was quenched with a 5-minute incubation in 3% hydrogen peroxide. Tissues were rinsed twice for 5 minutes in PBS and blocked for 15 minutes in 5% goat serum. After two additional 5-minute rinses in PBS, sections were incubated with a 1:100 dilution of ELOVL4 polyclonal antiserum or preimmune serum as a negative control for 1 hour at room temperature. Tissues were subsequently rinsed three times for 5 minutes in PBS and then incubated with 1 μg/ml of biotinylated goat anti-rabbit immunoglobulin secondary antibody (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. After a final PBS rinse, tissues were incubated with HRP-conjugated avidin (Vectastain Elite kit; Vector Laboratories) for 20 minutes. Tissue sections were equilibrated in 0.05 M Tris-HCl [pH 7.4], then developed with a diaminobenzidine (DAB) kit (Pierce Biotechnology), and the brown- or black-colored reaction product was visualized by light microscopy. For DNA counterstaining, sections were incubated with 30% methyl green for 50 seconds.

Animal Retina Sections. Mouse, feline, and rat specimens were perfused with 4% paraformaldehyde, and eyes were extracted and fixed overnight at 4°C. The bovine eyes obtained from an abattoir were immersed in 4% paraformaldehyde overnight. The anterior segment of the eye was removed. The eye cup was rinsed in buffer and embedded in paraffin. Specimens were sectioned at 5 to 7 μm. Tissue sections were dehydrated in xylene and rinsed in PBS. Rehydrated sections were treated with proteinase K (5000 ng/ml), as previously described.50 Immunohistochemical analysis was performed with a kit (Vectastain ABC kit; Vector Laboratories, Inc.), according to the manufacturer’s protocol. Rat and cat sections were analyzed with the ELOVL4 polyclonal antiserum at a 1:400 dilution, and mouse and cow sections were analyzed with the ELOVL4 affinity-purified antibodies at a 1:10,000 dilution. Control experiments were performed with a 1:400 dilution of the preimmune serum in place of the ELOVL4 antibodies. Results were visualized by light microscopy.

RESULTS

Multispecies Conservation of ELOVL4 DNA Sequences and mRNA Expression

To detect the presence of ELOVL4 sequence homologues and/or orthologues in a number of different species, we probed a Southern blot containing genomic DNA isolated from human, monkey, rat, mouse, dog, cow, rabbit, chicken, and yeast with ELOVL4-derived sequences. Hybridization signals were detected in all the species examined (Fig. 1), suggesting that the ELOVL4 gene is conserved across a variety of eukaryotic species.

Retinal expression of the ELOVL4 gene has already been demonstrated in human tissue samples.19,23 To investigate the expression of ELOVL4 mRNA in additional human eye tissues, we performed Northern blot analysis. The approximately 2.9- and 2.4-kb transcript sizes previously reported for the human ELOVL4 gene were consistently observed in different peripheral retina samples (Fig. 2); however, we detected two addi-
tional transcripts of roughly 4.5 and 0.25 kb. These bands persisted even after Cot-1 DNA suppression and high-stringency washing of blots. Similar hybridization signals were observed for human fovea tissue; however, the 2.9- and 2.4-kb transcripts appear to be absent in RPE, choroid, and ciliary body tissue samples (Fig. 2). This suggests that ELOVL4 or ELOVL4-like genes are differentially expressed among different human eye tissues. All four transcripts were detected in rhesus monkey peripheral retina and macular tissues, indicating that ELOVL4 gene expression is highly conserved between primate retinal tissues.

To extend these findings to additional species, retinal RNA samples derived from mouse, cow, and pig were similarly assessed by Northern blot analysis (Fig. 2). The approximately 4.5-kb transcript appeared in all the species, whereas the 2.9-kb transcript is clearly observed in the mouse and cow retina but was undetectable in the pig retina RNA. The 2.4-kb transcript readily detected in the primate retina samples is not as easily observed in the retinal RNA from the other species under the stringent hybridization and washing conditions used. The approximately 0.25-kb transcript also appeared in all samples, except for porcine retina. These results suggest that different species express a different complement of ELOVL4 transcripts that may share varying degrees of sequence conservation, which in turn would lead to differences in detection capabilities based on the hybridization conditions used.

ELOVL4 Protein Orthologues and Homologues

The translated human ELOVL4 mRNA sequence (GenBank Accession No. AF277094) was used to search protein sequence databases to identify related proteins found in other species. Putative proteins exhibiting 25% or greater identity at the amino acid level with the human ELOVL4 protein were classified as potential orthologues or homologues. Presumed ELOVL4 orthologues and the best-matched sequence homologues from each of a variety of species were selected and aligned using the Clustal W (version 1.82) program (Fig. 3). A wide range of species including monkey, mouse, rat, insects, fish, fungus, plant, nematode, and yeast varieties were found to contain ELOVL4 homologous protein sequences. True orthologues were identified in monkey and mouse, each with greater than 90% amino acid identity with the human protein. Ninety-one amino acids are strongly evolutionarily conserved, present among eight or more of the homologous sequences examined, and representing almost one third of the entire protein. These residues tend to lie in or adjacent to putatively functionally significant regions of the protein such as the dioxy iron-binding HXXHH redox center motif of the elongase enzyme and the transmembrane domains (Fig. 3). Seventy-two distinct sequences identified to date in 18 different organisms share at least 23% identity with the human ELOVL4 protein.

This significant level of protein sequence similarity between such diverse species is suggestive of a critical cellular role that has been maintained throughout evolution. A phylogenetic tree depicting the evolutionary relationship between these sequences was generated with the Clustal W (version 1.82) and PHYLIP (version 3.5) programs (Fig. 4). Belonging to distinct clades, each of the human proteins identified appears to be more related to gene products from other species than to other members of the human ELO protein family, whereas many of the putative nonhuman elongases exhibit greater intraspecies similarity and interspecies divergence. In most cases, the invertebrate ELO proteins group according to species, whereas the vertebrate ELOs span a number of distinct clusters (Fig. 4).

Despite sharing certain functional motifs including histidine clusters and ER membrane-spanning domains, fatty acid desaturases, another class of enzymes involved in the process of fatty acid elongation, were not found to be represented among the list of putative ELO proteins. Furthermore, no significant overall sequence similarity was detected between the human ELOVL4 protein and any of the known human desaturase gene products identified to date. Therefore, fatty acid elongases and desaturases represent two independent enzymatic protein families.

Immunodetection of ELOVL4 Protein in Mammalian Retinas

To confirm the existence of ELOVL4 protein homologues in multiple species, we used polyclonal antibodies raised against...
a peptide derived from the human sequence to probe immu-

noblots of total protein extracts prepared from the retinas of

various animals. A single intense band of approximately 37 kDa

was detected in mouse, bovine, feline, and rat retinal extracts

(Fig. 5). A similarly prominent band was detected in protein

extracts prepared from human retinal tissue. Competition as-

says performed using the ELOVL4 antibodies in the presence

and absence of the peptide antigen verified the specificity of

the antibodies for this 37-kDa band, which is consistent with

the predicted molecular weight of the human ELOVL4 protein

(36.8 kDa). Thus, we conclude that the ELOVL4 protein is

present in the retinas of different mammalian species and that
the amino acid sequence corresponding to the peptide antigen used for polyclonal antibody production is conserved between humans, mice, cows, cats, and rats, in which ELOVL4 protein species of the identical size are detected.

**Distribution of ELOVL4 in Mammalian Retinas**

The localization of ELOVL4 proteins in specific cell layers within the retinas of various mammalian species was examined by immunohistochemistry. In human retina tissue sections, strong ELOVL4 immunostaining was observed in the photoreceptor layer, particularly within the inner segments of the photoreceptor cells (Fig. 6A). Uniform staining was detected across the length of this retinal layer in the sections examined and was found in both rod and cone cells. Slight antibody reactivity was also noted in cell processes extending from the outer plexiform layer. Cell bodies were not observed to contain ELOVL4 protein, as evidenced by the absence of immunostaining in this subcellular region. In mouse, bovine, and feline retinal sections, a similar protein localization pattern was observed, with staining above background levels predominantly confined to the photoreceptor inner segment layer (Figs. 6B–E). The intensely pigmented RPE cell layer observed in the mouse, bovine, and feline sections makes it difficult to ascertain whether staining due to the ELOVL4 antibodies is present in this region. However, slight RPE staining was detected in sections derived from melanin-deficient albino rats, indicating localization of ELOVL4 proteins to RPE cells (Fig. 6E). Human retina sections also exhibited increased RPE staining in the presence of the ELOVL4 antisera (Fig. 6A), which is in accordance with the detection of ELOVL4 RNA transcripts in human RPE tissue (Fig. 2). No significant immunostaining was observed in control sections incubated with preimmune

**FIGURE 4.** Phylogenetic tree displaying evolutionary relationships between all known and predicted ELO protein family members found in the GenBank protein sequence database to date. Two- or three-letter species abbreviations precede the protein name or GenBank accession number for each sequence represented. Percentage identity between each sequence and human ELOVL4 (bold) is indicated in parentheses after each name or accession number. The rooted phenogram shown was generated by aligning amino acid sequences using the Clustal W program (ver. 1.82) followed by phylogenetic analysis with PHYLIP (ver. 3.5). Species abbreviations: Hs, *Homo sapiens* (human); Mf, *Macaca fascicularis* (monkey); Mm, *Mus musculus* (mouse); FPV, fowlpox virus; Ag, *Anopheles gambiae* (mosquito); Rn, *Rattus norvegicus* (rat); Sm, *Scophthalmus maximus* (turbot); Dm, *Drosophila melanogaster* (fruit fly); Pp, *Physcomitrella patens* (moss); Ma, *Mortierella alpina* (fungus); Sp, *Schizosaccharomyces pombe* (fission yeast); Sc, *Saccharomyces cerevisiae* (budding yeast); At, *Arabidopsis thaliana* (plant); Os, *Oryza sativa* (rice); Dd, *Dictyostelium discoideum* (amoeba); Ce, *Caenorhabditis elegans* (nematode); Lm, *Leishmania major* (trypanosome); Ig, *Isochrysis galbana* (microalgae).
Consistent with previous reports, we detect both the presumed full-length 2.9-kb mRNA transcript and a shorter 2.4-kb transcript in human retinal tissue. However, we also consistently observe the existence of additional transcripts that bind specifically to ELOVL4 coding sequences. Nucleic acid sequence database searches using the hybridization probe sequence as bait do not reveal any other known genes that are predicted to produce the transcript sizes detected. It is possible that these novel RNA species represent the products of genes that have yet to be defined as ELOVL4 homologues. Multiple paralogues of other genes involved in fatty acid elongation, such as desaturases, have been identified in various species, giving rise to physically and catalytically distinct enzymes. Furthermore, some members of the human ELO gene family share a greater degree of sequence similarity between them than others, suggesting the existence of protein “subfamilies” whose members may exhibit more similar functional characteristics as well. This observation, along with the ongoing discovery of novel human elongase genes (most recently, the ECE gene), may reveal the existence of multiple ELOVL4-like sequences arising from distinct genetic loci. These sequences may give rise to the approximately 4.5- and 0.25-kb transcripts appearing on our Northern blot analysis. The 2.4-kb mRNA species is thought to arise by alternative polyadenylation of the nascent ELOVL4 RNA transcript. Alternative promoter usage is another means by which different transcripts can be produced from a single genetic locus. Thus, both pre- and posttranscriptional processing of as yet unidentified ELOVL4-like genes and gene products may account for the results of Northern blot analysis observed. As these novel transcripts are detected in the retina of different species, the mechanism by which they arise appears also to be conserved. All four of the transcripts are detected in the retina of different species, the mechanism by which they arise appears also to be conserved. All four of the transcripts are seen in tissue derived from both the central and peripheral retina; however, there is more limited gene expression noted in other human ocular tissues. This finding resembles the expression profile of other retinal genes and indicates that retina-specific gene function may be imparted through differential rather than retina-specific gene expression.

Bioinformatic analysis reveals that ELOVL4 protein orthologues have been identified thus far in monkey (Umeda S, et al. ARVO Abstract 2807, 2002) and in mouse, exhibiting 97% and 95% amino acid identity, respectively, with the human

**FIGURE 5.** Western blot analysis of retinal protein extracts derived from various mammalian species demonstrating conserved ELOVL4 protein production. The 37-kDa protein corresponding to the predicted size of ELOVL4 was detected in mouse, bovine, feline, rat, and human retina tissues. Significant reduction in the intensity of the 37-kDa band was observed in human extracts when blots were incubated with primary antibodies in the presence of excess competing peptide antigen (+ peptide), demonstrating specificity of the ELOVL4 polyclonal antibodies.

**FIGURE 6.** Immunohistochemical analysis of mammalian retina sections demonstrating conserved localization of ELOVL4 protein in the inner segments of photoreceptor cells. Retinal sections were incubated with rabbit anti-ELOVL4 polyclonal antibodies (A–E) or preimmune serum (F–J) followed by subsequent detection and visualization with diaminobenzidine (brown) upon light microscopy. (A, F) Human retina sections; (B, G) mouse retina sections; (C, H) cow retina sections; (D, I) cat retina sections; (E, J) rat retina sections. Human retina sections were counterstained with methyl green to visualize cell nuclei (blue-green). RPE, retinal pigment epithelium; OS, photoreceptor outer segments; IS, photoreceptor inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. Bars, 50 µm.
protein. In addition, a wide variety of evolutionarily divergent species also harbor ELOVL4-homologous sequences, suggesting the importance of this gene or similar entities in cellular function. As expected, regions of the protein with the highest degree of conservation coincide with predicted functional motifs. Highly conserved amino acid residues also exist outside of these domains and indicate the significance of other protein regions that have yet to be defined within ELOVL4, such as potential substrate-binding, protein–protein interaction, and regulatory domains.

The ELOVL4 protein is a member of a large family of known and putative fatty acid elongases, sharing from 23% to 97% amino acid sequence identity with 72 different proteins identified in this family to date. Results of phylogenetic analysis indicate that, in general, the intraspecies divergence of vertebrate ELO proteins is greater than that of invertebrate ELOs. Proteins from moss, fungus, plant, nematode, and single-celled organisms cluster according to species, whereas mammalian ELO proteins tend to cluster according to protein family membership instead of species designations. This is evident for all the human ELO protein sequences examined, including ELOVL4, and suggests that the evolutionary divergence of ELOs into subfamilies occurred before the divergence of individual vertebrate species. This, in turn, supports the hypothesis that the genes encoding these proteins have evolved from common ancestral genes that play distinct roles in fatty acid biosynthesis and are part of a highly conserved metabolic mechanism.

The exclusion of fatty acid desaturases from the extensive list of ELOVL4 protein homologues identified indicates that physically distinct enzymes are responsible for catalyzing different reactions in the fatty acid elongation pathway in many species. Clearly, the HXXHH motifs, transmembrane domains, and putative ER-targeting signals are not sufficient to impart significant overall amino acid sequence homology between members of the elongase and desaturase protein families, as might be expected for enzymes that are involved in distinct catalytic activities and that have requirements for recognizing chemically different substrates. This contrasts with other lipid biosynthetic processes such as de novo fatty acid synthesis, in which the fatty acid synthase enzyme consists of multifunctional polypeptides in animal cells but distinct proteins in bacteria. However, it is believed that the reactions constituting the fatty acid desaturation and chain elongation pathway are catalyzed by a closely coordinated or concerted enzyme system.

To further the study of the ELOVL4 protein, we generated and describe herein for the first time polyclonal antibodies that specifically recognize the expected 37-kDa ELOVL4 protein in retinal extracts from different species. Immunohistochemical studies performed with these antibodies revealed conserved subretinal localization of the protein in photoreceptor cells and more specifically within the inner segments of both rods and cones. This finding is consistent with the results of in situ hybridization experiments previously reported. The cellular biosynthetic machinery is located in the inner segments of photoreceptor cells. Accordingly, enzymes involved in various biosynthetic processes in the retina have been localized to this subretinal layer. Thus, it is expected that ELOVL4, an enzyme putatively involved in lipid synthesis, is also found in this subcellular location. Furthermore, the process of fatty acid elongation in which the ELOVL4 protein is thought to function primarily occurs at the ER membrane. This, coupled with the putative ER-localization motif at the carboxyl terminus of the protein sequence predicts that ELOVL4 is found in the ER, which in turn is located in photoreceptor inner segments.

The disease mutations thus far identified in the ELOVL4 gene in STGD3/adMD-affected pedigrees are believed to lead to the production of a prematurely truncated protein without the ER-localization signal, rendering the enzyme dysfunctional as a result of aberrant protein targeting. Photoreceptor inner segment labeling by ELOVL4 antibodies described herein is the first demonstration of subcellular localization of this protein and is consistent with its putative location in the ER within cells.

Several of the disease genes that underlie atrophic macular degeneration phenotypes are expressed in both rod and cone photoreceptors, although the cone-rich macular region is the primary target in these conditions. It is interesting to note that the ELOVL4 gene is also expressed in both types of photoreceptor cells. The ABCA4, RDS, and RPGR genes, whose protein products have been detected in both rods and cones, are also mutated in disorders affecting the peripheral retina. However, no disease-associated sequence alterations in ELOVL4 have as yet been reported in patients with peripheral retina disorders, despite mutational analysis of the gene within such individuals. Elucidation of the pathologic mechanisms by which mutation of each of these genes leads to the development of diseases affecting different regions of the retina awaits further study.

It has been hypothesized that ELOVL4 may be an enzyme responsible for catalyzing an elongation step in the synthesis of docosahexaenoic acid (DHA), the most abundant polyunsaturated fatty acid species in vertebrate retinal lipids. It is also possible that this protein is involved in the synthesis of other essential fatty acids, the deficiency of which may lead to impaired retinal function. Alteration of the lipid environment of retinal cells has been shown to influence retinal protein function, photoreceptor cell development, and cell survival. Thus, dysfunction of a gene that results in aberrant lipid synthesis in the retina is predicted to have deleterious effects and may account for the pathogenesis observed in cases of STGD3/adMD and other retinal dystrophies in which ELOVL4 mutations are identified. Continued characterization of the physical and functional properties of ELOVL4 will provide valuable assistance toward uncovering its precise biological role in the retina, which in turn may lead to the development of potential therapeutic strategies for individuals who have the ocular disorders arising from mutations in the ELOVL4 gene.

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