Complement Factor H: Spatial and Temporal Expression and Localization in the Eye

Md Nawajes A. Mandal and Radha Ayyagari

PURPOSE. Complement factor H (CFH) is a component of the mammalian complement system, which regulates the alternative pathway of complement activation and protects the host cell from inappropriate complement activation. CFH is a key regulator of innate immunity, and CFH deficiency leads to membranoproliferative glomerulonephritis type II. A variation in human CFH, Y402H, has been shown to be associated with an increased risk for age-related macular degeneration. The authors describe studies on the spatial and temporal expression of the CFH gene and localization of this protein in ocular tissues to gain insight into its role in the eye.

METHODS. CFH expression in human and mouse tissues was studied by quantitative RT-PCR and Western blot analysis, and localization of CFH was studied by immunohistochemical analysis followed by fluorescence microscopy.

RESULTS. In human and mouse, CFH expression was found to be similar to the highest level of expression in the liver. In ocular tissue, CFH was detected in the distalmost optic nerve (3 mm) cut from the scleral surface of the eyeball, sclera, RPE-choroid, retina, lens, and ciliary body. In mouse, Cfh expression was observed from early embryonic stages, and in the eye its expression increased with age.

CONCLUSIONS. A significant level of CFH expression is maintained in different ocular tissues during development and aging. Sustained high levels of CFH expression in eye tissues suggest that this protein may play a role in protecting these tissues from indiscriminate complement activation and inflammatory insult. (Invest Ophthalmol Vis Sci. 2006;47: 4091–4097) DOI:10.1167/iovs.05-1655

The complement system in the blood of mammals is composed of more than 30 proteins that are primarily synthesized in the liver and that circulate in their inactive forms. When activated, these proteins produce various complexes in a cascade that participates in immunity to microorganisms, the processing of immune complexes, and the development of an appropriate and optimal cellular and humoral immune response. The complement system is activated by three distinct pathways: classical, alternative, and mannos-bind lectins (MBLs). The active complement cascade is a potent force and must constantly be kept in check to prevent indiscriminate complement activation on the host cell, which could lead to inflammation and cellular injury. This is particularly important for the alternative pathway, which is constantly active. Human complement factor H controls spontaneous activation of the complement cascade.2 Biologic function of CFH occurs through its interactions with C3 cleavage product C3b, which covalently binds to the surface of the cells during complement activation. Bound C3b forms a C3-activating enzyme, C3b-Bb, and together with other plasma proteins initiates a cascade that results in the production of several additional C3b molecules by cleaving the C3. Binding of factor H to C3b blocks the formation of the C3-activating enzymes and greatly accelerates the decay of any existing enzyme by factor I. Proteolytic cleavage of C3b by factor I occurs only when C3b is in complex with its cofactor, CFH.3–6

CFH is a key regulator of the complement system of innate immunity that maintains the optimum C3 level in circulation.7 Genetic alterations including absolute factor H deficiency have been associated with membranoproliferative glomerulonephritis (MPGN) and hemolytic uremic syndrome (HUS).8–11 CFH deficiency in humans, pigs, and knockout mice is shown to allow uninhibited activation of plasma C3 and thereby cause severe depletion of plasma C3.8,9,11–17 Recently, a known polymorphism in human CFH, T → C substitution in exon 9, which resulted in the substitution of an uncharged tyrosine with a positively charged histidine (Y402H), was shown to be associated with increased risk for age-related macular degeneration (AMD).18–22 The human and mouse CFH protein contains 20 repetitive units of 60 amino acids, referred to as the short consensus repeat (SCR) or the complement control protein (CCP) module, arranged in a continuous fashion like a string of 20 beads.7 The Y402H polymorphism is located on SCR 7, within the cluster of positively charged amino acids implicated in the binding of heparin, C-reactive protein (CRP), and M-proteins, and it may alter the binding properties of CFH to these proteins. Alteration in the binding properties of CFH is thought to be associated with the mechanism underlying the pathogenesis of CFH-mediated AMD.20,21

CFH was first identified as β1F-globulin by Nilsson and Mueller-Eberhard in 1965.22 This protein has been studied extensively for its role in immunity and cellular defense. Recent findings demonstrating the association of a CFH variant with AMD prompted us to study the role of CFH in ocular tissue and its physiology. Here we describe the spatial and temporal expression patterns of the CFH gene in human and mouse and the localization of this protein in mouse tissues with specific focus on ocular tissues to gain insight into the role of this protein in ocular physiology.

MATERIALS AND METHODS

Animal and Tissue Collection

Maintenance and care of the C57Bl/6 and BALB/c mice used in this study were in accordance with the National Institutes of Health Guidelines for the Use of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animals were maintained on a 12-hour light/12-hour dark cycle, and tissues were
collected at the end of a dark cycle at the appropriate age. We collected the eye, brain, heart, lungs, liver, spleen, kidney, skeletal muscle, testes, skin, cardiac veins and arteries, and uterus from 7-month-old mice to study the expression of CFh in different tissues. Eyeballs (6 to 8) were collected from 1-, 3-, 5-, 10-, 15-, 30-, 180-, and 300-day-old mice to study the expression dynamics of CFh in whole eye. To study the distribution of this gene expression in eye tissue and its expression during aging, six to eight eyeballs per age were obtained from 35-, 60-, 180-, 300-, 420-, 500-, and 600-day-old mice, and the distalmost 3-mm optic nerve cut from the scleral surface of the eyeballs (ON), posterior segment (PS), retina, iris-ciliary body, lens, and corneal tissues were collected after dissection. The proximal optic nerve with chiasm was also dissected after the brain was dissected from 180- and 300-day-old mice. Mouse embryonic cDNA was obtained from Clontech (Mountain View, CA).

After transcardiac perfusion of 90-day-old albino (BALB/c) mice, eyeballs and liver tissues were fixed in 4% paraformaldehyde and embedded in OCT, and cryosections were prepared for immunolocalization studies of CFh protein.

Human Tissue Collection

Human donor eyes were collected on ice within 8 hours of death (Midwest Eye Bank and Transplantation Center, Ann Arbor, MI), and we collected different tissues from those eyes within 16 to 20 hours of death. Retina, RPE, optic nerve (−5 mm distalmost, including the nerve head), sclera, and iris-ciliary body tissues were dissected from two 73-year-old donor eyes and were stored at −80°C. Cells from human primary cultures of RPE and lens epithelium (LE) generated from 69-year-old donor eyes were also harvested in reagent (TRIZol; Invitrogen, Carlsbad, CA) and used for RNA isolation. No clinical history of ocular diseases was reported in the medical report obtained from the eye bank for those donor eyes. RNA was isolated separately from each collected tissue. Similarly, normal human lymphocytes were isolated from donor blood and stored in TRIZol. Human major organ cDNAs from brain, liver, heart, spleen, pancreas, kidney, muscle, and placenta were obtained (BioChain Institute Inc., Hayward, CA) and were used for expression studies. Human retina quick-clone cDNA (Clontech) was also used for quantitative (q) RT-PCR.

RNA Isolation and cDNA Synthesis

RNA was isolated from all the collected tissues using TRIZol reagent according to the manufacturer’s protocol. To remove genomic DNA contamination from isolated RNA, total RNA was treated with RNase-free DNase (Promega, Madison, WI) and was purified with an RNeasy minikit (Qiagen, Valencia, CA). First-strand cDNA synthesis was carried out (SuperScript II First-Strand Synthesis System; Invitrogen) for RT-PCR. First-strand cDNA was used for RT-PCR and qRT-PCR. The purity of dissected mouse tissue was determined by amplifying tissue-specific genes such as Rhodopsin (Rds, Rom1) (specific for retina), and Ctrp5, Mfpr (specific for RPE and ciliary body) and by immunohistochemical analysis on the tissue-specific proteins rhodopsin, ABCA4, ELOVL4, CTRPS, MFRP, and TIMP3.

Real-Time Quantitative qRT-PCR

Forward and reverse primers for qRT-PCR were designed from the human and mouse CFH coding regions in such a way that they expanded over at least one intron to nullify the chance of amplification from residual genomic DNA contamination. Primer pairs were used for qRT-PCR of mouse (forward, 5′-GGTCCCCTCCACCTATGACA-3′; reverse, 5′-GTAGGAAATGCGGCTGAATC-3′) and human (forward, 5′-GGAAACCCTCAATGCAAAG-3′; reverse, 5′-AAGCTCTGTGTGGCCTGCC-3′) CFH, respectively. Primers for human CFH were designed from exons 20 and 22 and for the mouse gene from exons 14 and 15, which should amplify the major transcript of CFH rather than the truncated isoform, factor H-like 1 (FHl1), which is truncated after exon 9. Three sets of control primers derived from human and mouse β-actin, HGPRT, and RPL19 genes were used to normalize and validate CFH expression, as described in Ayyagari et al. Quantitative PCR (iQ SYBR Green Supermix; Bio-Rad, Hercules, CA) and melt-curve analysis (iCycler; Bio-Rad) were performed. Relative quantity of expression of CFH and Cfb in different samples was calculated by the comparative Ct (threshold cycle) value method. Expression data were calculated from three independent qRT-PCR reactions carried out for each sample, and mean values (± SD) were presented as fold over the most consistent housekeeping gene, HGPRT.

Protein Isolation and Western Blotting

Total protein was extracted from frozen human ocular tissues using the lysis buffer containing 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 with protease inhibitor cocktail (Sigma, St. Louis, MO). Isolated protein was quantitated using BCA reagent (Pierce Biotechnology, Inc., Rockford, IL) and sample was prepared in Laemmli sample buffer. Equal amounts of protein from each sample were resolved on reducing 10% SDS-PAGE. Western blot analysis was carried out using goat polyclonal anti-CFH antibodies (1:1000 dilution; Quidel Corp., San Diego, CA). Signal was detected using an enhanced chemiluminescence kit (ECL Kit; Amer sham Biosciences, Piscataway, NJ) according to the manufacturer’s instructions.

Immunofluorescence Microscopy

Localization of the CFH protein in the sections of mouse liver and eye were determined by immunofluorescence microscopy. Ten-micron-thick radial cryosections were hydrated with PBS followed by brief (10 minutes) treatment with protease inhibitor followed by (10 minutes) treatment with proteinase K (20 μg/mL). Blocking was carried out using 10% heat-inactivated horse serum at room temperature (RT) for 1 hour, followed by overnight incubation at 4°C with diluted primary antibodies (1:1500). Sections were then washed and incubated with secondary antibody (rabbit anti-goat IgG conjugated with Alexa Fluor 555; Invitrogen) for 1 hour at RT. After washing, slides were mounted in antifade mounting medium (ProLongGold; Invitrogen) containing DAPI for nuclear staining. Images were captured with a confocal microscope (LSM 510; Zeiss, Oberkochen, Germany).

RESULTS

Tissue Distribution and Expression of the CFH Gene in Human Ocular Tissues

The highest level of CFH mRNA was detected by qRT-PCR in human liver tissue (Fig. 1A), whereas low-level expression was observed in brain, lungs, heart, spleen, pancreas, kidney, muscle, and placenta (Fig. 1A).

The quality of RNA obtained from different human ocular tissues collected from postmortem samples was not uniform; hence, CFH expression in these tissues was studied using qualitative rather than quantitative measurement. Expression of CFH was evident in retina, sclera, and optic nerve tissues (Fig. 1B). High levels of expression of CFH were observed in primary cell lines derived from human RPE and lens epithelium (Fig. 1B).

Levels of expression of CFH protein (~155 kDa) were observed to be high in the human distalmost optic nerve and nerve head containing nerve fibers and retinal and pial arteries (Fig. 1C). This protein was also observed in human retina, ciliary body, sclera, and the RPE (Fig. 1C).

Human and Mouse CFH Are Homologous

To study the expression and localization of CFH in mouse tissues and in mouse eye, we first examined the homology of mouse CFH with human CFH. CFH is localized to human chromosome 1 at the 193.35- to 193.45-Mb (1q32, cytogenetic location) region, and mouse Cfb is localized to the 139.54- to 139.98-Mb (cytogenetic location, 74.1 cM) region of mouse...
chromosome 1. The genomic segment 155.3 to 203.9 Mb in human chromosome 1 is evolutionarily conserved across several species and syntenic to the 130.2- to 170.4-Mb region of mouse chromosome 1. The CFH gene is present in this syntenic region along with several other conserved flanking genes. CFH mRNA is 71% identical with Cfh mRNA, and human CFH is 61% identical with the mouse protein. Both these proteins contain multiple complement control protein (CCP) domains (n = 20), also known as short consensus repeats (SCRs) or SUSHI repeats at identical positions.

The Tyr402His polymorphism in CFH was shown to be associated with AMD. Most sequences of rat and mouse CFH deposited in the National Center for Biotechnology Information (NCBI) database contain tryptophan (W) at position 402 (Fig. 2), and the adjacent amino acid at position 401 is a tyrosine (Y). CFH sequences from different species were collected from the NCBI database (http://www.ncbi.nlm.nih.gov/) and compared using the multiple sequence alignment program ClustalW, available through EMBLEBI (http://www.ebi.ac.uk/Tools/sequence.html). Neither histidine nor tyrosine was found to be conserved at the 402 human homologous position by cross-species sequence alignment (data not shown).

Expression of Cfh in Mouse Tissues and in Embryos

As observed in human tissues, the highest level of Cfh expression was detected in mouse liver (Fig. 3A). Significant expression was also observed in arteries and veins, heart, lungs, and uterus (Fig. 3A). Eye, brain, spleen, kidney, muscle, skin, and testis showed a minimal level of expression (Fig. 3A).

Expression of this gene in mouse embryos during development was determined using the RNA isolated from whole embryos at E7, E11, E15, and E17. The Cfh transcript was first observed at E11, and the level of expression of this gene increased steadily until E17 (Fig. 3B).

Expression of Cfb in Mouse Postnatal Eyes and in Eye Tissues

Expression of Cfb was evident at P1 in the RNA isolated from whole eye, and levels of expression did not vary significantly up to P30 (Fig. 4A). In the adult mouse eye at P180, the level of Cfb was significantly higher than the expression observed at P30. At P900, the level of expression of Cfb was even higher than the levels observed at P180 (Fig. 4A).

To assess the expression of Cfb in different ocular tissues, mouse eyes were dissected and tissues were used for isolation of RNA. In 300-day-old mice, the highest level of Cfb expression was observed in the approximately 3-mm distal optic nerve cut from the surface of the sclera (ON) (Fig. 4B). This portion of the optic nerve contains axonic fibers, glial cells, astrocytes, and retinal arteries covered by a sheath containing numerous pial arteries. In contrast, the proximal optic nerve, including the chiasm dissected from the brain tissue of the mouse, showed very low levels of Cfb expression (data not shown). Significant levels of Cfb expression were observed in arteries and veins located in other parts of the body (Fig. 3A). These observations indicate that the high levels of Cfb observed in the distal portion of the optic nerve could mainly be attributed to the contribution of the vascular tissue. Significant levels of expression were observed in the eye posterior tissues that contained RPE, choroid, and sclera (Fig. 4B). Low level of expression was also observed in iris-ciliary body and lens (Fig. 4B). Minimal expression was observed in the retina, and there was no expression of Cfb in the cornea (Fig. 4B).

Expression of Cfb in Mouse Eyes during Postnatal Development and Aging

RNA was isolated from mouse ocular tissues from P35 to P600. Age-related variation in the expression of Cfb was studied in two ocular tissues that showed high levels of expression of this gene. At all ages, a very high level of expression was main-

**Figure 2.** Homology of human CFH protein with mouse and rat proteins at the codon 402 region. Seventy amino acids surrounding the AMD-linked variation of human are compared with mouse and rat sequence. Position 402 is shown on the top, and the composition of a typical conserved SCR or SUSHI domain is presented at the bottom. This region contains the 7th SCR domain of CFH protein.
tained in the optic nerve and eye posterior tissues (Fig. 5). The variation observed in Cfh expression in ocular tissues at different ages did not follow any particular pattern.

**Location of CFH in Mouse Eye**
In the mouse eye, significant CFH protein was detected in the optic laminar region (Fig. 6A) and the outer vascular sheaths of the optic nerve (Fig. 6B). CFH localization was also observed on the membranes of ciliary epithelial cells (Fig. 6C). CFH in the RPE cells was found to be significant, indicating the RPE was the major contributing tissue to the high level of CFH observed in eye posterior tissues by qRT-PCR (Fig. 6E). In the inner retina, CFH was detected in the outer plexiform layer, inner plexiform layer, and ganglion cell layers, and significantly in the limiting membranes (Fig. 6E, arrows). CFH-labeled cells in the inner plexiform layer could be migratory ganglion cells or astrocytes; additional studies are needed to establish the nature of these cells. CFH was also detected in the photoreceptor inner segments (Fig. 6E, arrows). No labeling was observed when anti–CFH antibodies were replaced by normal goat serum (Fig. 6D). Tissue distribution in mice and in humans indicates that the liver produces the highest level of CFH in the body. Immunohistochemical analysis of mouse liver sections with CFH antibodies revealed intense CFH protein labeling in hepatocytes (Fig. 6F-6H), which served as positive control.

**DISCUSSION**
Human and mouse CFH are homologous and are located in syntenic chromosomal regions. Consistent with earlier reports, the highest level of CFH expression was found in the liver of humans and mice. We also observed high levels of Cfb expression in the mouse cardiac veins. Low levels of CFH expression were observed in the brain, eyes, lungs, heart, spleen, kidneys, pancreas, and placenta. In the eye, we detected CFH expression in the optic nerve, retina, ciliary body, lens, and retinal pigment epithelium. In mice, Cfb expression was evident from early embryonic development, and a high level of expression was maintained in eye tissues with aging.

The complement system, a protein cascade involved in immune response, consists of several fluid-phase and cell membrane–associated proteins.6 The CFH, a member of this group of proteins, is a single polypeptide chain plasma glycoprotein that regulates complement activation in fluid-phase and on cellular surfaces. Factor H binds to C3b, accelerates the decay of the alternative pathway C3–convertase (C3b-Bb), and acts as a cofactor for the factor I–mediated proteolytic inactivation of C3b.3–5 Activation products of the complement cascade contribute to the production of other inflammatory mediators and can therefore promote tissue injury at sites of inflammation. The role of inflammation in the pathogenesis of AMD is well documented,27,28 and the association of AMD with members of the complement system, C-reactive protein (CRP), factor B (BF), complement component 2 (C2), and CFH has been reported.18,20,22,29,30 Smoking, which increases inflammation, is a potent risk factor for AMD and is also known to deplete the plasma level of CFH.29,31–33 Drusen deposits in the RPE-choroid interface are a hallmark of AMD. Activation of the complement cascade and local inflammation have been implicated in the formation of drusen.54–56 Drusen-like deposits indistin-

**FIGURE 3.** (A) Expression and distribution of Cfb gene in mouse tissues. Quantitative RT-PCR was performed with the primers that selectively amplify the Cfb gene. Three independent reactions were performed per sample per primer, and mean values (± SD) are presented over the values of mouse Hgprt gene that is amplified in parallel replica reactions for each sample. Sample abbreviations are similar to those in Figure 1. E, eye; Sk, skin; T, testis; Cav, cardiac arteries and veins; U, uterus. (B) Expression of Cfb in mouse embryos. RNA isolated from whole mouse embryo at embryonic day (E)11, E15, and E17 was used for qRT-PCR.

**FIGURE 4.** (A) Expression of Cfb in mouse eye with age. RNA isolated from mouse whole eye from P1 to P300 was used to measure the quantitative expression of the Cfb gene. (B) Distribution of Cfb in mouse eye tissues. Eyes from P300 mice were dissected in nuclease-free PBS, and a clean tissue pool of optic nerve (ON; ~3-mm distalmost optic nerve cut from the surface of the sclera), posterior segment (PS) containing RPE-choroid-sclera, retina (R), iris-ciliary body (I-CB), lens (L), and cornea (C) were collected for RNA isolation and cDNA synthesis.
Expression of the CFH gene was measured in the cDNAs prepared from mouse optic nerve (ON) and posterior segments (PS) obtained from mouse eyes from P35 to P600. Expression values are presented over the values of Hgprt on the y-axis.

FIGURE 5. Expression profile of Cfh in mouse eye tissues with age. Expression of the Cfh gene was measured in the cDNAs prepared from mouse optic nerve (ON) and posterior segments (PS) obtained from mouse eyes from P35 to P600. Expression values are presented over the values of Hgprt on the y-axis.

FIGURE 6. Localization of CFH protein in mouse ocular tissues. CFH protein localization was detected by anti-CFH antibodies. (A) Distal optic nerve with attached retina; CFH location is shown by arrows. (B) Distalmost optic nerve; CFH expression is predominantly localized to the sheaths (arrows). (C) Part of the anterior segment of the eye containing ciliary body and neural retina; CFH expression is shown by arrows and arrowhead. (D) Mouse retinal section in which the anti-CFH antibodies were replaced with normal goat serum; layers were marked and served as negative control. (E) Retinal section of albino mouse eye and CFH localization in different layers is shown by arrows. To focus the nucleus of the RPE layer, the nuclear staining (blue) of the outer nuclear layer (ONL) was allowed to become oversaturated in this image. (F–H) Mouse liver sections labeled with anti–CFH antibodies (red). (F, H) Nuclei are labeled with DAPI (blue). (G, H) Images are similar except for DAPI staining. Ret, retina; ON, optic nerve; CB, ciliary body; 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; GCL, ganglion cell layer.
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