CTRP5 Impairs Its Secretion

PURPOSE. In a prior study, a S163R mutation in the complement-1q tumor necrosis factor–related protein 5 (CTRP5/C1QTNF5) was reported to be associated with early-onset long anterior zonules (LAZ) and late-onset retinal degeneration (L-ORD). The ocular tissues involved in the phenotype are the retinal pigment epithelium (RPE) and lens in the anterior segment. The purpose of this study was to characterize the spatial and temporal expression of the mouse Ctrp5 gene, determine tissue and subcellular localization, and study the effect of the S163R mutation.

METHODS. The expression of the Ctrp5 gene in the mouse was studied by quantitative (q)RT-PCR and in situ hybridization. CTRP5 protein expression and distribution were studied by Western blot analysis, immunohistochemistry, and immunoelectron microscopy. Cellular location of wild-type and mutant CTRP5 in MDCK and COS-7 cells was determined by immunofluorescence and immunoblot analysis.

RESULTS. A significant level of Ctrp5 expression was detected in the adult mouse in the ciliary body (CB) and RPE, and expression started at a very early stage of embryogenesis. Immunohistochemical analysis showed CTRP5 protein in the apical processes of the RPE and forming a hexagonal lattice associated with the RPE lateral membranes. In the ciliary body, CTRP5 was localized to the apical aspects of the CE, the region between the bilayered ciliary epithelial cells. The membrane association of CTRP5 in the RPE and CE was further confirmed by immunoelectron microscopy. Furthermore, cultured cells were used to show that the CTRP5 is a secretory protein and that its secretion is impaired by the S163R mutation.

CONCLUSIONS. CTRP5, a secretory and membrane-associated protein, is localized to the lateral and apical membranes of the RPE and CB. Impaired secretion of the mutant protein may underlie the pathophysiology of L-ORD and LAZ. (Invest Ophtalmol Vis Sci. 2006;47:5505–5513) DOI:10.1167/iovs.06-0312

Macular degenerations form a heterogeneous group of diseases affecting the macula and causing loss of central vision. These diseases are clinically characterized by geographic atrophy or neovascularization of the choroid and may be accompanied by accumulation of lipofuscin or development of drusen in the central area of the retina.1 Age-related macular degeneration (AMD) alone affects more than 20% of the U.S. population older than 65 years.2–4 Genetic studies of familial forms of macular degeneration have thus far revealed 17 mapped loci, and genes for 10 of these have been cloned.5 In addition, several genes associated with susceptibility to AMD have been reported (FBLN5, ABCA4, TLR4, APOE, CFH, BF, LOC387715, and C2).6–13 Recent findings have also suggested potential involvement of complement pathway in the development of AMD.14–15 There is significant phenotypic overlap among the late-onset macular degenerations, which include Sorsby’s fundus dystrophy, adult-onset foveomacular dystrophy, late-onset retinal degeneration (L-ORD), and AMD.16–18 The functional roles of the genes associated with macular degenerations and the pathophysiologic of these diseases are not well understood. Some of the genes associated with monogenic macular degenerations are expressed in photoreceptors (ELOVL4, RDS, RPGR, ABCA4, and FSCN2) and are thought to have a role in maintaining the structure and/or physiological function of photoreceptor cells.19–23 The remaining genes associated with the macular degenerations are expressed in the RPE, with most being extracellular matrix or plasma membrane components (CTRP5, EFEMP1, FIBL-6, TIMP3, and VMD2).24–28 A founder mutation, S165R, in a conserved domain of the CTRP5 gene has been reported in families with L-ORD.19 Recently, we reported the same mutation in a family (UM.H389) with a complex dominant phenotype of late-onset progressive macular degeneration (L-ORD) and early-onset long anterior lens zonules (LAZ).24 This gene is expressed in the RPE and ciliary body, the two tissues of neuroectodermal origin that are involved in the pathologic courses of L-ORD and LAZ, respectively.24 In the human and mouse genomes, the CTRP5 gene is very closely associated with the membrane frizzled-related protein (MFRP) gene, and the complete CTRP5 open reading frame (ORF) is reported to reside in the 3’ untranslated region (UTR) of MFRP and, therefore, is thought to be dicistronic.29,30 A recessive mutation in the Mfrp gene causes retinal degeneration in the rd6 mouse.29,31 In addition, null mutations in the...
MFRP gene are reported to be associated with extremely small-sized eyes, nanophthalmos, and hyperopia in humans. However, the functional role of CTRP5 and its relationship to MFRP in the development and maintenance of normal physiology of these tissues is not known.

In the present study, we characterized the Ctrp5 gene in terms of its spatial and temporal pattern of expression and tissue distribution to gain insight into the role of this protein in ocular physiology. We found that CTRP5 is both associated with the cell membrane and secreted. We also observed that the L-ORD mutant of CTRP5 is associated with impaired secretion, which could be one of the major contributing factors of disease development.

**Materials and Methods**

**Animals and Tissue Collection**

The maintenance and care of the C57BL/6 and BALBc mice used in the study were in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All the animals were maintained on a 12-hour light-dark cycle, and tissues were collected at the end of the dark cycle at the appropriate age. We collected the retina, lens, ciliary body and iris, posterior segment (containing RPE, choroid, and sclera), and other tissues, including brain, heart, lungs, liver, spleen, kidney, skeletal muscle, testes, skin, and uterus from four 33-week-old C57BL/6 mice, to isolate RNA and measure expression of the Ctrp5 gene. Mouse embryonic cDNAs at embryonic days (E) 7 to E17 were obtained from cDNAclontech (Mountain View, CA). Eight to 10 eyes from four to five C57BL/6 mice were collected at postnatal days (P) 1, P3, P5, P10, P15, and P30 (P1E–P30E) to study the expression of these genes during postnatal development. Ten-micrometer-thick cryosections of C57BL/6 embryos at E14.5 and E18.5 and adult BALBc mouse (90-day-old) eyes were prepared as described earlier.

**Real-Time qRT-PCR**

Quantitative (q)RT-PCR was performed, and the data were analyzed according to previously published protocols. Prime to PCR reaction was performed according to the following protocols: 24,33 Primers for qRT-PCR were designed from regions of the Ctrp5 sequence that spanned at least one intron (Figs. 1A–1C). We used the expression of four genes—Gapdh, Hgprt, β-actin, and RpL19—as control genes to normalize Ctrp5 gene expression. Sequences of the primers used can be obtained from the authors by request. Mean (±SEM) relative expression levels were calculated by analyzing at least three independent samples with replica reactions and presented on a scale that represents the expression over the housekeeping gene Hgprt.

**In Situ Hybridization**

A cDNA clone, IMAGE: 876157, containing the full-length coding sequence of mouse Ctrp5 (1.3 kb) was used to generate probes for in situ hybridization (ISH; Fig. 1C). The Ctrp5 riboprobes (sense and antisense) were generated with a digoxigenin (DIG)-RNA labeling kit (Roche Diagnostics Corp., Indianapolis, IN). Eight to 10 eyes from four to five C57BL/6 mice were collected at postnatal days (P) 1, P3, P5, P10, P15, and P30 (P1E–P30E) to study the expression of this gene during postnatal development. Ten-micrometer-thick cryosections of C57BL/6 embryos at E14.5 and E18.5 and adult BALBc mouse (90-day-old) eyes were prepared as described earlier.

**Antibodies and Their Source**

To raise antibodies against the CTRP5 protein, we selected two antigenic peptides, 1GSPPLDNKIPSLCPGHI and 9SVPPRSASPAMKRESER. These peptides showed no homology with any other known proteins and had 100% homology with the human and mouse sequences (Fig. 1D). Antibodies were raised in rabbits against these two peptides and purified using protein G-agarose affinity column. Both antibody species showed similar results in Western blot and immunohistochemical analyses. Monoclonal anti-ezrin antibodies (clone 3C12; Sigma-Aldrich, St. Louis, MO); monoclonal anti-TIMP3 antibodies (Chemicon, Temecula, CA); monoclonal anti ZO-1 antibodies, anti-Xpress, anti-V5, and secondary antibodies conjugated to either Alexa Fluor 488 or Alexa Fluor 555 (Invitrogen-Molecular Probes, Carlsbad, CA); anti-rabbit, and antimouse secondary antibodies conjugated to horseradish peroxidase (HRP; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were obtained from the commercial sources indicated.

**Immunofluorescence Labeling of Tissues and Cells**

Immunostaining for CTRP5 protein was performed according to published procedures.34,35 The following dilutions of the primary antibodies were used: anti-CTR5P (1:2000), anti-ezrin (1:4000), anti-TIMP3 (1:2500), and anti ZO-1 (1:2000).

Madin-Darby canine kidney (MDCK) epithelial cells grown to confluence were fixed in absolute methanol at −10°C for 15 minutes and then incubated for 1 hour in blocking solution containing 5% horse serum and 1% IgG-free BSA (Jackson Immunoresearch Laboratories, West Grove, PA) in PBS (Sigma-Aldrich, St. Louis, MO), and further incubated for 1 hour at room temperature in primary antibody dilute in PBS containing 0.1% BSA. After PBS washes, the cells were incubated for 1 hour at room temperature in a secondary antibody diluted as just described and then were washed and mounted in anti-fade mounting medium containing DAPI (PromoGold; Invitrogen). Images were captured using a confocal microscope (LSM510; Carl Zeiss Meditec, Inc., Dublin, CA).

**Immunoelectron Microscopy**

The electron microscopic immunohistochemical analysis was performed as previously described, by using formalin-fixed retinal sections from mouse and human (65 years of age) eyes embedded in resin (Unicycl; Electron Microscopy Sciences, Fort Washington, PA). Thin sections were collected on 200-mesh nickel grids. The sections were blocked with 5% normal goat serum (Vector Laboratories, Burlingame, CA) and incubated in anti-CTR5P affinity-purified antibodies (1:50 dilution) at 4°C overnight, followed by incubation with a gold-conjugated secondary antibody (1:10 dilution, 10-nm gold particles; Sigma-Aldrich). Sections were rinsed and poststained with uranyl acetate and lead citrate before viewing and photography with an electron microscope (JEM1200EX II; JEOL, Tokyo, Japan).

**Generation of CTRP5 Constructs and Expression in COS-7 Cells**

Primers with BamHI and NotI restriction sites were designed to amplify full-length coding regions of CTRP5 using the human IMAGE clone 5262504 as a template. The sequences of these primers are available from the authors on request. The full-length CTRP5 fragment was ligated into the pcDNA3B vector (Invitrogen) to generate a construct with a V5 tag at the C-terminal end (CTRP5-V5; Fig. 1E). A V5-tagged human mutant of CTRP5 (S163R) was generated by site-directed mutagenesis using a kit (Invitrogen) according to the manufacturer’s protocol (Fig. 1F). All cloned DNA fragments were completely sequence verified. COS-7 and MDCK cells were transfected with expression constructs or control vectors.36 Mock transfections were performed using transfection reagents only. After 48 hours of transfection, cells and media were harvested for protein isolation.

**Western Blot Analysis**

Cell lysates were prepared in lysis buffer (50 mM Tris-HCl [pH 7.4], 0.15 M NaCl, 1 mM EDTA, 0.1% Triton X-100, and 0.1% [wt/vol] SDS), containing protease inhibitor cocktail (Sigma-Aldrich). Total protein extract was prepared from mouse whole eye, mouse liver, mouse lungs, and human RPE-choroid in lysis buffer containing protease inhibitor cocktail (Complete; Roche, Indianapolis, IN).
levels of followed by the iris-ciliary body (Fig. 2A). Very low or minimal Ctrp5 peptides for 1 hour at 37°C followed by Western blot analysis. competition experiments, antibodies were preadsorbed with the specific cases when samples were prepared with only SDS sample buffer. For SDS sample buffer) separated on 10% Tris-glycine gels except in some Protocol, plasma membranes are readily purified from crude mixtures by the technique of aqueous two-phase partition. The cocktail is incubated at 37°C to separate the hydrophobic proteins from the hydrophilic proteins through phase partitioning. When cultured mammalian cells are used for plasma membrane protein isolation, this procedure is reported to produce membrane fractions with more than 90% purity.39 cells are used for plasma membrane protein isolation, this procedure is validated by measuring the expression of RPE- and CE-specific genes, MFRP (accession no.: NM_015645, human; DQ002598, mouse). Bottom red line: the region from which the probes for ISH and Northern blot analysis were generated. (D) CTRP5 protein with its defined domains. Ca and Cb are the regions from which the peptide sequences were selected to raise antibodies. (E) CTRP5 protein with V5 tag at the C-terminal end. (F) The mutant CTRP5 protein (S163R) with V5 tag at the C-terminal end.

Membrane and hydrophilic fractions of the proteins from transfected cells were isolated (Mem-PER Eukaryotic Membrane Protein Extraction Kit; Pierce Biotechnology, Inc., Rockford, IL). In this protocol, plasma membranes are readily purified from crude mixtures by the technique of aqueous two-phase partition. The cocktail is incubated at 37°C to separate the hydrophobic proteins from the hydrophilic proteins through phase partitioning. When cultured mammalian cells are used for plasma membrane protein isolation, this procedure is reported to produce membrane fractions with more than 90% purity.39 All Western blot analyses were performed with reduced proteins (by adding 0.5% β-mercaptoethanol [BME] and 5 mM dithiothreitol [DTT] in SDS sample buffer) separated on 10% Tris-glycine gels except in some cases when samples were prepared with only SDS sample buffer. For competition experiments, antibodies were preadsorbed with the specific peptides for 1 hour at 37°C followed by Western blot analysis.

Results

Spatial and Temporal Expression of the Ctrp5 Gene in Mouse Tissue

Tissue Distribution of Ctrp5. Analysis of various tissues from 33-week-old C57BL/6 mice revealed highest levels of Ctrp5 gene transcripts in the posterior segment of the eye, followed by the iris-ciliary body (Fig. 2A). Very low or minimal levels of Ctrp5 transcripts were detected in the mouse brain, retina, lens, liver, lungs, heart, spleen, kidney, skeletal muscle, skin, testes, and uterus (Fig. 2A). The quality of cDNA isolated from retina and lens tissue expressing low amounts of Ctrp5 was validated by measuring the expression of RPE- and CE-specific genes, Efemp1 and Temp3 to test for potential contamination from RPE and CE (data not shown).

Embryonic Expression of Ctrp5. Expression of this gene during embryonic development was determined by qRT-PCR using the RNA isolated from whole mouse embryos between E7 and E17. The presence of Ctrp5 transcripts was first observed at E11 and continued up to E17 (Fig. 2B). Alterations observed in the level of Ctrp5 expression at different stages of embryonic development could be due to changes in the ratio of the tissue expressing the gene to the total size of the embryo (Fig. 2B).

Expression in Postnatal Eye. In whole eye, the level of Ctrp5 was highest at P1 (P1E), gradually declined up to P5, and then remained unchanged during further development up to P30 (Fig. 2C). We studied the expression of Ctrp5 in the iris-ciliary body and in the posterior segment, and abundant expression of this gene was observed in both tissues up to 20 months, the latest time point that we checked (data not shown). Further detailed analysis is necessary to investigate the age-related variation in expression of this gene in those tissues considering the early-onset of abnormal lens zonules and the late-onset of L-ORD in individuals carrying the CTRP5 mutation.

Cellular Pattern of Ctrp5 Expression. The specific tissues or cell types that express the Ctrp5 transcript were determined by ISH with a probe specific to the Ctrp5 sequence (Fig. 1C). In sections of eye and brain from pigmented mice at December 2006, Vol. 47, No. 12

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FIGURE 1. Structure of CTRP5 genes and constructs used in this study are presented by the following diagrams. (A) The genomic region of the human CTRP5 gene, which is homologous to the mouse Ctrp5 gene. Black and colored boxes represent the exons. The F and R arrows represent the region from which primers were designed for qRT-PCR analysis of Ctrp5. (B) CTRP5 transcript in which the whole CTRP5 ORF lies in the 3' UTR of the MFRP gene (accession no. AAI62823, human; NM_145613, mouse; GenBank). (C) CTRP5 transcript independent of MFRP (accession no.: NM_015645, human; DQ002598, mouse). Bottom red line: the region from which the probes for ISH and Northern blot analysis were generated. (D) CTRP5 protein with its defined domains. Ca and Cb are the regions from which the peptide sequences were selected to raise antibodies. (E) CTRP5 protein with V5 tag at the C-terminal end. (F) The mutant CTRP5 protein (S163R) with V5 tag at the C-terminal end.

FIGURE 2. (A) Quantitative expression (±SEM) of Ctrp5 in different tissues of adult mouse. (B) Expression of Ctrp5 (±SEM) during embryonic development at E7, E11, E15, and E17. (C) Expression profile of Ctrp5 (±SEM) during postnatal eye (P1E–P30E) development. LI, liver; LU, lungs; B, brain; ICB, iris-ciliary body; PS, posterior segment of eye including the RPE, choroid, and sclera; R, retina; L, lens; H, heart; SP, spleen; K, kidney; SM, skeletal muscle; S, skin; T, testis; U, uterus.

FIGURE 3. Localization of the Ctrp5 transcripts in the mouse embryonic eye and brain by ISH. Expression of Ctrp5 transcripts in the mouse eye obtained by ISH at E14.5 (A, B, arrows), and at E18.5 (C, arrows), in the developing choroid plexus at E14.5 (Fig. 3D, arrows). (A’–D’) The corresponding sections hybridized with sense riboprobe that served as negative control samples. L, lens; ICB, iris-ciliary body; cp, choroid plexus.
tion in the RPE and ciliary body, significant levels of expression was evident in the developing choroid plexus of 3-month-old albino BALBc mice with no pigmentation. Expression was prominent in cells of the presumptive ciliary body and iris stroma (Fig. 3A, arrows). At E18.5, cells and cranial paraxial mesoderm that mark the anlage of the ciliary processes were observed in the cells of the ciliary epithelium and RPE.24

FIGURE 4. Western blot analysis using anti-CTR5 antibodies. In all panels: lane 1: human RPE-choroid; lane 2: mouse eye; lane 3: mouse liver; lane 4: mouse lungs; lane 5: COS-7 cell lysate containing V5 tagged CTR5; and lane 6: media of COS-7 cells containing V5 tagged CTR5 proteins. (A) Western blot analysis was performed with anti-CTR5 antibodies. (B) Blot containing lanes 5 and 6 of (A) was stripped and reprobed with anti-V5 antibodies. Lanes 5' and 6' are the same as lanes 5 and 6 of (A). (C) Duplicate blot of (A) was probed with preadsorbed anti-CTR5 antibodies. (D) Blot containing lanes 5 and 6 of (C) was stripped and reprobed with anti-V5 antibodies. Lanes 5' and 6' are the same as lanes 5 and 6 of (C). (E) Blots probed with preimmune serum. (F) Blot containing lane 5 of (E) was stripped and reprobed with anti-V5 antibodies. Right: molecular size (kDa).

CTR5 was detected (purple) in the layer of neural crest cells and cranial paraxial mesoderm that mark the anlage of the ciliary body and iris stroma (Fig. 3A, arrows). At E18.5, CTR5 expression was prominent in cells of the presumptive ciliary body and iris (Figs. 3B, 3C, arrows). At these same embryonic ages, expression was evident in the developing choroid plexus within the lateral ventricles of developing brain (Fig. 5D, arrows).

Between P3 and P15, the period of development and differentiation of the anterior chamber of the eye, the expression of CTR5 is localized to ciliary processes (data not shown). In the eyes of 3-month-old albino BALBc mice with no pigmentation in the RPE and ciliary body, significant levels of expression of CTR5 were observed in the cells of the ciliary epithelium and RPE.24

Distribution of CTR5 Protein in Ocular Tissues

Characterization of Antibodies. We used affinity-purified polyclonal antibodies raised against CTR5 to detect protein bands corresponding to the expected size of human and mouse CTR5 (~25 kDa) in extracts of human RPE-choroid and mouse eye (Fig. 4A; lanes 1 and 2, respectively) by Western blot analysis. A second 37-kDa band was detected in both the tissues (Fig. 4A; lane 1 and 2, respectively). Neither species of CTR5 was detected in mouse liver or lung extract (Fig. 4A, lanes 3 and 4, respectively). The 25-kDa band corresponds to the expected size of CTR5 and the additional 37-kDa band may represent CTR5 proteins. A commercially available antibody against CTR5 protein was also reported to detect a 37-kDa band (product no. 3571; ProSci Inc., Poway, CA). The C1q group of proteins is known to exist in highly posttranslationally modified forms, and the 37-kDa band detected by the CTR5 antibodies may represent a posttranslationally modified form of the CTR5 protein.18 Anti-CTR5 antibodies recognized bands of ~29 kDa in extracts of COS-7 cells transfected with CTR5-V5 and media in which these cells were grown (Fig. 4A, lanes 5 and 6, respectively). Anti-V5 antibodies recognized bands of the same size when the blot containing lanes 5 and 6 in Figure 4A was stripped and reprobed with V5 antibodies (Fig. 4B, lanes 5', 6'), indicating that both the antibodies recognize the same protein. Western blot analysis of a set of duplicate blots with CTR5 antibodies preadsorbed with antigenic peptides indicated that the intensity of CTR5 protein bands was significantly reduced or the bands were eliminated (Fig. 4C). When the blot containing lanes 5 and 6 of Figure 4C was reprobed with anti-V5 antibodies, a strong presence of V5-tagged CTR5 protein was detected, further supporting the competitive blocking of the CTR5 antibodies with corresponding antigenic peptide (Fig. 4D, lanes 5', 6'). Preimmune serum obtained from the same rabbit did not recognize any proteins close to the size of the CTR5 (Fig. 4E, lanes 5', 6'). These observations indicate that the polyclonal peptide antibodies generated against CTR5 specifically recognize the CTR5 protein.

Cellular Localization of CTR5 Protein. The expression pattern of CTR5 protein in mouse embryonic and adult eye tissue was examined by immunofluorescence microscopy. Consistent with the localization of the Ctrp5 transcript, the CTR5 protein was also detected in cranial paraxial mesodermal cells that developed into the ciliary body in the section of E14.5 eye (data not shown).

In the posterior segments of the adult mouse eye, CTR5 was observed in a hexagonal lattice on the lateral plasma membranes of the RPE (Figs. 5A–C, arrowheads) and also at the RPE apical processes (Figs. 5B, 5E, arrows) and in the ganglion cells (Figs. 5A, arrowheads). The CTR5 protein colocalized with ezrin (orange color; Figs. 5C–5E, arrows and arrowheads), a marker of the apical processes of the RPE, but not with TIMP3, a marker for Bruch’s membrane (data not shown).

Based on the qRT-PCR and ISH analyses, the other region of the eye expected to have significant expression of CTR5 protein is the ciliary body, a bilayer consisting of pigmented and nonpigmented ciliary epithelial cells. Immunofluorescence microscopy showed that CTR5 is localized to the apoposed apical plasma membrane surfaces of these pigmented and nonpigmented cells (Fig. 5G, arrows). As the apical membranes of pigmented and nonpigmented CE layers are close to each other, it is difficult to determine whether CTR5 is localized to one of them or both. Similar to the pattern observed in RPE cells, CTR5 protein showed punctate distribution in CE. Antibodies raised against different epitopes showed a similar pattern of expression of CTR5 in both RPE and ciliary body. In control experiments, no labeling was observed when the primary antibodies were replaced by preimmune serum (Fig. 5F).

In the lens, the CTR5 protein was distributed in a punctate pattern that appeared to be localized to the plasma membrane of the lens epithelial cells (Figs. 5H–5K, arrows). These results indicate that the CTR5 protein is associated...
FIGURE 5. Localization of CTRP5 proteins in the adult mouse eye. By immunofluorescence microscopy, CTRP5 antibody (green) labeling was detected in the sections of mouse eye (A, arrowbeads). Localization on the hexagonal RPE membrane is shown by arrowbeads (B, C) and on the RPE apical processes by arrows (B, C). CTRP5 colocalization with ezrin is shown in (C–E, arrows and arrowbeads). In (A–C), apical labeling of CTRP5 appeared to be differential which is due to the variation in the optical planes selected for capturing images with the confocal microscope. CTRP5 immunolabeling (green) in the anterior segment of the same mouse eye section is shown in (G, arrows). When the anti-CTRP5 antibodies were replaced with preimmune rabbit serum, no labeling was observed in the RPE (data not shown) and the ciliary bodies (F). (H–K) CTRP5 (green) localization to the lens epithelia. Nuclei are stained in red with propidium iodide, and the boxed region in (J) is enlarged in (J) and (J) is enlarged in (K). IS, photoreceptor inner segment. Scale bar, 50 μm. (L) CTRP5 labeling with the antibody (CTRP5b) raised against peptide b (Cb of Fig. 1) which is similar to the labeling of the second antibody (G) raised against peptide a (Ca of Fig. 1). CTRP5b antibody preadsorbed with its peptide antigen and used for labeling mouse section containing ciliary body tissue (M).

FIGURE 6. CTRP5 localization as a membrane-associated protein. (A, arrows) Immunostaining of the RPE section from albino mouse with anti-CTRP5 antibodies shows a punctate hexagonal localization of CTRP5 (B, green, arrows), and colocalization with ZO-1 protein on the lateral membrane of the RPE (C, D, red, arrows, arrowbeads). MDCK cells colabeled with anti-CTRP5 (red) and anti-ZO-1 antibodies (E-G, green). Arrows: lateral membrane localizations; arrowbeads: apical membrane localizations. MDCK cells transiently producing V5-tagged CTRP5 protein were labeled with anti-V5 antibodies (red). Arrows: membrane localization; arrowbeads: intracellular and/or apical localization. Scale bar: 50 μm.
with the plasma membrane. A significant reduction was observed in the immunoreactivity of CTRP5 antibodies in the ciliary body tissue when the antibodies were preadsorbed with antigenic peptides (Figs. 5L, 5M). Polarized distribution of other RPE expressed proteins has been reported previously.40

Further support of the plasma membrane association of the CTRP5 protein came from immunocytochemical analysis of mouse RPE sections in which CTRP5 showed a hexagonal punctate membrane localization (Fig. 6A, arrows). This pattern of CTRP5 staining was colocalized with a known membrane-associated tight junctional protein ZO-1 (Figs. 6B–6D; arrows for CTRP5 and arrowheads for ZO-1). Subsequently, we tested the distribution of CTRP5 in MDCK cells, prototypical epithelial cells that form tight junctions and expresses both CTRP5 and ZO-1 proteins (tested by RT-PCR and Western blot analysis; data not shown). Immunocytochemistry of confluent MDCK cells with anti-CTRP5 and anti-ZO-1 antibodies indicated that CTRP5 colocalizes with ZO-1 on the lateral membranes at the site of tight junctions between adjacent cells (Figs. 6E–6G, arrows). In addition, CTRP5 was localized to the apical membranes of MDCK cells in a punctate pattern (Fig. 6E, arrowheads). When MDCK cells were transfected with V5-tagged CTRP5 protein and immunolabeled with anti-V5 (red) and anti-ZO-1 (green) antibodies, localization of the CTRP5 protein was found to be associated with plasma membrane (Fig. 6H, arrows), in addition to intracellular and/or apical localization (Fig. 6H, arrowheads). The transfection efficiency of MDCK cells was found to be low.

When evaluated by immunoelectron microscopy, the anti-CTRP5 antibodies were found to label the plasma membranes of both the human (Fig. 7A) and mouse RPE (Fig. 7B). The highest concentration of immunolabeling was observed at the apical surface of the cells in the area where two adjacent cells juxtapose, possibly near the membrane junctions (Figs. 7A, 7B, arrows). Apical processes were also heavily labeled (Fig. 7A, arrows). Using light microscopy, we observed predominant CTRP5 localization on apical membranes of CE (Fig. 5G) but could not resolve localization on lateral membranes. However, by immunoelectron microscopy we observed immunopositive labeling on the lateral membrane between two adjacent pigmented cells of CE (Figs. 7C, human CE; 7D, mouse CE). Immunoelectron microscopy provides confirmatory evidence for the localization of CTRP5 to the apical and lateral membranes of RPE and CE.

**Existence of CTRP5 as a Secretory Protein and Effect of L-ORD Mutation on its Secretion**

Immunohistochemical analysis of CTRP5 localization in the previous section suggested association with the plasma membrane. The known primary structure of the CTRP5 protein contains a signal peptide at the N-terminal end, which suggests that it could be a secretory protein. CTRP5 is a member of the short-chain collagens and the C1q-TNF superfamily of proteins. The TNF proteins are known to exist as type-II membrane proteins as well as secreted proteins.41 Immunoelectron microscopy detected a pattern of CTRP5 labeling in RPE and ciliary body tissues that appeared to be secretory vesicles that were filled with CTRP5 protein (Figs. 7A–D, arrowheads), suggesting its likely nature as a secretory protein.

To evaluate further the properties of CTRP5 protein, membrane and hydrophilic fractions of the lysate of COS-7 cells expressing the CTRP5-V5 were isolated separately, and the media in which these cells were grown were collected. Immunoblot analysis with anti-V5 antibodies detected the presence of CTRP5-V5 fusion protein in the membrane and hydrophilic fractions of the transfected cell lysates as well as in the medium (Fig. 8A, lanes 1, 2, and 3). These results support that CTRP5 exists not only as a membrane-associated protein but also as a protein secreted into the medium.

When COS-7 cells were transfected with a S163R mutant construct of CTRP5, the expected 29-kDa band was observed in the cell extract (Fig. 8A, lane 4) but not in the media in which these cells were cultured (Fig. 8A, lane 5). This was consistent in multiple independent experiments and suggested that the processing or turnover of L-ORD mutant CTRP5 is altered, which prevents its secretion and provides an important clue toward our understanding of the mechanism of disease development.

By expressing the gC1q domain polypeptides of CTRP5, Hayward et al.,18 showed that gC1q peptides can form multimers. We expressed the complete CTRP5 protein with a C-terminal V5 tag, which is naturally processed and secreted (Fig. 8A, lanes 1-3). The Western blot analysis showed the presence of monomeric protein in the cell extracts (Fig. 8A, lanes 1, 2). In addition, we observed a significant presence of CTRP5 protein that did not enter into the gel but remained at the top of the lane (Fig. 8A, lanes 1, 2). To investigate further the higher-molecular-weight species of the CTRP5 protein in the cell, in the media, and when it is mutated, the protein extracts were subjected to two types of treatments: boiling with only SDS, and boiling with SDS and reducing agents (BME and DTT). Only monomeric (29 kDa) CTRP5 bands were detected under reduced conditions in the membrane fraction (Fig. 8B, lane 1), in the hydrophilic fraction (Fig. 8B, lane 3), in the media (Fig. 8B, lane 5), and also in the fraction containing mutant CTRP5 (Fig. 8B, lane 7). With SDS alone, an additional band of 50 kDa was observed in the wild-type protein fractions that may represent a dimeric form of the CTRP5 protein that is partially resistant to detergent but not to the reducing agents (Fig. 8B, lanes 2, 4, 6; vertical arrows). The membrane and

**FIGURE 7.** Immunelectron microscopic assignment of CTRP5 location in human and mouse RPE and CE. Arrows: CTRP5 localization (black dots) in human (A) and mouse (B) RPE. Arrows: CTRP5 localization on the membrane between the pigmented cells of human CE (C) and mouse CE (D). The cluster of dots (arrowbeads) in (A–D) may indicate the secretory vesicle filled with CTRP5 proteins. Scale bar, 500 nm.
hydrophilic fractions of wild type CTRP5 protein seemed to form stronger higher order multimers as evidenced from the lanes having significant amount of proteins that could not enter into the gel (Fig. 8B, lanes 1-4; horizontal arrow). Of note, the higher-molecular-weight complex of CTRP5 was not observed in the culture medium, suggesting that the secretory form of CTRP5 may exist as a monomer or dimer (Fig. 8B, lanes 5, 6; arrowheads). Monomeric mutant (S163R) CTRP5 was observed under reducing conditions. In contrast to the wild type protein, neither monomer nor dimer was detected under nonreducing conditions, and the mutant CTRP5 protein remained at the top of the gel. These observations suggest that the wild-type CTRP5 may exist in different higher order hetero- or homo-oligomeric complexes or posttranslationally modified forms and the nature of the mutant CTRP5 differs from the wild-type protein. Therefore, when mutant protein interacts with wild-type protein in the heterozygous condition, it may alter the properties of the wildtype protein and that may explain the dominant negative phenotype of L-ORD. A detailed further investigation of the nature and function of these different forms of CTRP5 and their interaction may provide useful information.

**DISCUSSION**

The LAZ and retinal degeneration observed in patients with the S163R mutation in CTRP5 show significant variation in age of onset. Abnormal anterior lens zonules were observed around age 20, whereas retinal changes were observed in the fourth or fifth decade. Delayed dark adaptation was reported as an early symptom of retinal change, whereas loss of central vision, accumulation of drusen, and choroidal neovascular membrane (CNVM) were observed in the late stage of disease. Some of these patients also had glaucoma that developed at a late stage. We have established that Ctrp5 is expressed at very early stages of development, and continued expression is maintained in adult mouse eyes. This protein exists in membrane-bound and secreted forms and is expressed predominantly in RPE, CE, and lens, the tissues associated with the pathologic nature of L-ORD and abnormal lens zonules. The spatial and temporal expression patterns of CTRP5 suggest that this protein may play a critical role in the normal development and physiology of these ocular tissues.

CTRP5 is a member of the C1q-tumor necrosis factor (TNF) superfamily, which is involved in processes as diverse as host defense, inflammation, cell differentiation, and apoptosis. C1q is the key subcomponent of the classic complement activation pathway involved in innate immunity and IgG- or IgM-acquired immunity. C1q is also involved in other immunologic processes including clearance of apoptotic cells and phagocytosis of bacterial cells. It has been hypothesized that the deposits commonly found in AMD between Bruch’s membrane and the RPE cell layer may act as a stimulus for local activation of the complement system. The accumulation of subretinal deposits may lead to local ischemia and activation of RPE cells, causing them to release angiogenic stimuli that initiate choroidal neovascularization, a common feature of AMD. Proteins involved in the complement cascade and retinal autoantibodies were detected as constituents of drusen. Recent findings suggest that immunologic factors are involved in the pathogenesis of age-related macular degeneration (AMD). Members of the complement system, complement factor H (CFH), C-reactive protein (CRP), factor B and complement component 2 are associated with age-related macular degeneration. Late-onset retinal degeneration associated with a missense mutation in the CTRP5 gene shares several phenotypic features with AMD, including subretinal deposits, drusen, and CNV membranes. Therefore, it is plausible that CTRP5 with the C1q domain also may participate in the pathogenesis of macular degeneration by activating the complement system.

The CTRP5 protein also has sequence and structural similarities to collagens VIII and X, which have diverse functions including cell adhesion and polarity. We observed the polarized expression of CTRP5 protein on the plasma membranes of ciliary...
epithelial and retinal pigment epithelial cells (Fig. 5). The localization of membrane-associated CTRP5 to cell junctions and its colocalization with the tight junction protein ZO-1 (Fig. 6) suggests a potential role for this protein in cell–cell communication, cell adhesion, or cell polarity. This predicted role is consistent with the observation that the CTRP5 mutation results in abnormal adhesion between RPE and Bruch’s membrane, leading to subretinal deposits.42 Similarly, mutant CTRP5 expressed in the ciliary and lens epithelium affects normal zonule anatomy and probably affects the general physiology of these tissues, resulting in altered ocular pressure with age. Additional studies are needed to establish the relationship between CTRP5 mutations and glaucoma in LORD and the effects of this mutation on the function of the ciliary body and lens.

The TNF ligands are synthesized as type-II membrane proteins, and some of these are proteolytically cleaved and secreted. The TNF family of proteins plays a major role in inflammation, and some of these are membrane-bound forms.43 The TNF family of proteins includes TNF-α and TNF-β and their receptors, TNFR1 and TNFR2, respectively. The TNF family of proteins is involved in various cellular signaling pathways, including NF-κB signaling.56,57 We and others have shown that the CTRP5 interacts with MFRP.54,55 MFRP is a transmembrane protein that contains extracellular CUB domains, and it is thought that the CUB domain of MFRP interacts with C1q domain of CTRP5, suggesting recruitment of exogenous (secretory) CTRP5 to the membrane through this interaction. MFRP also has an intracellular frizzled or CRD domain and frizzled-containing proteins are shown to be involved in various cellular signaling pathways, including Wnt signaling.56 Therefore, it is likely that either secreted, membrane-associated, or intracellular CTRP5 responds to various cellular stimuli or inflammatory signals that also involve MFRP. The absence of secreted CTRP5 due to the S163R mutation may affect these responses of RPE cells to different stimuli and generate local inflammation that leads to the formation of drusen, subretinal deposits, and choroidal neovascularization. Impairment of secretion due to mutations in proteins that are constitutively secreted may affect these responses more profoundly.57,58 Like other C1q-TNF superfamily proteins, CTRP5 protein also have membrane-associated and secreted forms (Figs. 5–8). The significant presence of Ctrp5 transcripts in the choroid plexus, an intraventricular secretory epithelial formation that secretes the cerebrospinal fluid (Fig. 3D), also supports its secretory nature.55

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References


