Impaired Binding of the Age-related Macular Degeneration-associated Complement Factor H 402H Allotype to Bruch’s Membrane in Human Retina

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Age-related macular degeneration (AMD) is the predominant cause of blindness in the industrialized world where destruction of the macula, i.e. the central region of the retina, results in loss of vision. AMD is preceded by the formation of deposits in the macula, which accumulate between the Bruch’s membrane and the retinal pigment epithelium (RPE). These deposits are associated with complement-mediated inflammation and perturb retinal function. Recent genetic association studies have demonstrated that a common allele (402H) of the complement factor H (CFH) gene is a major risk factor for the development of AMD; CFH suppresses complement activation on host tissues where it is believed to bind via its interaction with polyanionic structures. We have shown previously that this coding change (Y402H; from a tyrosine to histidine residue) alters the binding of the CFH protein to sulfated polysaccharides. Here we demonstrate that the AMD-associated polymorphism profoundly affects CFH binding to sites within human macula. Notably, the AMD-associated 402H variant binds less well to heparan sulfate and dermatan sulfate glycosaminoglycans within Bruch’s membrane when compared with the 402Y form; both allotypes exhibit a similar level of binding to the RPE. We propose that the impaired binding of the 402H variant to Bruch’s membrane results in an overactivation of the complement pathway leading to local chronic inflammation and thus contributes directly to the development and/or progression of AMD. These studies therefore provide a putative disease mechanism and add weight to the genetic association studies that implicate the 402H allele as an important risk factor in AMD.

AMD affects ~50 million people worldwide and can be subdivided into two types, neovascular (“wet”) and atrophic (“dry”), which have different disease characteristics (1). Both of these forms of AMD are preceded by the accumulation of extracellular deposits, including drusen, between the RPE and Bruch’s membrane, a multilaminar extracellular matrix that separates the retina from the blood vessels in the choroid. These deposits, which lead to visual impairment, are associated with local chronic inflammation at the RPE-Bruch’s membrane interface that results from an overactivation of the complement system (2), a major effector system in innate immunity that acts as a front line defense against microorganisms and also has an important role in the clearance of cellular debris from host tissues. A common polymorphism (Y402H) (3) in the gene encoding the complement regulator factor H has been found to be strongly associated with the development of AMD (4–7). About 35% of people of European descent carry the 402H disease allele in which a His residue substitutes for a Tyr at position 402 in the preprotein. However, other risk and protective CFH alleles have been identified (reviewed in Ref. 8) along with polymorphisms in other complement components, e.g. in factor B (9).

Factor H, a serum glycoprotein that is mainly produced in the liver, acts as a regulator of the alternative complement pathway. In this regard, it plays an important role in host recognition, preventing damage from the random deposition of C3b on host surfaces that, otherwise unchecked, would lead to opsonization and inflammation (8). CFH is believed to bind to polyanions on host tissues (supplemental Fig. S1), positioning it to act as a co-factor for the proteolytic deactivation of C3b by factor I (10, 11). One such family of polyanionic molecules thought to be involved in this recognition pathway are the glycosaminoglycans (GAGs), i.e. ubiquitous polysaccharides found on the cell surface and in the extracellular matrix of mammalian tissues. Although it is well established that CFH interacts with heparin (11–18) (a GAG only secreted by mast cells), there are few, if

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** The abbreviations used are: AMD, age-related macular degeneration; RPE, retinal pigment epithelium; CFH, complement factor H; flCFH, full-length complement factor H; CFHBS, CFH-binding sites; GAG, glycosaminoglycans; CCP, complement control protein domain; HS, heparan sulfate; DS, dermatan sulfate; ANOVA, analysis of variance.

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Reduced Binding of Factor H 402H Variant to Bruch's Membrane

TABLE 1
Details of eyes used in this study: donor sex, age, and CFH Y402H genotype

| Donor Group | Donor* | Sex | Age | Genotype | Days postmortem |
|-------------|--------|-----|-----|----------|-----------------|
| A           | M11913D | F   | 79  | H/Y      | 3               |
|             | M12103A | F   | 82  | H/Y      | 1               |
|             | M12104B | M   | 83  | Y/Y      | 1               |
|             | M12105C | M   | 90  | Y/Y      | 1               |
|             | M12106C | M   | 84  | H/Y      | 1               |
|             | M1183A  | M   | 46  | Y/Y      | 1               |
|             | M1184A  | M   | 72  | H/H      | 2               |
|             | M1186A  | M   | 69  | H/H      | 2               |
|             | M12102C | M   | 59  | H/H      | 2               |
|             | M12106D | M   | 84  | H/Y      | 1               |
|             | M12107C | M   | 81  | H/Y      | 1               |
|             | M12109C | M   | 81  | H/Y      | 1               |
|             | M12189C | M   | 64  | H/Y      | 2               |
|             | M12190D | M   | 58  | H/Y      | 2               |

* Two eyes from each donor were collected.

** Genotype determined for both eyes from each donor where 402Y/402Y, 402H/402Y, and 402H/402H are referred to as Y/Y, H/Y, and H/H respectively.

† The number of days macula were collected and fixed after death. No statistical significance in the data is observed between donors collected 1 or 2 days after postmortem (donor groups A and B; Student’s t test). For CCP6–8 variants: 402H RPE p = 0.2183, 402Y RPE p = 0.2671, 402H Bruch’s p = 0.4288, 402Y Bruch’s p = 0.06214; and for flCFH variants: 402H RPE p = 0.2444, 402Y RPE p = 0.5677, 402H Bruch’s p = 0.2517, 402Y Bruch’s p = 0.7332.

any, direct data demonstrating its binding to related GAGs in tissues (e.g. heparan sulfate (HS)), although this is often assumed. In this regard, CFH, which is composed of 20 contiguous complement control protein (CCP) modules (19) (also referred to as short consensus repeats), has two major binding sites for heparin in CCPs 7 and 20 (12, 13, 15–18). Importantly, the Tyr/His coding change at residue 402, located in CCP7, has a major effect on the heparin binding properties of CFH (12), and a structural explanation for this has been postulated (13). The Y402H polymorphism also affects the binding of CFH to DNA, necrotic cells, and host proteins (20, 21), and in some cases, recognition of bacterial pathogens (22, 23). At present, it is not known how these functional differences of the 402H and 402Y CFH variants contribute to AMD initiation/progression.

EXPERIMENTAL PROCEDURES

Fluorescent Labeling of Proteins—The 402Y and 402H variants of CFH (in the context of either the full-length protein (flCFH) (24) or a recombinant CCP6–8 construct composed of CCP domains 6–8 (12)) were labeled with Alexa Fluor 488 and Alexa Fluor 594, respectively, using Alexa Fluor protein labeling kits (Molecular Probes, Paisley, UK). The fluorophore labeling was also reversed on the CCP6–8 variants for control experiments. In the case of the CCP6–8 constructs, labeling was in the presence of a heparin oligomer of defined length to avoid the modification of GAG-binding residues. 1 mg/ml CCP6–8 (~50 μM), in a total volume of 500 μl, was preincubated with 2 mg of heparin dp24 (Iduron, Manchester, UK), i.e. at an ~6-fold molar excess, in 20 mM HEPES, 130 mM NaCl, pH 7.3 at room temperature for 1 h. To the CCP6–8/dp24 mixture, or flCFH (1 mg/ml in 500 μl PBS), 50 μl of 1 M sodium bicarbonate was added, and these solutions were then transferred to vials containing preweighed reactive dye and mixed in the dark at room temperature for 1 h. Free dye was removed from fluorescently labeled protein on a PD10 column equilibrated and run in 10 mM potassium phosphate, 150 mM NaCl, pH 7.2, 0.2 mM azide. The heparin dp24 was removed from the labeled CCP6–8 proteins by exhaustive dialysis against PBS supplemented with 1 M NaCl in 10-kDa molecular mass cut-off snakeskin dialysis tubing (Pierce, Cramlington, UK) in the dark at 4 °C. The degree of labeling for the flCFH and CCP6–8 proteins was determined from the absorbance at 280 nm and either 494 nm (Alexa Fluor 488) or 590 nm (Alexa Fluor 594) using the formulae

\[
\text{Protein concentration (m) = } \frac{A_{280 \text{ or } 494 \text{ or } 590 \text{ nm}} - (A_{494 \text{ or } 590 \text{ nm}} at 350 \text{ nm}) \times 0.56}{\text{Molar extinction coefficient of protein}}
\]

(Eq. 1)

\[
\text{Moles of dye per mole of protein} = \frac{A_{494 \text{ or } 590 \text{ nm}} at 350 \text{ nm}}{73,000 \times \text{protein concentration (m)}}
\]

(Eq. 2)

where 0.56 is the correction factor for the Alexa Fluor dyes at 280 nm and 73,000 is the molar extinction coefficient for the Alexa Fluor dyes. The molar extinction coefficients for the 402H variant were calculated to be 43,960 and 241,480 for CCP6–8 and flCFH, respectively, whereas the corresponding values for the 402Y variant were determined to be 45,240 and 242,460 respectively, using the method described previously (25). The CCP6–8 and flCFH 402H/402Y variants, which were labeled with 1.5 and 4 moles of dye per mole of protein, respectively, were stored at ~80 °C.

The effect of fluorophore labeling on the CCP6–8 and flCFH proteins (i.e. for both 402H and 402Y) was assessed by affinity chromatography on a 1-mL HiTrap Heparin column (GE Healthcare, Hatfield, UK) (12) equilibrated in PBS (Oxoid, Basingstoke, UK) at 1 ml/min. The CCP6–8 and flCFH proteins were loaded onto the column (at 50 and 25 μg, respectively), which was washed for 10 min and then proteins were eluted with a 20-min gradient (0–1 M NaCl in PBS). As shown in supplemental Fig. S2, this modification of the CFH proteins had no effect on their heparin binding properties, as determined by the salt strength necessary to elute them from the affinity column.

Tissue Preparation and Genotyping—Posterior segments from 14 human donors (Table 1) were obtained from the Manchester Royal Eye Hospital Eye Bank and lightly fixed in 4% (v/v) paraformaldehyde for 2 h at room temperature. The macula regions were excised using an 8-mm diameter biopsy punch and further fixed in 4% (v/v) paraformaldehyde for 16 h at 4 °C, preserved in OCT cryo-protectant (RA Lamb Ltd., Eastbourne,
Reduced Binding of Factor H 402H Variant to Bruch’s Membrane

UK and stored at −80 °C. Macular tissue sections (7 μm) were mounted on polysine-coated microscope slides (Menzel-Gläser, Braunschweig, Germany) and, if not used immediately, stored at −80 °C.

Retinal tissue (20 mg) was digested overnight in restriction buffer (Qiagen buffer G2, as supplied by the manufacturer) containing proteinase K (50 mg/ml) at 56 °C on a shaking hot block. These samples were homogenized and then clarified by centrifugation at 3,000 × g for 10 min at 4 °C. DNA was extracted from the supernatants using a BioRobot EZ1 DNA workstation (Qiagen, Crawley, UK) in accordance to the manufacturer’s instructions.

Primer sequences were designed flanking the polymorphic nucleotide (rs1061170) of CFH (forward primer, 5′-CTTTAGTTGTCCTCAGTTATAC-3′; reverse primer, 5′-CTGACCCAGGATGGCCAGGCACTCTC-3′). Retinal DNA (25 ng) was amplified by 30 cycles of PCR (45 s at 94 °C; 45 s at 55 °C; and 1 min at 72 °C, but with 10 min of extension used in the final step) using 20 pmol of the forward and reverse primers, 0.75 mM dNTPs, and 0.1 units of Taq DNA polymerase in 67 mM Tris-HCl (pH 8.0), 3.7 mM MgCl₂, 6.7 μM EDTA, 16 mM (NH₄)₂SO₄, 0.085 mg/ml BSA. PCR products were purified using Montage PCR centrifugal filter devices (Millipore, Watford, UK), and direct sequencing was performed using the BigDye™ Terminator Version 3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK) on a fluorescent sequencer (ABI 3777).

Tissue Pretreatments and Staining—Prior to staining and tissue pretreatments, the slides were incubated with chilled (−20 °C) histological grade acetone for 20 s before being thoroughly washed with PBS. The sections (either untreated or pretreated as described below) were incubated with 100 μl of blocking buffer (1 mg/ml BSA, 1% (v/v) goat serum, 0.1% (v/v) Triton X-100 in PBS) at room temperature for 1 h and washed thoroughly in PBS. The sections were then incubated simultaneously for 16 h at 4 °C with the Alexa Fluor-labeled 402H and 402Y proteins (20 μg/ml of each in 100 μl of blocking buffer), in the context of either the CCP6–8 construct or the full-length CFH, and then washed with PBS prior to the addition of DAPI (100 μl/well; 0.3 μM final concentration) for 5 min at room temperature. “Blank” controls were incubated with blocking buffer alone. Following washing in PBS, the slides were mounted with VECTASHIELD (Vector Laboratories, Peterborough, UK).

Some sections (in all cases sequential to untreated controls) were pretreated prior to staining with GAG-degrading enzymes (20 units/ml of each enzyme; Sigma, Poole, UK) for 1.5 h at 37 °C; this was performed after fixation in acetone and followed by washing with PBS prior to the blocking step. In this regard, sections were treated with either a mixture of heparinase I, II, and III (“Heparinase” with a capital letter is used to refer to the mixture) and/or chondroitinase B or chondroitinase ABC lyase (all from Flavobacterium heparinum) or hyaluronidase (Streptomyces hyalurolyticus). In alternative experiments, the labeled proteins were preincubated with a 10-fold molar excess of various GAG preparations (i.e. unfractonated heparin corresponding to the 4th International Standard (25), 2,6-O-desulfated heparin prepared from the 2nd International Standard (26–28), or chondroitin-4-sulfate (Calbiochem, Nottingham, UK)) for 1 h at room temperature prior to their use in tissue staining.

Endogenous CFH was detected using a mixture of OX23 and OX24 monoclonal antibodies (i.e. against human CFH (29)) in human eye sections that were either untreated or pretreated with Heparinase and chondroitinase B as described above. The antibodies (10 μg/ml of each in 100 μl of blocking buffer) were incubated with eye sections for 16 h at 4 °C, washed with PBS, and then washed using a 1:5,000 dilution of Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes) in PBS for 2 h at room temperature; DAPI staining and mounting were performed as described above.

Microscopy and Data Analysis—Images were collected on an Olympus BX51 widefield microscope using a 20×/0.3 Plan Fln objective and captured using a CoolSNAP ES camera (Photometrics) through MetaVue Software (Molecular Devices). Specific band pass filter sets for DAPI, FITC, and Texas Red were used to prevent bleed-through from one channel to the next. Images were then processed and analyzed using ImageJ64 (version 1.40g; rsb.info.nih.gov/ij).

The relative fluorescent intensities of the RPE, Bruch’s membrane, and choroidal arterioles were determined. Briefly, the grayscale images of both red and green channels (i.e. for either the test sections or the blank controls) were overlaid, and the ellipse selection tool (in ImageJ64) was used to create an overlapping network of circles enclosing the RPE or Bruch’s membrane (care was taken to ensure no overlap and good separation between these different regions), whereas a box was used to select individual choroidal blood vessels. This allowed the determination of fluorescent intensities specific to these macular features following subtraction of background auto-fluorescence from blanks (i.e. sequential to the test sections). Tissue sections from five separate donors were analyzed to obtain mean values (± S.E.), and the statistical significance of 402H versus 402Y binding was determined using the paired Student’s t test within KaleidaGraph (v 4.0), where p < 0.05 was considered to be significant. To assess any potential effect of CFH genotype on the localization of exogenously applied protein (either CCP6–8 or flCFH), fluorescent intensities specific to the RPE and Bruch’s membrane for three separate donors of each possible genotype (i.e. homozygous 402Y/402Y, heterozygous 402H/402Y, and homozygous 402H/402H; n = 9 total) were compared using one-way analysis of variance (ANOVA) analysis within KaleidaGraph; p < 0.05 was considered to be significant.

Microtiter Plate-based GAG Binding Assays—The GAG binding properties of the 402H and 402Y variants of CCP6–8 were determined using a microtiter plate assay essentially as described previously (12). The CCP6–8 proteins (unlabeled) were immobilized at 1 μg/well in 100 μl of PBS on MaxiSorp plates (NUNC, Kastrup, Denmark), and the binding of biotinylated-GAGs (i.e. chondroitin-4-sulfate, chondroitin-6-sulfate (both from Calbiochem), dermatan sulfate from porcine mucosa (12), and heparan sulfate from bovine kidney (Sigma)) was determined at a range of concentrations (1–10 μg/well). The GAGs were biotinylated as described before (12). Plates were developed for 10 min, and absorbance values at 405 nm
Reduced Binding of Factor H 402H Variant to Bruch’s Membrane

RESULTS AND DISCUSSION

Differential Binding of 402Y and 402H in Bruch’s Membrane—Here we have investigated whether the Y402H polymorphism affects the interaction of CFH with its binding sites in normal macula in the human eye (from donors without AMD). To determine the effect of this coding change in isolation from other CFH polymorphisms, we used constructs comprising CCP6–8 with either a Tyr or a His at position 402 (used previously for both functional and structural studies (12, 13, 20, 22)) and compared this with the variants in the context of the full-length CFH protein (24). The 402H and 402Y variants (in both CCP6–8 and flCFH) were differentially labeled with red and green fluorophores, respectively, allowing their simultaneous detection in eye tissues and thus the visualization of CFH-binding sites (CFHBS). As can be seen from Fig. 1, this analysis indicated that there are significantly fewer binding sites in the Bruch’s membrane and choroidal blood vessels for the 402H variant when compared with the 402Y form (p < 0.05), but there is a similar level of binding of the two variants within the RPE (i.e. in the context of both the CCP6–8 construct and the flCFH proteins). It should be noted that an essentially identical result was obtained when the fluorophores were swapped on the 402H and 402Y variants (in the context of the CCP6–8 construct), showing that the differential binding of the two variants is independent of the fluorophore used (see supplemental Fig. S3). Furthermore, the binding of labeled CCP6–8 was shown to be specific by competition studies (supplemental Fig. S4). Here sections were co-incubated with the Alexa Fluor-labeled proteins in the absence or presence of the equivalent unlabeled protein at 1:1 or 1:10 molar ratios, giving rise to ~50 and ~80–90% reductions, respectively, in the binding of the labeled proteins.

Thus, the data in Fig. 1C (averaged from five donors) clearly demonstrate that there are more CFHBS for the 402Y variant when compared with the 402H form within Bruch’s membrane: i.e. 2- or 3-fold higher, respectively, when using the flCFH or CCP6–8 proteins as probes. This is in contrast to the similar level of binding seen for the 402H and 402Y variants to RPE (Fig. 1), which is consistent with previous studies on human RPE cells in culture (30); we also observed equivalent 402H and 402Y binding to leukocytes within the choroid (Fig. 1A), but this has not been investigated further. Similar to that seen for the Bruch’s membrane, blood vessels of the choroid had a significantly higher level of 402Y-binding sites (green staining) when analyzed in the context of either CCP6–8 or the flCFH proteins (Fig. 2).

The striking differences in the levels of the binding sites for the disease and non-disease variants of CFH within certain regions of human macula could be a major contributor to AMD pathology (discussed in more detail below). In this regard, the differential binding to the Bruch’s membrane may be of particular importance given that this is the site of drusen formation/accumulation. Therefore, this region of the macula (along with the adjacent RPE) provided the major focus for our further investigations.

HS and DS Are Binding Sites for CFH in Bruch’s Membrane and RPE—Given that the Y402H polymorphism affects the interaction of CFH with heparin (12, 13, 30), we investigated whether the differential binding of the 402H/402Y variants to sites in macula involved sulfated GAGs; this was done using the CCP6–8 constructs because these contain a single heparin-binding site and their GAG binding properties have been extensively characterized (12, 13). As can be seen from Fig. 3 (and supplemental Fig. S5), pretreatment of tissue sections to remove HS (with Heparinase enzymes) reduced binding by 70% in both RPE and Bruch’s membrane, whereas digestion of dermatan sulfate (DS) with chondroitinase B caused a reduction of ~20–45%, respectively. When these enzyme pretreatments were combined, almost all of the binding of the CCP6–8 proteins was abolished (Fig. 3B), indicating that HS and DS glycosaminoglycans constitute major binding sites for the AMD-associated region of CFH. Both HS and DS proteoglycans have been shown previously to be constituents of the Bruch’s membrane (31), and this has been supported by our recent staining for HS and DS with phage display and “stub” antibodies, respectively.

The above results are consistent with the finding that the 402H and 402Y variants (in CCP6–8) interact with both DS and HS (Fig. 4), i.e. in assays where the binding of biotinylated GAGs to immobilized proteins was assessed. From Fig. 4, it can be seen that both the 402H and 402Y variants show saturable binding to these GAGs. For DS, previous studies have shown that CFH binds via CCP6–8 (12, 32); however, we reported that CFH did not interact with HS (12), which is at odds with the results described here. In Clark et al. (12), the HS was immobilized on an allylamine-coated surface, where such immobilization of GAGs has been demonstrated to rely on ionic interactions (33). Thus, it seems likely that the passive absorption of HS to the allylamine surface masked its ability to bind CFH (12); e.g. sulfates on HS necessary for mediating the interaction with CFH (i.e. implied from our studies on heparin binding (12, 13)) are utilized in the immobilization of HS to the plasma polymerized microtiter plate. From the data in Fig. 4, it is apparent that the CCP6–8 construct interacts with HS, and, thus, it is likely that the discrepancy from our previous study (12) arises from the different assay systems used.

Additional enzyme pretreatments of the tissue sections, along with competition experiments with well characterized GAG preparations, indicate that neither chondroitin sulfate nor hyaluronan is required for CFH binding in the macula (Table 2). For instance, chondroitinase ABC lyase, which digests chondroitin sulfate in addition to DS, did not remove more CFHBS when compared with chondroitinase B alone, and pretreatment with hyaluronidase had little effect on binding of the 402H and 402Y variants (supplemental Fig. S6). Furthermore, preincubation of the CFH proteins with chon-
droitin-4-sulfate did not significantly reduce their binding to sites in macula, whereas heparin caused an \( \sim 60-70\% \) reduction (supplemental Fig. S7); the lack of significant competition by chondroitin-4-sulfate is consistent with the poor binding seen for chondroitin sulfate in solid-phase assays (Fig. 4). These data are consistent with our previous studies showing that CCP6–8 (402Y) does not interact with immobilized chondroitin sulfate or hyaluronan (32).

FIGURE 1. Identification of 402H- and 402Y-binding sites in human macula. The 402H and 402Y variants (in the context of recombinant CCP6–8 or flCFH) were conjugated with Alexa Fluor 594 (red) and 488 (green), respectively, and used to label sections obtained from five human eyes (without AMD); nuclei were stained with DAPI (blue). Merged images (A) and corresponding grayscale data (B) are illustrated for one representative donor eye (a 402H/402Y heterozygote), where the scale bar corresponds to 100 \( \mu \)m (here and in all other figures); yellow/orange staining (e.g. on the RPE) is indicative of regions to which both 402H and 402Y variants bind equivalently. C, the relative fluorescent intensities of the RPE and Bruch’s membrane were determined for the five different eyes, and the autofluorescence from blanks was subtracted; the autofluorescence observed in the red channel is due to the accumulation of lipofuscin granules within the RPE (7). Data are shown as mean values \( \pm \) S.E., where statistical analysis of the 402H versus 402Y binding data was determined using the paired Student’s \( t \) test. The five donors (donor group A) used here and throughout the rest of the study (unless indicated otherwise) corresponded to three heterozygous (402H/402Y) and two homozygous 402Y/402Y samples (Table 1).
402H and 402Y Bind to Different HS Structures in Human Macula—Our previous studies on the CCP6–8 region of CFH indicated that the His and Tyr variants are likely to recognize different heparin structures (12, 13); the binding of 402Y is much less sensitive to changes in sulfation than 402H (i.e. the former has a broader specificity). For example, removal of the 2-O- and 6-O-sulfates from unfractionated heparin had markedly different effects on the binding of these proteins, i.e. 2,6-O-desulfated heparin binds better to 402Y than 402H (12). Here we found that 2,6-O-desulfated heparin could compete for the binding of 402Y to both Bruch’s membrane and RPE (~70% reduction) but did not compete 402H binding (Fig. 3C and supplemental Fig. S5). These data (and equivalent results for the full-length proteins; not shown) demonstrate that the 402H

FIGURE 2. Visualization of 402H- and 402Y-binding sites in choroidal blood vessels. CFH-binding sites were detected in large choroidal blood vessels using the 402H and 402Y variants in the context of either CCP6–8 (A) or flCFH (B). Merged images for 402H (red), 402Y (green), and DAPI (blue), illustrated for one donor eye (corresponding to the same 402H/402Y heterozygote section used in Fig. 1), are shown with the corresponding grayscale images for 402H and 402Y underneath. The white arrows denote 50-μm sections through representative blood vessels (from outside to inside) from which the grayscale values (fluorescent intensities) shown in C and D were determined. E and F, the relative fluorescent intensities were determined for five donor eyes, and the autofluorescence from blanks was subtracted; eight and seven vessels in total were analyzed for the CCP6–8 (E) and flCFH (F), respectively. Data, which are shown as mean values ± S.E., were compared using the paired Student’s t test.
Reduced Binding of Factor H 402H Variant to Bruch’s Membrane

FIGURE 3. Heparan sulfate and dermatan sulfate represent major CFH-binding sites in human macula. Fluorescently labeled 402H and 402Y variants of CFH (in the context of the CCP6–8 construct) were used to stain eye sections essentially as in Fig. 2, except that sections were pretreated with GAG-digesting enzymes (A and B) or the labeled proteins were preincubated with a defined GAG preparation (C). A, merged images illustrated for one representative donor eye (a 402Y/402Y homozygote) with and without enzymatic pretreatment using Heparinase and/or Chondroitinase B. B, the relative fluorescent intensities of the RPE and Bruch’s membrane were determined for the five donor eyes where sections were treated as described in A, and the autofluorescence from blanks was subtracted (grayscale values are provided in supplemental Fig. S5). Data are shown as mean percentage of binding (n = 5; ± S.E.) relative to untreated sections (dotted lines). C, merged images for the same donor as in A where the 402H and 402Y proteins were preincubated with or without 2,6-O-desulfated heparin (2,6-O-deS) prior to staining and the corresponding mean percentage of binding (relative to the absence of competitor; dotted line) for the five donor eyes (± S.E.). Grayscale values are provided in supplemental Fig. S5. Statistical analysis between pairs of data (in B and C) was performed using the paired Student’s t test.
and 402Y variants bind to distinct structural features (e.g. sulfation patterns) within the HS present in human macula. These features are likely to be highly tissue-specific, given that there was only a small difference seen in the relative binding of the two CFH variants to HS purified from a different source (i.e. bovine kidney; Fig. 4).

Moreover, because HS is a highly heterogeneous GAG (i.e. with a hugely diverse range of possible sequences (34, 35)), it seems reasonable to suggest that the differential binding seen for these CFH variants may also result from differences in the HS found in different regions of the macula. Furthermore, these data indicate that 2-O- and/or 6-O-sulfates are essential for the binding of the 402H variant to sites within Bruch’s membrane and RPE.

Contribution of CCPs 6–8 to the Binding of CFH to Human Macula—The differences in binding patterns for the 402H and 402Y variants (throughout the tissue) were essentially identical whether examined in the context of either recombinant CCP6–8 constructs or flCFH purified from homozygous individuals (Fig. 1), which have single or multiple heparin-binding sites, respectively. Thus, the interaction of CFH with GAGs in human macula appears to be largely mediated via the CCP6–8 region where the His/Tyr coding change is located. Although we cannot rule out that other GAG-binding sites in CFH (e.g. in CCP20 (18), which has been implicated as the major binding site for the interaction of CFH in other tissues/cells, such as kidney/endothelial cells (36–38)) could also play a role, polymorphisms in CCP19–20 known

**TABLE 2**

Summary table showing percentage binding of CCP6–8 402H/402Y variants to macula tissue either after enzymatic pre-treatment or in the presence of competitors.

The table shows percentage of binding (%) ± S.E., where *n* = 5 eye samples from different donors.

| Location          | CFH variant | Competition                     | Enzymatic treatment |
|-------------------|-------------|---------------------------------|---------------------|
|                   |             | Unfractionated (4IS) heparin    |                      |
|                   |             | Chondroitin–4–sulfate           |                      |
|                   |             | 2,6-O Desulfated heparin        |                      |
|                   |             | Hyaluronidase                   | Chondroitinase ABC   |
| RPE               | 402H        | 37.76 ± 13.71                   | 101.07 ± 9.78        |
|                   | 402Y        | 35.08 ± 7.71                    | 33.79 ± 9.63         |
| Bruch’s membrane  | 402H        | 26.49 ± 10.15                   | 111.43 ± 5.49        |
|                   | 402Y        | 28.85 ± 4.90                    | 35.03 ± 7.55         |

**FIGURE 4.** 402H and 402Y variants of CFH bind directly to dermatan sulfate and heparan sulfate. The 402H and 402Y variants of CFH (in the context of the CCP6–8 construct) were immobilized onto microtiter plates, and the binding of biotinylated GAGs was determined over a range of concentrations. A, chondroitin-4-sulfate (C4S); B, chondroitin-6-sulfate (C6S); C, DS, D, HS. Data (402H, circles; 402Y, squares) are plotted as mean absorbance (A405 nm) determined from two independent experiments (*n* = 8; ± S.E.).
to affect heparin binding have not been found to be associated with AMD (7, 8). Thus, tissue-specific differences in GAG structures may alter the relative contribution to binding by the CCP7 and CCP20 heparin-binding sites. Importantly, removal of HS and DS reduced the binding of flCFH to the RPE and Bruch’s membrane by 40–50% (supplemental Fig. S8), demonstrating that these GAGs are major determinants in the interaction of CFH within macula. However, other non-GAG ligands (e.g. proteins), binding mostly outside of the CCP6–8 region, are also likely to make a significant contribution.

Endogenous CFH Associates with HS/DS in Human Macula—Analysis of endogenous CFH in macula tissue samples (Fig. 5) demonstrates that it has an essentially identical localization to that of the CFHBS detected with exogenously added protein, e.g. it is present on the RPE, in Bruch’s membrane, and in the choroid. Removal of HS and DS caused a significant reduction in staining (~40 and ~25% in Bruch’s membrane and RPE, respectively) indicating that endogenous CFH interacts with HS/DS within these locations. Importantly, this also provides direct evidence that CFH does interact with sulfated GAGs within host tissues.

**FIGURE 5.** Endogenous CFH associates with GAGs in Bruch’s membrane and RPE within human macula. Endogenous CFH was detected (using OX23/ OX24 monoclonal antibodies and the appropriate Alexa Fluor 488-labeled secondary antibody) in human eye sections from five heterozygous individuals (donor group C; Table 1) either untreated or following treatment with Heparinase and chondroitinase B. A, merged images for untreated, treated, and ‘blank’ sections illustrated for a single donor eye (a 402Y/402H heterozygote). B, the relative fluorescent intensities of the RPE and Bruch’s membrane (determined for the donor sections as described above and with the autofluorescence from blanks subtracted), where data are shown as gray values (n = 5; ± S.E.). C, data from B are shown as the mean percentage of binding (n = 5; ± S.E.) relative to untreated sections (dotted line). Statistical analyses of these data relative to untreated controls were done using the paired Student’s t test.

**TABLE 3**

One-way ANOVA analysis of the influence of CFH genotype on 402H and 402Y variant binding

|                | CCP6-8 | BCFP | H/Y heterozygous | H homozygous | Y homozygous | Mean   | std   | s.e.m. | One Way ANOVA P value |
|----------------|--------|------|------------------|--------------|--------------|--------|-------|--------|-----------------------|
|                |        |      |                  |              |              |         |       |        |                       |
| RPE            |        |      |                  |              |              |         |       |        |                       |
| 402H           | 49.989 | 8.783| 40.637           |              |              | 111.704| 65.061| 64.957 | 107.125               |
| 402Y           | 44.043 | 47.568| 56.905           |              |              | 32.876 | 121.974| 55.444 | 77.353                |
| Bruch’s membrane | 29.006 | 25.222| 47.322           |              |              | 11.130 | 17.895| 78.598 | 39.076                |
|                |        |      |                  |              |              | 163.457| 40.134| 44.926 | 71.592                |
| Y homozygous   |        |      |                  |              |              | 127.563| 134.634| 131.076 | 129.538               |
|                |        |      |                  |              |              | 147.599| 136.045| 141.488 | 140.546               |
|                |        |      |                  |              |              | 71.625 | 56.734| 67.811 | 75.486                |
|                |        |      |                  |              |              | 119.160| 133.127| 190.739 | 143.833               |
| 402H           |        |      |                  |              |              | 145.156| 153.521| 161.324 | 127.563               |
| 402Y           |        |      |                  |              |              | 162.965| 145.547| 196.035 | 147.599               |
|                |        |      |                  |              |              | 87.359 | 41.994| 113.163 | 71.625                |
|                |        |      |                  |              |              | 201.190| 146.079| 208.229 | 119.160               |
|                  |        |      |                  |              |              |         |       |        |                       |
|                  |        |      |                  |              |              |         |       |        |                       |

* To address the possibility that endogenously expressed CFH masks the recognition of CFHBS by the exogenously applied proteins to the tissue sections and that this will differ between individuals of different CFH genotype, we performed ANOVA analysis of nine donors: three from each of 402Y/402Y, 402H/402Y, and 402H/402H genotype, which correspond to donor groups ‘A’ and ‘B’ in Table 1. As demonstrated here, none of the genotypes affects the binding of 402H or 402Y CFH proteins (in the context of either CCP6–8 or flCFH) to the Bruch’s membrane or RPE.

† Analysis of these nine donor samples by the Student’s t test shows a significant difference in binding for 402H and 402Y in the Bruch’s membrane (p = 0.00032 and p = 0.01313 for CCP6–8 and flCFH, respectively), whereas there was no significant difference in their binding to the RPE (p = 0.07516 and p = 0.86330, respectively).
One consequence of endogenous CFH being present within human macula is that it could potentially mask the binding sites for exogenously added protein, where this might be influenced by the genotype of the donor (e.g. given the differential binding specificities of the 402H and 402Y variants). To address this possibility, we performed ANOVA analysis of 402H and 402Y binding (in the context of both CCP6–8 and flCFH) to tissue from three donors of each CFH genotype (i.e. 402Y/402Y, 402H/402Y, and 402H/402H). This statistical analysis revealed that the binding of CFH to Bruch’s membrane and RPE is independent of the CFH genotype (Table 3); supplemental Fig. S9 shows a comparison of CCP6–8 and flCFH binding to tissue sections from the three genotypes. Therefore, masking by endogenous CFH is unlikely to be responsible for the differential binding observed for the exogenously added 402H and 402Y proteins.

It should be noted that analysis of the CFHBS in the nine donors (groups ‘A’ and ‘B’ combined) described in Table 3 gave an essentially identical result to that seen for donor group A alone in Fig. 1. In this regard, there was a statistically significant difference in the binding of 402H and 402Y (in the context of both CCP6–8 and flCFH) to Bruch’s membrane but no significant difference in their binding to RPE (see footnote † in Table 3).

Differential Binding of the CFH Variants Provides a Potential Disease Mechanism for AMD—The data reported here provide strong evidence that CFH does associate with sulfated GAGs within host tissues where these interactions may underpin its role in immune surveillance. Importantly, we have shown that the AMD-associated 402H variant of CFH binds less well than the 402Y form to HS/DS GAGs within Bruch’s membrane when these are exogenously added as probes. However, it should be noted that, as yet, we have not examined whether this fundamental difference in the GAG binding properties of the 402H/402Y allotypes (e.g. see Refs. 12 and 13) affects the localization of the endogenous CFH variants. If this is the case, then the much poorer binding of the disease-associated variant to Bruch’s membrane is likely to make a major contribution to the initiation and development of AMD due to impaired regulation of the complement alternative pathway at this site. For example, increased complement turnover at the Bruch’s membrane would lead to chronic local inflammation at the RPE/chorioid interface, which is believed to be a major feature of the pathology of AMD (39). Thus, the finding that the 402H and 402Y variants differentially recognize sulfated polysaccharides within macula and that this may influence their localization at the site of AMD pathology provides a potential disease mechanism for this major form of blindness.

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REFERENCES

1. Coleman, H. R., Chan, C. C., Ferris, F. L., 3rd, and Chew, E. Y. (2008) Lancet 372, 1835–1845
2. Hageman, G. S., Luther, P. J., Victor Chong, N. H., Johnson, L. V., Anderson, D. H., and Mullins, R. F. (2001) Prog. Retin. Eye Res. 20, 705–732
3. Day, A. J., Willis, A. C., Ripoche, J., and Sim, R. B. (1988) Immunogenetics 27, 211–214
4. Edwards, A. O., Ritter, R., 3rd, Abel, K. J., Manning, A., Panhuysen, C., and Farrer, L. A. (2005) Science 308, 421–424
5. Klein, R. J., Zeiss, C., Chew, E. Y., Tsai, J. Y., Sackler, R. S., Haynes, C., Henning, A. K., SanGiovanni, J. P., Mane, S. Y., Mayne, S. T., Bracken, M. B., Ferris, L. F., Ott, J., Barnstable, C., and Hoh, J. (2005) Science 308, 385–389
6. Haines, J. L., Hauser, M. A., Schmidt, S., Olson, L. M., Gallins, P., Spencer, K. L., Kwan, S. Y., Noureddine, M., Gilbert, J. L., Schnett-Boutaud, N., Agarwal, A., Postel, E. A., and Pericak-Vance, M. A. (2005) Science 308, 419–421

17. Hageman, G. S., Anderson, D. H., Johnson, L. V., Hancox, L. S., Taiber, A. J., Hardisty, L. L., Hageman, J. L., Stockman, H. A., Borchardt, J. D., Gehrs, K. M., Smith, R. J., Silvestri, G., Russell, S. R., Kraver, C. C., Barbazetto, I., Chang, S., Yunnuzzi, L. A., Barile, G. R., Merriam, J. C., Smith, R. T., Olsh, A. K., Bergeron, J., Zernant, J., Merriam, J. E., Gold, B., Dean, M., and Allikmets, R. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 7227–7232

18. Barlow, P. N., Hageman, G. S., and Lea, S. M. (2008) Adv. Exp. Med. Biol. 632, 117–142

19. Montes, T., Tortajada, A., Morgan, B. P., Rodriguez de Cordoba, S., and Harris, C. L. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 4366–4371

20. Kazatchkine, M. D., Fearon, D. T., Silbert, J. E., and Austen, K. F. (1990) J. Exp. Med. 172, 1209–1215

21. Meri, S., and Pangburn, M. K. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 3982–3986

22. Clark, S. J., Higman, V. A., Mullroy, B., Perkins, S. J., Lea, S. M., Sim, R. B., and Day, A. J. (2006) J. Biol. Chem. 281, 24713–24720

23. Prosser, B. E., Johnson, S., Roversi, P., Herbert, A. P., Blaum, B. S., Tyrell, J., Jowitt, T. A., Clark, S. J., Tarelli, E., Uhrin, D., Barlow, P. N., and Roversi, P., Johnson, S., Day, A. J., and Lea, S. M. (2007) J. Exp. Med. 204, 2277–2283

24. Pangburn, M. K., Atkinson, M. A., and Meri, S. (1991) J. Biol. Chem. 266, 16847–16853

25. Blackmore, T. K., Sadlon, T. A., Ward, H. M., Lublin, D. M., and Gordon, D. L. (1996) J. Immunol. 157, 5422–5427

26. Blackmore, T. K., Hellwege, J., Sadlon, T. A., Higgs, N., Zipfel, P. F., Ward, H. M., and Gordon, D. L. (1998) J. Immunol. 160, 3342–3348

27. Giannakis, E., Jokiranta, T. S., Male, D. A., Ranganathan, S., Ormsby, R. J., Fischetti, V. A., Mold, C., and Gordon, D. L. (2003) Eur. J. Immunol. 33, 962–969

28. Herbert, A. P., Uhrin, D., Lyon, M., Pangburn, M. K., and Barlow, P. N. (2006) J. Biol. Chem. 281, 16512–16520

29. Ripoche, J., Day, A. J., Harris, T. J., and Sim, R. B. (1988) Biochem. J. 249, 593–602

30. Goldberg, A. P., Trouw, L. A., Clark, S. J., Sjölander, J., Heinegård, D., Sim, R. B., Day, A. J., and Blom, A. M. (2007) J. Biol. Chem. 282, 10894–10900

31. Laine, M., Jarva, H., Seitsonen, S., Haapasalo, K., Raitanen, I., Jokiranta, T. S., Hageman, G. S., Immonen, I., and Miinniemi, S. (2007) J. Immunol. 178, 3831–3836

32. Schneider, M. C., Prosser, B. E., Caesar, J. J., Kugelberg, E., Li, S., Zhang, Q., Quorashi, S., Lovett, J. E., Deane, I. E., Sim, R. B., Roversi, P., Johnson, S., Tang, C. M., and Lea, S. M. (2009) Nature 458, 890–893

33. Haapasalo, K., Jarva, H., Siljamäki, T., Tewodros, W., Vuopio-Varkila, J., and Jokiranta, T. S. (2008) Mol. Microbiol. 70, 583–594

34. Hakobyan, S, Harris, C. L., van den Berg, C. W., Fernandez-Alonso, M. C., Gioiochea de Jorge, E., Rodriguez de Cordoba, S., Rivas, G., Mangione, P., Pepys, M. B., and Morgan, B. P. (2008) J. Biol. Chem. 283, 30451–30460

35. Gill, S. C., and von Hippel, P. H. (1989) Anal. Biochem. 182, 319–326

36. Mullroy, B., Gray, E., and Barrowcliffe, T. W. (2000) Thromb. Haemost. 84, 1052–1056

37. Mullroy, B., Forster, M. J., Jones, C., Drake, A. F., Johnson, E. A., and Davies, D. B. (1994) Carbohydr. Res. 255, 1–26
Reduced Binding of Factor H 402H Variant to Bruch’s Membrane

28. Ostrovsky, O., Berman, B., Gallagher, J., Mulloy, B., Fernig, D. G., Delehedde, M., and Ron, D. (2002) J. Biol. Chem. 277, 2444–2453
29. Sim, E., Palmer, M. S., Puklavec, M., and Sim, R. B. (1983) Biosci. Rep. 3, 1119–1131
30. Ormsby, R. J., Ranganathan, S., Tong, J. C., Griggs, K. M., Dimasi, D. P., Hewitt, A. W., Burdon, K. P., Craig, J. E., Hoh, J., and Gordon, D. L. (2008) Invest. Ophthal. Vis. Sci. 49, 1763–1770
31. Call, T. W., and Hollyfield, J. G. (1990) Exp. Eye Res. 51, 451–462
32. Marson, A., Robinson, D. E., Brookes, P. M., Mulloy, B., Wiles, M., Clark, S. J., Fielder, H. L., Collinson, L. J., Cain, S. A., Kiely, C. M., McArthur, S., Buttle, D. J., Short, R. D., Whittle, J. D., and Day, A. J. (2009) Glycobiology 19, 1537–1546
33. Mahoney, D. J., Whittle, J. D., Milner, C. M., Clark, S. J., Mulloy, B., Buttle, D. J., Jones, G. C., Day, A. J., and Short, R. D. (2004) Anal. Biochem. 330, 123–129
34. Esko, J. D., and Selleck, S. B. (2002) Annu. Rev. Biochem. 71, 435–471
35. Clark, S. J., Bishop, P. N., and Day, A. J. (2010) Biochem. Soc. Trans., in press
36. Ferreira, V. P., Herbert, A. P., Hocking, H. G., Barlow, P. N., and Pangburn, M. K. (2006) J. Immunol. 177, 6308–6316
37. Oppermann, M., Manuelian, T., Iózsi, M., Brandt, E., Jokiranta, T. S., Heinen, S., Meri, S., Skerka, C., Götze, O., and Zipfel, P. F. (2006) Clin. Exp. Immunol. 144, 342–352
38. Pickering, M. C., de Jorge, E. G., Martinez-Barricarte, R., Recalde, S., Garcia-Layana, A., Rose, K. L., Moss, J., Walport, M. J., Cook, H. T., Rodriguez de Córdoba, S., and Botto, M. (2007) J. Exp. Med. 204, 1249–1256
39. Anderson, D. H., Radeke, M. J., Gallo, N. B., Chapin, E. A., Johnson, P. T., Curiel, C. R., Hancox, L. S., Hu, J., Ebright, J. N., Malek, G., Haeuser, M. A., Rickman, C. B., Bok, D., Hageman, G. S., and Johnson, L. V. (2010) Prog. Retin. Eye Res. 29, 95–112