Dominant Negative Mechanism Underlies Autosomal Dominant Stargardt-like Macular Dystrophy Linked to Mutations in ELOVL4*

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ELOVL4 (elongation of very long chain fatty acids 4) is a member of the ELO family of proteins involved in the biosynthesis of very long chain fatty acids. Protein truncation mutations in ELOVL4 have been identified in patients with autosomal dominant Stargardt-like macular degeneration. To determine whether a dominant negative mechanism is responsible for the autosomal dominant inheritance pattern of this disease, we studied the subcellular localization and interaction of wild type and mutant ELOVL4 in COS-7 and HEK 293T cultured cells by immunofluorescence and co-immunoprecipitation. Wild type ELOVL4 containing an endoplasmic reticulum retention sequence was localized to the endoplasmic reticulum as expected. In contrast, disease-associated C-terminal truncation ELOVL4 mutants accumulated as large inclusions exhibiting aggresome-like characteristics in a juxtanuclear position within COS-7 cells. When the wild type and mutant proteins were co-expressed in cultured cells, wild type ELOVL4 co-purified with mutant ELOVL4 on an immunopurity column and co-localized with the mutant protein in aggresome-like inclusions adjacent to the nucleus. These results indicate that wild type and mutant ELOVL4 form a complex that exhibits an abnormal subcellular localization found for individually expressed mutant ELOVL4. From these studies, we conclude that disease-linked C-terminal truncation mutants of ELOVL4 exert a dominant negative effect on wild type ELOVL4, altering its subcellular localization. This dominant negative mechanism contributes to the autosomal dominant inheritance of Stargardt-like macular dystrophy.

Macular degeneration is a heterogeneous group of retinal disorders that represent a major cause of blindness in the developed world. Macular degeneration is characterized by a marked decrease in central vision associated with a loss in visual acuity and degeneration of photoreceptor and retinal pigment epithelial (RPE) cells in the central retina known as the macula. In a number of cases of macular degeneration, yellow flecks are seen in the fundus resulting from the accumulation of fluorescent lipofuscin deposits in the RPE cells. One such macular degeneration is Stargardt disease (STGD1; OMIM 248200), the most common form of inherited early onset macular degeneration (1). This autosomal recessive disorder has been linked to mutations in ABCA4, an ATP-binding cassette transporter implicated in the removal of retinal derivatives from photoreceptor disc membranes (2–4). Mutations in this same gene have also been implicated in a number of other retinal degenerative diseases, including retinitis pigmentosa, cone-rod dystrophy, and age-related macular degeneration (5–8). The gene responsible for two related forms of autosomal dominant macular degeneration known as Stargardt-like macular dystrophy (OMIM 600110) and autosomal dominant macular dystrophy (OMIM 600100) was recently shown to encode a membrane protein known as ELOVL4 for elongation of very long chain fatty acids 4 (9). The protein consists of 314 amino acids (calculated molecular mass of 36.8 kDa) and is expressed primarily in rod and cone photoreceptor cells (10, 11). A 5-bp deletion (790-794delAAACTT) was found to segregate with the disease (9), and a second mutation, two 1-bp deletions separated by 4 nucleotides (789delT and 794delT), was found in an unrelated pedigree in the same location (12). Both of these mutations cause a frameshift resulting in an almost identical truncated protein product. More recently, a third mutation was identified, a single base substitution encoding a premature stop codon (Y270X) that results in a truncated protein lacking the C-terminal 45 amino acids (13).

The cellular function of ELOVL4 is presently unknown. The strongest clue as to the function of ELOVL4 is its homology (35% amino acid identity) with members of the yeast ELO gene family, known to be involved in the biosynthesis of very long chain fatty acids (14). Other members of this gene family have been studied in rodents, where they are necessary for fatty acid elongation (15, 16). The homology of these fatty acid elongation proteins is striking, with human ELOVL4 containing all three features typical for members of this protein family: hydrophobic core, a dideoxy binding motif (HXXHH) (17), and a dilysine endoplasmic reticulum (ER)-retention motif (KXXX) (18). Several diseases have been associated with defects in long chain fatty acid elongation including adrenoleukodystrophy (19) and adrenomyeloneuropathy (20). The identification of pathogenic mutations in ELOVL4 implicates fatty acid biosynthesis in macular dystrophies for the first time. The lipid environment in photoreceptors is crucial for their normal function, and alterations in the lipid content have been observed in several retinal degenerative disorders (21–26). It is possible that ELOVL4 may be involved in the biosynthesis of docosahexaenoic acid from dietary α-linolenic acid (27), where docosahexaenoic acid has been shown to account for 33–50% of the fatty acid content of vertebrate photoreceptor outer segments (28, 29).

Previous studies have suggested that the subcellular localization of wild type (WT) ELOVL4 may be crucial for its putative function. Heterologous expression of ELOVL4 in several cell lines has confirmed that
ELOVL4 is predominantly localized to the ER, the site of very long chain fatty acid biosynthesis. The effect pathogenic mutations in ELOVL4 have on the localization of the heterologous protein, however, has produced conflicting reports. One study showed that the truncated mutant proteins resulting from the 5- and 2-bp deletions were retained in the Golgi (30), possibly in the trans-Golgi network. Other studies have reported that disease-associated ELOVL4 mutants do not co-localize with the Golgi but instead are present as cytoplasmic aggregates (13, 31).

It is unclear at present what underlies the mechanism of disease in patients with mutations in ELOVL4. To better understand the functional role of ELOVL4 in retinal cell biology and in the development of autosomal dominant macular degeneration, we have examined the biochemical properties and localization of WT and disease-associated mutants of ELOVL4 in a cell culture model and in retina tissue. In this study, we show that ELOVL4 is a membrane glycoprotein that exists as a multisubunit complex. Disease-associated ELOVL4 mutants interact with WT ELOVL4, resulting in an abnormal localization of WT ELOVL4 in cells. Our studies indicate that pathogenic ELOVL4 mutants exert a dominant negative effect on WT ELOVL4, and this mechanism is responsible for the autosomal dominant inheritance pattern of Stargardt-like macular dystrophy.

**EXPERIMENTAL PROCEDURES**

**Construction of ELOVL4 Plasmids**—Full-length ELOVL4 cDNA was isolated from human retinal RNA by reverse transcription-PCR, using the first strand cDNA synthesis kit (Amersham Biosciences). Restriction endonuclease recognition sites were introduced using PCR. The cDNA was cloned into the KpnI and BamHI sites of the pCEP4 vector (Invitrogen) and the BamHI site of the pEGFP-C1 vector (Clontech, Palo Alto, CA) in frame with the C terminus of enhanced GFP. Immunofinity tags were added to the N terminus of ELOVL4 in the pCEP4 vector using modified primers. 3F4-ELOVL4 contained a 9-amino acid 3F4 epitope tag (YDLPHPRT), and Myc-ELOVL4 contained a 10 amino acid Myc tag (EQKLISEEDL). The ELOVL4 mutants T22A, Y270X, acid Myc tag (EQKLISEEDL). The ELOVL4 mutants T22A, Y270X, 5-bp deletion (790_794delAACTT), and 2-bp deletion (789delT and 794delT) were generated using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s instructions using the WT ELOVL4 in pEGFP-C1 and 3F4-ELOVL4 in pCEP4 as templates. All of the constructs were verified by DNA sequencing.

**Generation of ELOVL4 Monoclonal Antibodies**—The C-terminal 46 amino acids of human ELOVL4 were cloned in frame with the C terminus of glutathione S-transferase in the pGEX-4T-1 vector (Amersham Biosciences). Mice were immunized with fusion protein that had been purified on glutathione-agarose beads (Sigma). Hybridoma cell lines were generated as previously described (32) and screened for specificity to ELOVL4 using Western blots of purified 3F4-ELOVL4. The epitopes of antibodies were mapped using the SPOTS kit (Sigma Genosys, The Woodlands, TX) according to the manufacturer’s protocol. Synthetic peptides (9 amino acids long, 2-amino acid overlap) spanning the C-terminal 46 amino acids of ELOVL4 were coupled to a cellulose membrane, and their immunoreactivity was detected by enhanced chemiluminescence.

**Immunofluorescence Labeling of Human Retina**—Human retina tissue was processed for immunofluorescence as described previously (33). Sections were blocked and permeabilized for 20 min at 22 °C in PB (0.1 M phosphate buffer, pH 7.4) containing 10% (v/v) normal goat serum and 0.2% (v/v) Triton X-100 and incubated with Elo 6F1 hybridoma supernatant (1:5) or purified Elo 6F1 (1:100) overnight in PB containing 2.5% (v/v) normal goat serum (antibody buffer) at 22 °C. Double-labeled sections were also incubated with JH 492 (1:6000), a polyclonal antibody specific for green/red cone opsin generously provided by Jeremy Nathans. Sections were washed with PB-T (PB containing 0.1% (v/v) Tween 20) and labeled for 1 h with Alexa 488-conjugated goat anti-mouse Ig and Alexa 594-conjugated goat anti-rabbit Ig secondary antibody (1:2000; Molecular Probes, Burlington, ON) in antibody buffer at 22 °C. Control samples were incubated with the secondary antibody only. Labeled retinal sections were visualized using a Zeiss LSM 510 Meta confocal microscope.

**Cell Culture and Transfection**—HEK 293T and COS-7 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium with 10% (v/v) bovine growth serum (Invitrogen). HEK 293T cells were transfected in one 10-cm dish with 30 μg of plasmid DNA using calcium phosphate (34) and harvested 24 h post-transfection. For co-transfection of Myc- and 3F4-tagged proteins, 15 μg of each plasmid were combined and added to one 10-cm dish. For control single transfections, 15 μg of empty pCEP4 vector was co-transfected with 15 μg of the ELOVL4 plasmid to ensure that the DNA concentrations were kept constant.

**Immunofluorescence Labeling of Cells**—HEK 293T and COS-7 cells were grown on glass coverslips and transfected using calcium phosphate. The pCEP4 and pEGFPC1 plasmid constructs were co-transfected in a 5:1 ratio. Twenty-four h post-transfection, cells were washed in PB and fixed for 10 min in methanol at −20 °C or for 20 min in 4% paraformaldehyde in PB at 22 °C. Cells were then blocked and permeabilized for 30 min at 22 °C in PB containing 10% (v/v) normal goat serum and 0.1% (v/v) Triton X-100 and treated with primary antibodies for 1 h at 22 °C in antibody buffer. The Rim 3F4 antibody (35) was diluted at 1:2, and Elo 6F1 and 9E10 antibody (for the detection of the Myc epitope) were used at a 1:5 dilution. The following dilutions of antibodies were also used: monoclonal antibodies Vimentin clone V9 (1:100), β-COP clone MaD (1:50) (Sigma), and golgin-97 (1 μg/ml) (Molecular Probes); polyclonal rabbit anti-calnexin 1:200 (Stressgen, Victoria, Canada) and rabbit anti-Myc 1:100 (Sigma). Goat anti-mouse Ig and goat anti-rabbit Ig secondary antibodies coupled with either Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) or Alexa 488, were added in antibody buffer for 1 h at 22 °C at 1:1000. Immunofluorescent labeling was visualized using a Zeiss Axioplan2 fluorescence microscope.

**Immunoprecipitation of Heterologously Expressed ELOVL4 Proteins**—Purified Rim 3F4 antibody was coupled to CNBr-activated Sepharose 4B beads as described previously (35). Transfected HEK 293T cells were harvested from the dishes washed with 20 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TBS); and solubilized with 18 mM CHAPS in TBS with protease inhibitors for 30 min at 22 °C. The cell extract was centrifuged at 120,000 × g for 10 min, and the soluble fraction (precolumn) was incubated for 1 h with Rim 3F4-Sepharose beads prewashed with column buffer (12 mM CHAPS in TBS). The unbound fraction was retained, and the immunoaffinity matrix was washed extensively in an Ultrafree filter unit (Millipore Corp., Billerica, MA) with column buffer. The bound protein was eluted from the beads with 60 μl of competing Rim 3F4 peptide (0.2 mg/ml) in column buffer followed by further elution with 60 μl of 1% (w/v) SDS in TBS. The fractions were analyzed by Western blotting.

**Endoglycosidase Digestion**—The total membrane fractions of bovine retina and transfected HEK 293T cells were isolated as described previously (36). Membranes were treated with N-glycosidase F and endoglycosidase H (New England Biolabs, Beverly, MA) according to the manufacturer’s instructions but with modifications. Briefly, washed membranes from bovine retina or HEK 293T cells were resuspended in 20 μl of 20 mM Tris-HCl, pH 7.4, incubated with 2 μl of 10× denaturing buffer for 10 min at 32 °C, and subsequently added to either 3 μl of 10× G5 buffer, 3 μl of 20 mM Tris-HCl, pH 7.4, and 2 μl of endoglycosidase H or 3 μl of 10% Nonidet P-40, 3 μl of 10× G7 buffer, and 2 μl of N-glycosidase F. Control samples were set up in parallel without the
enzyme. After a 2-h incubation at 32 °C, the samples were added to an equal volume of SDS-PAGE loading buffer, and 20 μl of each sample was analyzed by SDS-PAGE and Western blotting.

**Gel Filtration Chromatography of ELOVL4**—Bovine retinal membranes or HEK 293T membranes expressing 3F4-ELOVL4 were solubilized in TBS containing 18 mM CHAPS and 1 mM dithiothreitol on ice for 30 min. The samples were centrifuged at 100,000 × g for 15 min at 4 °C and loaded onto a Superdex 200 10/30 GL column (Amersham Biosciences), equilibrated with TBS containing 12 mM CHAPS and 1 mM dithiothreitol. The elution was performed using the same buffer at 0.5 ml/min. Fractions (250 μl) were collected and analyzed by dot blotting and Western blotting using Rim 3F4 and Elo 8E4 antibodies. The column was calibrated using thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), transferrin (81 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and myoglobin (17.6 kDa). The void volume was determined using blue dextran (2000 kDa; Amersham Biosciences). The amount of ELOVL4 immunoreactivity in each fraction was determined by densitometry and expressed as a percentage of the total immunoreactivity.

**Sucrose Velocity Sedimentation**—Bovine ELOVL4, 3F4-ELOVL4, and 3F4-ELOVL4-Del were applied to a 5–20% (w/w) linear sucrose gradient containing 12 mM CHAPS and 1 mM dithiothreitol in TBS at 214,000 × g and 4 °C for 6 h. Bovine ELOVL4 and 3F4-ELOVL4 membranes were solubilized as for gel filtration. 3F4-ELOVL4 and 3F4-ELOVL4-Del were immunoprecipitated as described above. Three drop fractions were collected from the bottom of each gradient and analyzed by dot blotting with Elo 8E4 and Rim 3F4 antibodies. Relative immunoreactivity of each fraction was quantified by densitometry.

**SDS-PAGE and Western Blotting**—Samples denatured in SDS-PAGE buffer (10 mM Tris-HCl, pH 6.8, 4% SDS, 20% sucrose, and 4% β-mercaptoethanol) were separated by SDS-PAGE on 12% resolving gels and electroblotted onto Immobilon-FL membranes (Millipore) for antibody labeling. Following blocking for 30 min at 22 °C in 0.5% skimmed milk in PBS, blots were incubated with hybridoma supernatants for 1 h at 22 °C in 0.5% skimmed milk in PBS containing 0.05% Tween 20 (PBS-T) at the following dilutions: Rim 3F4 (1:20), Elo 6F1 (1:10), Myc 9E10 (1:10), and Elo 8E4 (1:5). Subsequently, the blots were labeled for 1 h at 22 °C with anti-mouse Ig conjugated with either Alexa 680 or LI-COR IRDye™ 800 (Rockland, Gilbertsville, PA) diluted 1:15,000 in 0.5% skimmed milk in PBST containing 0.02% SDS. Bands were visualized by infrared scanning using the LI-COR Odyssey® system (Lincoln, NE). Concanavalin A labeling was carried out as described previously (36).

**RESULTS**

**Monoclonal Antibodies against ELOVL4**—Monoclonal antibodies Elo 6F1 and Elo 8E4 were raised against the C terminus of human ELOVL4 (amino acid residues 268–314) expressed as a glutathione S-transferase fusion protein. Both antibodies labeled a 32-kDa protein in human retinal extracts and membranes from HEK 293T cells expressing the human ELOVL4 protein (Fig. 1A). Only the Elo 8E4 antibody labeled a 32-kDa protein in bovine retinal membranes. Neither antibody labeled a corresponding protein in mouse retinal extracts.

The predicted molecular mass of ELOVL4 is 36.8 kDa, slightly larger than the 32-kDa protein band detected by Western blotting. To confirm that the 32-kDa protein is ELOVL4, membrane proteins from HEK 293T cells expressing the Myc-ELOVL4 protein were separated on SDS gels, and Western blots were labeled with the 9E10 antibody to detect the N-terminal Myc tag and the Elo 6F1 and Elo 8E4 antibodies (Fig. 1B). A single band migrating with the same apparent molecular weight was detected with each antibody in Myc-ELOVL4-transfected but not mock-transfected cells. No bands were detected when the secondary antibody was used alone or when the Elo 6F1 and Elo 8E4 antibodies were preadsorbed with the glutathione S-transferase fusion protein prior to labeling confirming the specificity of the antibodies (data not shown).

The epitopes of the ELOVL4 monoclonal antibodies were identified using synthetic peptides. The binding site for the Elo 6F1 mapped to the sequence SEKQLMI (amino acids 294–300), and Elo 8E4 mapped to the sequence QKNKGAK (amino acids 306–312).

**Distribution of ELOVL4 in Human Retina**—The expression pattern of ELOVL4 in human retina was examined by immunofluorescence using the Elo 6F1 antibody (Fig. 2A). Intense labeling was observed in the photoreceptor inner segment layer. The inner segment signal appeared to be confined to the myoid region adjacent to the outer limiting membrane, where the ER is abundant. Staining of the photoreceptor cell bodies in the outer nuclear layer was also observed, extending to the synapses in the outer plexiform layer. No staining was observed in the photoreceptor outer segments or the layers of the inner retina, but the ganglion cells were moderately labeled.

High acuity vision is mediated by cone photoreceptors in the central region of the retina. Since Stargardt macular dystrophy affects cone-dominant central vision, it was of interest to determine whether ELOVL4 was present in cone photoreceptors of the central retina. To determine whether ELOVL4 is present in these cones, we compared the cellular localization of ELOVL4 with green/red cone opsin in a cone-rich area of the central retina known as the parafovea (Fig. 2B). Labeling was observed in the inner segment of cone as well as rod photoreceptors, most evident when examined at higher magnification (Fig. 2B, Merge, inset). However, ELOVL4 labeling in cones appeared less intense than in rod photoreceptors. No labeling was observed when the primary antibodies were omitted (data not shown).

**ELOVL4 Is Modified by N-Linked Oligosaccharides**—Integral membrane proteins are commonly subjected to co-translational core N-glycosylation (37). To determine whether ELOVL4 is glycosylated, membranes from bovine retina and HEK 293T cells expressing 3F4-ELOVL4 were treated with the endoglycosidase enzymes N-glycosidase F and endoglycosidase H and subsequently analyzed by Western blotting (Fig. 3A). After treatment with either of the glycosidases, both native (bovine retina) and expressed ELOVL4 (3F4-ELOVL4) showed a slightly increased mobility, suggesting that ELOVL4 is N-linked glycosylated. Both native and expressed ELOVL4 were sensitive to endoglycosidase H.
as well as N-glycosidase F (Fig. 3A), indicating that the oligosaccharide modification is a simple hybrid or high mannose addition, since complex oligosaccharide side chains are usually insensitive to endoglycosidase H cleavage.

Inspection of the ELOVL4 amino acid sequence revealed a single consensus N-linked glycosylation motif (NX(T/S)) at amino acids 20–22 (NDT) that was highly conserved in ELOVL4 orthologues (10). To confirm that this motif is glycosylated, a T22A mutation was introduced into the N-linked consensus sequence of 3F4-ELOVL4. As shown in Fig. 3B, the T22A had the same mobility as the deglycosylated WT protein, and no detectable shift in the 3F4-ELOVL4-T22A protein was observed upon glycosidase treatment. To further confirm the glycosylation state of ELOVL4, the WT and mutant proteins were purified on Rim 3F4-Sepharose and analyzed by Western blotting. The wild type and T22A mutant protein were purified with the Rim 3F4 antibody or concanavalin A. Only the purified wild type protein was labeled with concanavalin A. The slower migrating bands observed in the eluates correspond to the expected molecular weight of an ELOVL4 dimer.

These results indicate that the N-terminal affinity tags used in this study do not alter the ER distribution of ELOVL4.

The localization of nonglycosylated 3F4-ELOVL4-T22A was examined in COS-7 cells (Fig. 4B). The subcellular distribution of the protein was comparable with WT ELOVL4. Furthermore, the T22A mutant protein also co-localized with calnexin, a transmembrane protein resident in the ER, the immunofluorescence signals overlapped (Fig. 4A). When the cells were double-labeled with Rim 3F4 and Myc-ELOVL4, the tagged WT ELOVL4 labeled with the Elo 6F1 antibody exhibited a similar ER distribution (data not shown). The subcellular localization of ELOVL4 mutants was confirmed by co-immunoprecipitation studies using WT ELOVL4 tagged at the N terminus with a 3F4 epitope tag (3F4-ELOVL4) and a Myc epitope tag (Myc-ELOVL4). We first determined whether the individually expressed epitope-tagged proteins could interact to form a stable complex. Cells individually expressing the 3F4-ELOVL4 and Myc-ELOVL4 were combined post-transfection, solubilized in CHAPS, and immunoprecipitated with Rim 3F4-Sepharose matrix (Fig. 5A). To confirm that the 3F4 and Myc affinity tags used in this study do not interfere with the localization of ELOVL4, we visualized the distribution of these constructs by immunofluorescence microscopy. Both 3F4-ELOVL4 and Myc-ELOVL4 expressed in COS-7 cells localized in a perinuclear and reticular pattern throughout the cell (Fig. 4A). The untagged WT ELOVL4 labeled with the Elo 6F1 antibody exhibited a similar ER distribution (data not shown). The localization of ELOVL4 mutants was confirmed by co-immunoprecipitation studies using WT ELOVL4 tagged at the N terminus with a 3F4 epitope tag (3F4-ELOVL4) and a Myc epitope tag (Myc-ELOVL4). We first determined whether the individually expressed epitope-tagged proteins could interact to form a stable complex. Cells individually expressing the 3F4-ELOVL4 and Myc-ELOVL4 were combined post-transfection, solubilized in CHAPS, and immunoprecipitated with Rim 3F4-Sepharose matrix (Fig. 5A). To confirm that the 3F4 and Myc affinity tags used in this study do not interfere with the localization of ELOVL4, we visualized the distribution of these constructs by immunofluorescence microscopy. Both 3F4-ELOVL4 and Myc-ELOVL4 expressed in COS-7 cells localized in a perinuclear and reticular pattern throughout the cell (Fig. 4A). When the cells were double-labeled with calnexin, a transmembrane protein resident in the ER, the immunofluorescence signals overlapped (Fig. 4A). The untagged WT ELOVL4 labeled with the Elo 6F1 antibody exhibited a similar ER distribution (data not shown). The possibility that the unglycosylated protein is misfolded and retained in the ER as part of a misfolded protein response, such as ER-associated degradation (38), and is not exhibiting a normal distribution pattern cannot be ruled out.

ELOVL4 Exists as a Multisubunit Complex—To determine whether WT ELOVL4 exists as a monomer or multimeric complex, we carried out co-immunoprecipitation studies using WT ELOVL4 tagged at the N terminus with a 3F4 epitope tag (3F4-ELOVL4) and a Myc epitope tag (Myc-ELOVL4). We first determined whether the individually expressed epitope-tagged proteins could interact to form a stable complex. Cells individually expressing the 3F4-ELOVL4 and Myc-ELOVL4 were combined post-transfection, solubilized in CHAPS, and immunoprecipitated with Rim 3F4-Sepharose matrix (Fig. 5A). To
was found in the precolumn and unbound fractions but not in the bound, eluted fractions. In contrast, 3F4-ELOVL4 was efficiently immunoprecipitated and present predominantly in the eluted fractions (Fig. 5A). These results indicate that individually expressed 3F4 and Myc-tagged ELOVL4 do not associate together to form a complex.

The inability of individually expressed 3F4 and Myc-tagged ELOVL4 proteins to interact with each other may be due to the fact that they exist as monomers or that they already exist as a multisubunit complex. We therefore determined whether 3F4-ELOVL4 and Myc-ELOVL4 associate when co-expressed. HEK 293T cells were co-transfected with the two constructs, solubilized in CHAPS, and applied to a Rim 3F4-Sepharose column. The precolumn and eluted (peptide and SDS-eluted) fractions were analyzed by Western blotting (Fig. 5B). Most of 3F4-ELOVL4 was present in the eluted fractions. A significant fraction (~50%) of Myc-ELOVL4 co-immunoprecipitated with 3F4-ELOVL4, indicating that ELOVL4 exists as a multisubunit complex (Fig. 5B). When Myc-ELOVL4 alone was applied to Rim 3F4-Sepharose, no protein was detectable in the eluted fractions, indicating that the Myc-ELOVL4 does not bind nonspecifically to the Rim 3F4-Sepharose matrix (Fig. 5B).

To further estimate the size of the ELOVL4 complex, we analyzed the CHAPS-solubilized ELOVL4 complex by gel filtration chromatography. Isolated membranes from bovine retina were solubilized in CHAPS and fractionated on a Superdex 200 size exclusion column. Bovine ELOVL4 from retina eluted just after the void volume as a relatively broad peak, indicating that the CHAPS-solubilized ELOVL4 exists as a high molecular weight, heterogeneous detergent-protein complex (Fig. 6). A similar profile was observed for 3F4-ELOVL4 from solubilized membranes of transfected HEK 293T (Fig. 6).

Interaction of WT and Mutant ELOVL4—Mutations in ELOVL4 are associated with an autosomal dominant pattern of inheritance, raising the possibility of a dominant negative disease mechanism. Since WT ELOVL4 self-associates to form a multisubunit complex, we investigated whether WT and disease-linked ELOVL4 mutants also associate. Myc-ELOVL4 and the 5-bp deletion mutant 3F4-ELOVL4-Del were co-transfected into HEK 293T cells and purified using Rim 3F4-Sepharose. The 3F4-ELOVL4-Del mutant was found predominantly in the eluted fractions (Fig. 7A). A significant proportion of Myc-ELOVL4 co-immunoprecipitated with the mutant protein. This finding suggests that WT and mutant ELOVL4 interact and form a stable complex. The site of interaction is not within the C-terminal 51 amino acids of ELOVL4, since these residues are absent from the truncated mutant protein.
To determine whether the \( N \)-linked glycosylation of ELOVL4 is important for the assembly of the ELOVL4 complex, co-immunoprecipitation studies were carried out with WT Myc-ELOVL4 (glycosylated) and 3F4-ELOVL4-T22A mutant (unglycosylated) co-expressed in HEK 293T cells (Fig. 7B). Approximately 50% of the Myc-ELOVL4 was detected in the eluted fractions from the 3F4-Sepharose matrix, similar to the levels of Myc-ELOVL4 that co-precipitated with WT 3F4-ELOVL4. No Myc-ELOVL4 was detectable in the eluted fractions when Myc-ELOVL4 was expressed individually and applied to Rim 3F4-Sepharose (Fig. 7B). These results indicate that subunit assembly of the ELOVL4 complex is independent of the glycosylation state of ELOVL4.

We further determined whether the pathogenic mutations in ELOVL4 affect the hydrodynamic properties of the protein-detergent complex by velocity sedimentation. Fig. 8A shows that ELOVL4 from CHAPS-solubilized bovine retina and 3F4-ELOVL4 from CHAPS-solubilized HEK 293T cells both sedimented as a relatively broad peak, consistent with gel filtration studies. Immunoprecipitated WT 3F4-ELOVL4 and mutant 3F4-ELOVL4-Del were also analyzed (Fig. 8B). Similar sedimentation profiles were observed, suggesting that the CHAPS-extracted mutant ELOVL4, like WT ELOVL4, consists of a heterogeneous mixture of complexes.

Localization of Disease-linked ELOVL4 Mutants—Previous studies have suggested that disease-linked ELOVL4 mutant protein does not localize to the ER (13, 30, 31), ostensibly due to the absence of the \( \text{KXXX} \) C terminus ER retention motif. However, the distribution of the mutant protein has been controversial. One group reported that the mutant is confined to the \( \text{trans}-\text{Golgi} \) network (30), whereas another studies showed that it is present in the cytoplasm as large aggregates or inclusions (13, 31). We have investigated the localization of the three identified disease-linked variants in COS-7 and HEK 293T cells with either an \( N \)-terminal GFP or 3F4 tag.

In contrast to the ER localization of the WT protein (Fig. 9, A and B), the 5-bp deletion mutant GFP-ELOVL4-Del was present in a juxtanuclear position (Fig. 9, C and D) in the majority of cells, suggestive of its presence within an intracellular inclusion or within the Golgi. To further investigate the mislocalized mutant protein, GFP-ELOVL4-Del and GFP-ELOVL4 were double-labeled with Golgi-specific markers \( \beta \)-COP and golgin-97. The WT protein showed no overlapping localization with the \( \text{trans}-\text{Golgi} \) network protein golgin-97 but a small amount of co-localization with \( \beta \)-COP, a protein involved in membrane trafficking between the ER and Golgi and between different membranes of the Golgi (39). This could also represent a concentration of ER staining in this locale, rather than Golgi localization. The mutant protein showed partial co-localization with both golgin-97 and \( \beta \)-COP (Fig. 9, C and D). However, the \( \beta \)-COP staining became scattered (Fig. 9D), indicating that the morphology of the Golgi apparatus had been disrupted in these cells. In cells expressing lower levels of GFP-ELOVL4-Del, the mutant protein was distributed throughout the ER (data not shown), perhaps representing newly synthesized polypeptides on their way through the biosynthetic pathway or mutant protein retained in the ER for degradation (40).

Mutant ELOVL4 appeared to share similarities with intracellular inclusion bodies known as aggresomes (41–43). One of the most common features of aggresome formation observed in cell culture models is the rearrangement of the intermediate filament network. We therefore performed double labeling experiments using GFP-ELOVL4 fusion proteins and vimentin, a major component of intermediate filament cytoskeleton. WT GFP-ELOVL4 did not co-localize with vimentin in COS-7 (Fig. 10A) or HEK 293T cells (data not shown). In cells transfected with GFP-ELOVL4-Del, the intermediate filaments were severely
disrupted and collapsed to form a structure that co-localized with the mutant ELOVL4. This characteristic redistribution of vimentin has been observed in many aggresome-like structures (41, 43–45) and suggests that mutant ELOVL4 may form aggresome-like inclusions rather than accumulate in the Golgi.

Co-localization of WT and Mutant ELOVL4 Mutants to Inclusion Bodies—As indicated above, WT and disease-linked ELOVL4 mutants interact to form a complex when co-expressed in cultured cells. To determine whether mutant ELOVL4 affects the ER localization of WT ELOVL4, we examined the distribution of WT and mutant ELOVL4 in co-transfected COS-7 cells. When GFP-ELOVL4 and Myc-ELOVL4 were co-expressed, both proteins localized to the ER (Fig. 11A), consistent with the expected distribution of WT ELOVL4. However, when Myc-ELOVL4 was co-transfected with the GFP-ELOVL4-Del mutant, the Myc-ELOVL4 protein accumulated in a juxtanuclear position that partially co-localized with the Golgi markers (Fig. 11C and D), and dispersed the β-COP staining (D). Scale bar, 5 μm.

To confirm that the aberrant co-localization of the WT and mutant proteins was not influenced by the presence of the large GFP tag of the fusion protein, we also tested the co-expression of 3F4 and Myc-tagged proteins. We used an equal concentration of the two plasmids to reflect the levels of heterozygous ELOVL4 alleles in patients with autosomal dominant macular degeneration. When the two WT constructs were co-expressed in COS-7 cells, both proteins co-localized in the usual distribution pattern (Fig. 11D). However, when 3F4-ELOVL4-Del was co-expressed with Myc-ELOVL4, both proteins were detected in the juxtanuclear inclusion (Fig. 11E). The same phenomenon was observed in cells co-transfected with WT and the 2-bp deletion or Y270X mutant ELOVL4 in either COS-7 or HEK 293T cells (data not shown). These studies indicate that the interaction of mutant ELOVL4 with WT ELOVL4 alters the subcellular localization of the WT protein.

DISCUSSION

C-terminal truncation mutations in ELOVL4 have been identified in patients with an autosomal dominant form of macular degeneration known as Stargardt-like macular dystrophy; however, the mechanism underlying this retinal disease is presently unknown. The dominant disease phenotype in affected individuals could arise from a toxic gain of function of the mutant protein, a dominant negative effect of the mutant allele on the WT allele, or both or simply haploinsufficiency as in the
case of selected mutations in peripherin/rds linked to autosomal dominant forms of retinitis pigmentosa and macular dystrophy (46, 47). To address this issue, we have studied the cellular and subcellular localization and interaction of WT and disease-associated ELOVL4 variants by immunofluorescence and co-immunoprecipitation techniques using newly developed monoclonal antibodies and epitope-tagged constructs.

The localization of ELOVL4 and Elovl4 mRNA in developing mouse retina using immunohistochemistry (11) and in situ hybridization (10) indicates that ELOVL4 is predominantly expressed in ganglion cells of embryonic and postnatal retina and subsequently switches to photoreceptors later in development. In studies of adult human retina and retina from other species, ELOVL4 staining is predominantly found within the inner segments of rod and cone photoreceptors (30, 48). Here we have demonstrated that ELOVL4 is expressed not only in photoreceptors of the peripheral retina but also in rod and cone photoreceptor cells of the macular and parafoveal regions of the retina, the area of the retina associated with autosomal dominant Stargardt-like disease (49, 50). The most intense immunostaining was found in the myoid region of the inner segments, but staining extended throughout the cell bodies to the synaptic terminals. We also observed significant labeling of the ganglion cell layer using the Elo6F1 monoclonal antibody, a finding not described previously. There has been no report of ganglion cell involvement in patients with ELOVL4-mediated autosomal dominant macular dystrophy to date, so the significance of ELOVL4 expression in ganglion cells of adult retina requires further study.

Currently, there is little published information on the structural properties of ELO family members from yeast or mammals. In this study, we have used endoglycosidase digestion and site-directed mutagenesis to demonstrate that ELOVL4 contains a single N-linked oligosaccharide near its N terminus. This modification is not essential for protein expression or localization, since the T22A glycosylation-deficient mutant is expressed at WT levels and, like the WT protein, localizes to the ER. We also examined the oligomeric state of ELOVL4 by carrying out co-immunoprecipitation experiments with heterologously expressed ELOVL4 proteins co-purified on immunoaffinity resins when co-expressed in HEK 293T cells, indicating that WT ELOVL4 forms a stable multisubunit complex. The glycosylation-deficient T22A ELOVL4 mutant also co-purified with WT ELOVL4, suggesting that the glycosylation state of ELOVL4 is not important for self-association. We further investigated the oligomerization of ELOVL4 by gel filtration chromatography and found that the protein-detergent complex of both native bovine ELOVL4 and heterologously expressed ELOVL4 exist as heterogeneous high molecular weight complexes. Some of the heterogeneity could be due to protein aggregation, because these proteins are extremely hydrophobic. To date, there is little information on the biochemical properties of mammalian elongase enzymes, and the oligomerization state of these enzymes remains to be determined. However, our analysis showing that detergent-solubilized ELOVL4 exists as a heterogeneous high molecular weight complex is in general agreement with studies on various plant elongases showing that these detergent-solubilized enzymes are also highly heterogeneous as measured by gel filtration chromatography (51–53). Further studies are needed to determine whether the high molecular weight ELOVL4 complexes observed by gel filtration chromatography and velocity sedimentation are active in the elongation of very long chain fatty acids.

The formation of oligomeric complexes may be necessary for the functioning of ELOVL4 as a putative elongase enzyme in the biosynthesis of very long chain fatty acids.

Importantly, we have shown that disease-associated ELOVL4...
mutants co-assembled with WT ELOVL4 by co-immunoprecipitation. As a result of this interaction, WT ELOVL4 no longer localized to the ER but instead was recruited by the mutant ELOVL4 to inclusions in a juxtanuclear position within the cell, the same subcellular localization observed for individually expressed ELOVL4 mutants. Thus, the mutant ELOVL4 through its interaction with WT ELOVL4 exerts a dominant negative effect on the cellular trafficking of WT ELOVL4, causing it to mistarget to inclusion bodies in cells. The ER localization of WT ELOVL4 is presumably essential for its putative function in the elongation of very long chain fatty acids, since the ER has been identified as the site of very long chain fatty acid biosynthesis (54).

There has been some disagreement on the subcellular localization of individually expressed disease-linked ELOVL4 variants lacking the ER retention motif. One earlier study suggested that mutant ELOVL4 is retained in the trans-Golgi network (30). Other studies have shown no co-localization of mutant ELOVL4 with Golgi markers but, instead, showed the presence of dense aggregates of mutant ELOVL4 within the cytoplasm (13, 31). Our results are generally consistent with the latter study. We found that the mutant ELOVL4 partially overlapped with markers for the Golgi and trans-Golgi network, but the mutant protein appeared to displace the Golgi staining, particularly β-COP. A similar phenomenon has been described for misfolded proteins sequestered in inclusion bodies formed around the microtubule-organizing center, known as aggresomes (41, 43). Consistent with these previous studies, we also found that mutant ELOVL4 caused a characteristic collapse in the distribution of vimentin, suggesting that ELOVL4 does not necessarily accumulate in the Golgi but instead may form aggresome-like inclusion bodies. Ultrastructural analysis by electron microscopy should provide further insight into the exact milieu of the accumulated mutant protein.

There have been no developmental abnormalities observed in patients harboring mutations in ELOVL4, suggesting that either one functional allele is sufficient for fulfilling the developmental requirement of ELOVL4 protein or that ELOVL4 is not required for early development of photoreceptors. The mechanism underlying the progression of the disease in affected individuals is therefore open to speculation. Intracellular inclusions have been described in cell culture models of retinitis pigmentosa (43, 45) but have not been reported in degenerating photoreceptors in human patients or animal models of retinitis pigmentosa. One explanation could be that photoreceptors are very efficient at eliminating misfolded proteins, thereby preventing their accumulation. Alternatively, the presence of misfolded mutant protein could be more toxic to photoreceptors than cultured cells and cause the photoreceptors to die before the accumulated protein can be detected. Indeed, Karan et al. (31) have reported an increased incidence of apoptosis in cells heterologously expressing mutant ELOVL4 compared with WT protein. Whether a similar mechanism exists in photoreceptors is unclear.

The mislocalization of ELOVL4 may not be directly toxic to photoreceptors and the development of Stargardt-like macular dystrophy could be due to the consequences of altered ELOVL4 function. Each gene in the ELO family is thought to encode a single enzyme component of complex enzymatic systems that function in the elongation of long chain fatty acids (54). ELOVL4 may be involved in the condensation reaction steps involved in docosahexaenoic acid biosynthesis; however, the putative functional role of ELOVL4 in lipid metabolism, particularly in the retina, has yet to be established. The removal of misfolded mutant ELOVL4 by photoreceptor quality control machinery could lead to the removal of mislocalized WT ELOVL4, thus diminishing the levels of functional ELOVL4 residing in the ER, and/or the complex containing mutant and WT ELOVL4 may be nonfunctional. Since photoreceptors are constantly renewing their outer segments, the metabolic requirement for very long chain fatty acids synthesized in part by ELOVL4 may leave the photoreceptors vulnerable to damage. The lack of ELOVL4 function could lead to the altered lipid composition of outer segments, either by the accumulation of its substrate(s) or by a deficiency of its elongated fatty acid product. This in turn could lead to the altered function of other outer segment proteins (55–57), leading to photoreceptor degeneration.

The phenotype of patients with ELOVL4-associated autosomal dominant Stargardt-like disease is broadly similar to the phenotype of patients with ABCA4-associated autosomal recessive Stargardt disease (STGD1), including the presence of yellow flecks in the central retina at the level of the RPE cells and atrophy of the central photoreceptors and RPE cells. ABCA4 is a member of the ATP-binding cassette (ABC) family of transport proteins (1, 35). It has been suggested to function in the removal of retinylidene-PE from disc membranes following the photobleaching of rhodopsin (2–4). Absence or decrease in ABCA4 activity leads to an accumulation of N-retinylidene-PE in disc membranes and the formation of toxic dihydroxylic fluorophores including A2E derivatives that accumulate as fluorescent lipofuscin deposits in RPE cells as a result of phagocytosis of outer segments. The activity of ABCA4 has been shown to be strongly dependent on the lipid environment (57). It is possible that loss in ELOVL4 activity in patients with autosomal dominant Stargardt-like disease may adversely affect the activity of ABCA4 in disc membranes, thereby leading to the production of A2E-containing lipofuscin deposits in the RPE as found in individuals with autosomal recessive Stargardt disease and ABCA4 knock-out mice (2).

Recently, heterozygous mice expressing the 5-bp deletion mutation in ELOVL4 have been reported (58). These mice, like ABCA4 knock-out mice, showed an accumulation of lipofuscin containing A2E and its isomers in the RPE cells. However, the mutant ELOVL4 transgenic mice, unlike ABCA4 knock-out mice, showed more rapid photoreceptor degeneration that was related to the expression levels of the mutant protein, with the fastest rate of disease development detected in mice with increased transgene expression. These data are consistent with the conclusion of this study that the mutant ELOVL4 allele exerts a dominant negative effect. The finding that mutant ELOVL4 transgenic mice exhibit more rapid photoreceptor degeneration than ABCA4 knock-out mice, however, suggests that the dominant negative effect of the mutant protein on WT ELOVL4 activity may affect other photoreceptor functions.

This is the first study to demonstrate that mutant ELOVL4 interacts with WT ELOVL4, and this interaction leads to aberrant trafficking of the WT protein to inclusion bodies, possibly aggresomes, within cells. A major challenge is to understand how C-terminal truncation of ELOVL4 affects protein targeting and activity, leading to autosomal dominant Stargardt-like macular dystrophy.

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