The Factor H Variant Associated with Age-related Macular Degeneration (His-384) and the Non-disease-associated Form Bind Differentially to C-reactive Protein, Fibromodulin, DNA, and Necrotic Cells*

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Recently, a polymorphism in the complement regulator factor H (FH) gene has been associated with age-related macular degeneration. When histidine instead of tyrosine is present at position 384 in the seventh complement control protein (CCP) domain of FH, the risk for age-related macular degeneration is increased. It was recently shown that these allotypic variants of FH, in the context of a recombinant construct corresponding to CCPs 6–8, recognize polyanionic structures differently, which may lead to altered regulation of the alternative pathway of complement. We show now that His-384, corresponding to the risk allele, binds C-reactive protein (CRP) poorly compared with the Tyr-384 form. We also found that C1q and phosphorylcholine do not compete with FH for binding to C-reactive protein. The interaction with extracellular matrix protein fibromodulin, which we now show to be mediated, at least in part, by CCP6–8 of FH, occurs via the polypeptide of fibromodulin and not through its glycosaminoglycan modifications. The Tyr-384 variant of FH bound fibromodulin better than the His-384 form. Furthermore, we find that CCP6–8 is able to interact with DNA and necrotic cells, but in contrast the His-384 allotype binds these ligands more strongly than the Tyr-384 variant. The variations in binding affinity of the two alleles indicate that complement activation and local inflammation in response to different targets will differ between His/His and Tyr/Tyr homozygotes.

In the western world, age-related macular degeneration (AMD) is the leading cause of natural blindness in the elderly (1), affecting 50 million individuals worldwide. As the mean age of the population increases it is expected that the incidence will rise even further (2). Epidemiology reveals a complex etiology with not only age but also sex, diet, and ethnic background as underlying factors for AMD predisposal. As with every multifactorial disease there are certainly also genetic predispositions. For example, C-reactive protein (CRP) levels appear to be higher in AMD patients (3), and recently attention has been drawn to a polymorphism in the factor H (FH) gene present in Caucasians (4). FH is the major soluble inhibitor of the alternative pathway of the complement system. Alternative pathway activation results from a failure to appropriately regulate the constant low level spontaneous turnover of the abundant complement component C3 to C3(H2O). Complement non-activating cells and other host surfaces are protected from alternative pathway complement attack through binding of FH, which is believed to have a strong affinity for polyanionic structures such as glycosaminoglycans as well as glycoproteins containing sialic acid residues (5–7). FH is therefore able to discriminate between self and non-self surfaces. FH inhibits the alternative pathway by binding C3b and reducing the formation and activity of the alternative pathway C3-convertase (C3bBb). It also accelerates the decay of this convertase and works as a cofactor for the serine proteinase factor I in the degradation of C3b (8). FH is a ~150-kDa plasma protein that consists of 20 tandemly arranged complement control protein (CCP) domains. Recently, the T1277C polymorphism in the FH gene with a Tyr to His alteration at amino acid 384 located in CCP7 in the mature protein (9), alternatively referred to as H402Y (10, 11), has been found to account for ~50% of the attributable risk of AMD in Caucasians (10–12). Current data suggest that a single copy of the His-384-encoding allele (homozygous) confers 2–4-fold increased risk of developing AMD, whereas two copies of this allele (homozygous) confer 5–7-fold increased risk.

*This work was supported by grants from the U.S. Immunodeficiency Network, Swedish Foundation for Strategic Research, Swedish Medical Research Council, Foundations of Österlund, Kock, and Borgström, King Gustav V’s 80th Anniversary Foundation, NIAMS, National Institutes of Health Grant S01 AR050926, and by research grants from Lund University (Tissues in Motion) and the University Hospital in Malmö. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Recipient of postdoctoral stipends from the foundations of Wenner-Gren and Anna-Greta Crafoord.
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4 The abbreviations used are: AMD, age-related macular degeneration; BSA, bovine serum albumin; FH, factor H; C4BP, C4b-binding protein; CRP, C-reactive protein; CCP, complement control protein; FMOD, fibromodulin; LPS, lipopolysaccharide; PC, phosphorylcholine.
FH has been shown to be associated with drusen (insoluble deposits accumulating between retinal pigment epithelium and Bruch’s basement membrane) in AMD patients (10) together with deposited complement factors such as C3b and components of the membrane attack complex (13). Therefore, a hypothesis was put forward suggesting that abnormal complement activation and inflammation in drusen contributes to AMD (14). At present it is unclear which substances could be activating complement in drusen but potential candidates include apoptotic cells, nuclear fragments, advanced glycation end products, CRP and glycosaminoglycans. In this regard, it was recently demonstrated that the His-384 and Tyr-384 polymorphic variants have differential heparin binding properties (5) and that they interact with sulfated glycosaminoglycans via a binding site in CCP7. In the case of unfractionated heparins, the His-384 construct showed higher binding compared with Tyr-384. The desulfation of heparin had a much greater effect on the interaction with His-384 (5). These differences are likely to reflect the differential ability of the variants to associate with polyanionic structures on surfaces activating complement. This finding is particularly relevant because the structure and expression of proteoglycans drastically change in aging and disease, representing a possible explanation as to why AMD is a disease of advanced age (15, 16). Interestingly, FH deficiency is also associated with type II membranoproliferative glomerulonephritis, a rare renal disease in which drusen present in complications in the eye have a similar composition to those found in AMD (17).

CRP also binds to the CCP7 region of FH (18, 19). Other ligands of FH, such as DNA (20) and the extracellular matrix proteoglycan fibromodulin (FMOD) (21), could potentially also bind at this site. Here we show that the His-384 variant of FH binds CRP less efficiently than the non-disease-associated form. We identify CCP6–8 to be a binding site for FMOD and, similar to the CRP interaction, Tyr-384 appears to bind FMOD better than the histidine variant. Interestingly, the His-384 variant of FH bound DNA and necrotic cells better than Tyr-384, which potentially could influence complement activation and opsonization in some areas of the drusen.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Full-length FH was purified from pooled human plasma of 12 donors (22). The His-384 and Tyr-384 variants of FH were expressed, in the context of a construct corresponding to CCPs 6–8, and purified as described previously (5). Proper folding of the expressed proteins was verified by one-dimensional NMR spectroscopy and determination of disulfide bonds by mass spectrometry of trypsin digests. These proteins were biotinylated using EZLink N-hydroxysuccinimide-LC Biotin (Pierce) as described (5). FMOD was isolated from bovine articular cartilage (23); recombinant CRP was obtained from Calbiochem and lipopolysaccharide (LPS) from Sigma. C4BP-PS complex (24) and C1q (25) were purified from human plasma as described. Phosphorylcholine-bovine serum albumin (PC-BSA) was prepared by coupling p-aminophenyl-phosphorylcholine to bovine serum albumin according to the procedure described by Padilla et al. (26). Deglycosylated FMOD was prepared by treatment with N-glycosidase F (Roche Applied Science) as previously described (21).

**Protein/Protein Binding Assays**—CRP or FMOD was coated overnight at 4 °C onto microtiter plates (Maxisorp, Nunc) at a concentration of 10 µg/ml in 75 mM sodium carbonate buffer, pH 9.6. LPS was coated in the same manner at a concentration of 5 µg/ml. Wells treated only with coating buffer or 1% (w/v) BSA (Sigma) in coating buffer served as negative controls. Between each step the wells were washed extensively with 50 mM Tris-HCl, 150 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5. All wells were blocked with 1% (w/v) BSA in phosphate-buffered saline (Invitrogen) for 1 h at 37 °C. Y384CCP6–8 and H384CCP6–8 or full-length FH were added at increasing concentrations in the binding buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM CaCl2). In the experiments with FMOD, 50 µg/ml BSA was included in the binding buffer. For the competition assays FH was added at 3 µg/ml in the binding buffer containing increasing concentrations of C1q or C4BP-PS. The amount of bound protein was assessed using a goat polyclonal anti-FH antibody (catalogue number A312; Quidel) followed by horseradish peroxidase-labeled anti-goat secondary antibodies (Dako) and the OPD development kit (Dako). Detection of biotinylated protein was performed using a StreptABComplex/horseradish peroxidase kit from Dako, and in both cases absorbance at 490 nm was measured to quantify protein binding.

**Protein/DNA Binding Assay**—The pcDNA3-vector (30 ng; Invitrogen), linearized using EcoRI (Fermentas), was incubated with 5 µg of His-384, Tyr-384, or full-length FH (positive control) in the binding buffer (as described above) in a total volume of 20 µl for 30 min at 37 °C. The negative control contained no protein. 1 µl of DNA loading buffer (0.25% (w/v) bromphenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol in deionized water) was added, and the samples were run on an agarose gel (0.8% (w/v); Cambrex) containing ethidium bromide (Sigma) and visualized by UV light. Changes in DNA migration served as the means of evaluating the presence of DNA-protein complexes.

**Cells and Induction of Necrosis**—Jurkat T cells (ATCC) were grown in RPMI supplemented with glutamine, penicillin, streptomycin, and 10% (v/v) heat-inactivated fetal calf serum (all from Invitrogen). Necrosis was induced by heat whereby the cells were brought to a concentration of 10^6/ml and incubated at 56 °C for 30 min in RPMI 1640 without fetal calf serum (27).

**Flow Cytometry**—Binding of FH variants to necrotic cells was analyzed using flow cytometry. FH binding was analyzed by incubating cells with increasing concentrations of FH variants (0–10 µg/ml) in flow cytometry binding buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2), with shaking for 30 min at room temperature. After washing twice in the same buffer, cells were stained with goat anti-FH polyclonal antibodies (Quidel) for 30 min at room temperature. This was followed by matched fluorescein isothiocyanate-labeled secondary antibodies (Dako).

**Surface Plasmon Resonance (Biacore)**—The interaction between CRP or fibromodulin and the polymorphic variants of FH was analyzed using surface plasmon resonance (Biacore 2000; Biacore). Three flow cells of a CM5 sensor chip were
activated, each with 20 μl of a mixture of 0.2 M 1-ethyl-3-(3-di- 
dimethylaminopropyl) carboadiimide and 0.05 M N-hydroxy-sul- 
fosuccinimide at a flow rate of 5 μl/min, after which CRP (20 
μg/ml in 10 mM sodium acetate buffer, pH 4.0) was injected 
over flow cell 2 to reach 4000 resonance units. Unreacted 
groups were blocked with 20 μl of 1 M ethanolamine, pH 8.5. 
Flow cell 3 was treated in a similar fashion but, instead of CRP, 
FMOD at 20 μg/ml was added to the buffer. A negative control 
was prepared by activating and subsequently blocking the sur-
face of flow cell 1. The association kinetics were studied for 
various concentrations of His-384 and Tyr-384. The flow buffer 
was 10 mM Hepes-KOH, pH 7.4, supplemented with 150 mM 
NaCl, 2.5 mM CaCl₂, and 0.005% Tween 20. Protein solutions 
were injected for 300 s during the association phase at a con-
stant flow rate of 30 μl/min. The sample was first injected over 
the negative control surface and then over immobilized CRP or 
FMOD. Signal from the control surface was subtracted. The 
dissociation was followed for 200 s at the same flow rate. In all 
experiments, 30 μl of 2 M NaCl was used to remove bound 
ligands during a regeneration step. BiaEvaluation 3.0 software 
was used to analyze sensorgrams obtained, and rate affinity 
constants were calculated using a steady state model.

RESULTS AND DISCUSSION

One current hypothesis for involvement of FH in AMD is 
that the risk-associated allele has impaired ability to inhibit 
complement activation leading to inflammation in macular tis-

eue (14). Further support for the role of complement in AMD is 
provided by the finding that variations in factor B and C2 also 
are associated with AMD (28). FH may be of special importance 
for protection of the macula basement membrane because, in 
contrast to cellular structures, it most probably lacks other 
complement inhibitors.

In most situations the complement system acts as a double-
edged sword. Excessive complement activation leads to inflam-
mation and exacerbates tissue injury. At the same time con-
trolled, low level activation of complement is necessary for 
removal of dying cells and various forms of debris. Therefore, 
the FH H384Y polymorphism could be associated with either a 
decrease or an increase in its ability to inhibit complement.

These opposite effects on complement activation could occur 
simultaneously in different locations within the same tissue. To 
better understand the contribution of FH to the AMD disease 
process and any involvement of molecules present in the local 
environment, we have analyzed the interactions between recombinant His-384 and Tyr-384 variants of FH and some of 
its known ligands.

To allow direct comparison of the functional differences 
between the His-384 and Tyr-384 forms of FH, we have pro-
duced the two variants in the context of recombinant con-
structs comprising CCPs 6 – 8, as described previously (5). This 
circumvents problems that arise when using FH purified from 
plasma, even when this is derived from individuals homozygous 
for the Y384H variants, because it has been reported recently 
that Y384H is not the only polymorphism in FH affecting AMD 
(29), i.e. such preparations would constitute a complex mixture 
of FH variants that could lead to difficulties when interpreting 
the resulting data. The use of constructs containing just three 
CCP domains has allowed us to focus on the Y384H variants 
and determine the effect of this polymorphism on the interac-
tion with known FH ligands.

The His-384 Variant of FH Binds CRP Less Efficiently than the 
Tyr-384 Form—CRP is a known activator of the classical path-
way of complement (30), to which the alternative pathway 
erves as an amplification loop. It is implicated in AMD because 
circulating CRP levels are high in patients suffering from this 
disease (3). CRP binds to FH, and it has been proposed that this 
interaction may control CRP-mediated complement activation 
in order to protect host tissues from complement-mediated 
damage (31). Because the binding site for CRP has been local-
ized to CCP7 (18, 19), we investigated whether the two allotypic 
variants of CCP6 – 8 of FH bound to CRP with different affinity. 

There are reports stating that commercial recombinant CRP 
may be contaminated with LPS (32). Because a direct interac-
tion between LPS and FH could cause aberrant results in our 
binding assays, we tested whether FH binds to LPS and we 
found that this is not the case (data not shown).

A direct binding assay where CRP was immobilized on a plas-
tic surface was used to assess interactions of the FH variants 
with CRP. The Tyr-384 variant bound significantly better than 
His-384 (p < 0.03 for all data points above zero), and this was 
evident in a dose-dependent manner (Fig. 1A). To verify that 
our results reflected true differences in the interaction of CRP 
with His-384 and Tyr-384 and not differential recognition of 
the two allotypic variants by the polyclonal antibodies used for 
detection, we repeated the experiment with biotinylated pro-
teins. Using a selected range of concentrations we show the 
same trend of lower CRP binding with the His-384 variant (Fig. 
1B). Furthermore, we analyzed the interactions using surface 
plasmon resonance (Biacore), and the data from these experi-
ments were used to calculate affinity constants. His-384 binds 
CRP with a K_D of 19 μM, and the corresponding value for Tyr-
384 is 12 μM (Fig. 2). There were clear differences in binding 
shown by Biacore analysis; however, the absolute K_D values 
calculated should be treated with some caution because it was 
impossible to use an ideal range of analyte concentrations in 
this analysis. The disease-associated variant of FH has impaired
ability to interact with CRP, which may potentially lead to increased complement activation by CRP, perhaps to the point of development of chronic inflammation in the affected tissue. FH interacts with dying cells, and this binding has been reported to be increased in the presence of CRP (33). Decrease in FH-CRP interaction could lead to less deposition of FH on dying cells, which in turn could potentially result in excessive complement activation on such debris. In this regard, given that AMD is a condition that takes 10–20 years to develop, it is to be expected that only a relatively subtle disruption in a biochemical mechanism is necessary for progression of the disease process. The differences we have determined for the allotypes of FH could certainly be sufficient to alter the balance of complement activation and immune clearance over this time scale.

**FH Binds PC but Does Not Compete with C1q and PC for the Binding to CRP**—Next, we wanted to further elucidate the binding characteristics of the CRP-FH interaction. A preferred approach for this was to examine the ability of FH to compete for binding to CRP with known ligands of CRP. This would provide important information regarding the location of the binding site for FH on CRP. The two faces of CRP are the A face where C1q and FcγR bind and the B face where PC binds (34). Using competition assays we found that full-length FH does not compete with C1q for the binding to the A face of CRP (Fig. 3A). C4b-binding protein (C4BP), in turn, very weakly inhibits FH binding to CRP (not shown) (35). We recently published data that C4BP to some extent competes with C1q for the binding to CRP (36). Taken together, these data imply that the C1q- and FH-binding sites are non-overlapping but may be adjacent. However, both C4BP and C1q are large molecules (both ~500 kDa) that can easily cause steric hindrances. Noteworthy in this context is also the possibility that an apparent lack of competition may be due to considerable differences in the affinity of the two ligands for the coated molecule.

Surprisingly, when we attempted to analyze whether FH binds to face B of CRP we found that there is a direct interaction between FH and PC; at physiological ionic strength, FH is capable of directly interacting with immobilized PC conjugated to BSA (Fig. 3B). Because FH already interacted strongly with PC-BSA-coated plates, it was not possible to assess whether FH binds CRP attached to PC (36). Binding of FH to immobilized CRP was not affected by the addition of phosphatidylcholine in solution (not shown), implying that FH does not bind face B of CRP.

Taken together, the issue of CRP binding to its ligands is complicated. We here address the matter in a number of ways showing that FH binds CRP independently of C1q. Furthermore, FH binds directly to PC, the main cell surface CRP ligand. We hypothesize that the binding site for FH on CRP may be located on the rim of the CRP pentamer, slightly overlapping with the C4BP interaction site situated on the periphery of the A face of CRP. This, however, requires further investigation.

**The Tyr-384 Variant of FH Interacts with FMOD with Higher Affinity than His-384, and the Binding Is Mediated by the Polypeptide Chain of FMOD**—FMOD is a member of the family of small leucine-rich repeat proteoglycans and was first
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FIGURE 4. FHCCP6–8 binds FMOD, and the His-384 variant binds FMOD less efficiently than the Tyr-384 variant. A, FMOD was coated in microtiter plate wells at a concentration of 10 μg/ml. BSA was used as negative coating control. The His-384 and Tyr-384 variants or full-length FH were incubated in fluid phase, and bound protein was detected with a specific polyclonal antibody. In panels A and B background signal was subtracted from the original values. Data are shown as mean values (n = 3) ± S.D., and in panel B asterisks indicate significance accordingly. **, p < 0.01; ††, p < 0.001.

The glycosylation state of FMOD (e.g. keratan sulfate attachment) may modify its interactions with its ligands. For example, it is not known whether FH binds the polypeptide of FMOD or rather its keratan sulfate chains. To address this question, we deglycosylated FMOD using N-glycosidase F, an amidase that removes N-linked oligosaccharides (including keratan sulfate). We found that the deglycosylated FMOD bound FH significantly better than the keratan sulfate-containing form (Fig. 4A). We also examined the interaction using Biacore, and the data showed that His-384 binds FMOD with a $K_D$ of 35 μM, whereas the corresponding value for Tyr-384 was 16 μM (Fig. 5). Throughout the experimentation we saw a clear trend toward higher affinity binding for the tyrosine compared with the histidine variant of FH CCP6–8. This trend is the same as the one observed for the interaction between CRP and FH. We speculate that these two separate binding events cooperate toward a more pro-inflammatory disease progression, because complement regulation on CRP and FMOD will be hampered in patients with the His-384 allotype.

The extracellular matrix of the eye shares a number of structural components with hyaline cartilage. FMOD is described as a 59-kDa protein (23) bound to collagen fibers in cartilage (37). The extracellular matrix of the eye shares a number of structural components with hyaline cartilage. FMOD is found in sclera (38), and it is expressed by epithelial cells of the eye (39). FMOD often contains one or two polyanionic keratan sulfate chains, distributed among the four potential substitution sites that are present in the leucine-rich region (40). An additional anionic domain is in the N-terminal region, containing up to nine tyrosine sulfate residues (41, 42). It was previously reported that FMOD binds C1q, which leads to complement activation (21). FMOD also binds FH, causing inhibition of the alternative pathway and thus a decrease in membrane attack complex formation (21). In the latter case, it is not clear which parts of the two molecules are involved in the interaction, although binding to C1q and FH, respectively, appears to engage different sites on FMOD. Using a direct binding assay we now show that FMOD interacts directly with the CCP6–8 region of FH and that the affinity is increased in the Tyr-384 variant compared with the His-384 variant (Fig. 4). When detecting biotinylated FH variants bound to immobilized FMOD we recorded significantly stronger signals for Tyr-384 at all concentrations tested (Fig. 4B). In the assay detecting unlabeled FH with a specific polyclonal antibody there is significant difference (p < 0.02) for two concentration points and the binding curves meet at the highest concentration (Fig. 4A). We also examined the interaction using Biacore, and the data showed that His-384 binds FMOD with a $K_D$ of 35 μM, whereas the corresponding value for Tyr-384 was 16 μM (Fig. 5). Throughout the experimentation we saw a clear trend toward higher affinity binding for the tyrosine compared with the histidine variant of FH CCP6–8. This trend is the same as the one observed for the interaction between CRP and FH. We speculate that these two separate binding events cooperate toward a more pro-inflammatory disease progression, because complement regulation on CRP and FMOD will be hampered in patients with the His-384 allotype.
His-384 FH Binds DNA More Efficiently than the Tyr-384 Variant—DNA is able to activate human complement (43). It was previously shown that complement inhibitor C4BP interacts with DNA and that there is more complement activation in human serum depleted of C4BP than in normal serum when DNA is added (27). In the case of C4BP, DNA may be one of the ligands attaching this inhibitor to necrotic cells. FH binds DNA (20) and possibly functions in a similar way. To qualitatively evaluate the ability of the two polymorphic FH variants to interact with DNA we incubated a linearized plasmid with either protein in the fluid phase. We then analyzed these samples by agarose gel electrophoresis to assess the presence of protein-DNA complexes (i.e. DNA with altered migration speeds). When comparing with full-length FH (positive control) and DNA only (negative control), we observed that both variants bound DNA but to different degrees. The His-384 variant retained DNA in its protein-complex form more efficiently than Tyr-384 (Fig. 6). In fact, DNA incubated with the Tyr-384 variant migrated mainly in its non-complexed form. Furthermore, we observed DNA-protein complexes in the samples containing the FHCCP6–8 variants (particularly with His-384) that migrated toward the cathode. This is most likely due to formation of larger positively charged protein-DNA complexes. The CCP6–8 constructs have a pI of ~7.9, consistent with this, whereas full-length FH has a pI of <7.0. These results show that the His-384 construct interacts more tightly with DNA than the Tyr-384 form, which exhibits only weak binding in this assay system. At present the molecular mechanism underlying the DNA-FH interaction is not clear. It is, however, plausible that there are multiple interaction sites in FH for DNA as has been observed for many other FH ligands. It is now apparent that at least one of the DNA-binding sites is localized to CCP6–8, probably within CCP7. It is possible that enhanced binding of FH to DNA exposed on necrotic cells present in drusen could lead to reduced complement-mediated opsonization, giving rise to impaired phagocytosis in the case of the His-384 disease-associated allotype.

FHCCP6–8 Binds Necrotic Cells—Late apoptotic and necrotic cells bind C1q and are able to activate complement to some extent (44). Previously, it has been proposed that these cells also capture complement inhibitor C4BP in order to allow phagocytosis of dying cells via C1q and C3b receptors but limiting inflammation (27). Furthermore, FH is able to interact with dying cells (45), but the molecular site of interaction has not been defined yet. We have rendered Jurkat T cells necrotic and analyzed the binding of the FH variants by flow cytometry. Fig. 7 shows that His-384 binds necrotic cells better than Tyr-384; a significant difference was detected at the higher concentrations tested. It appears therefore that the disease-associated variant is likely to accumulate on necrotic cells at a higher concentration than the Tyr-384 allotype, which may lead to increased inhibition of complement, thus reducing the phagocytosis of necrotic debris. Indeed, it has been shown that cellular debris derived from retinal pigment epithelium becomes sequestered between retinal pigment epithelium basal lamina and Bruch’s membrane and may provide a chronic inflammatory stimulus (46).

Taken together, it appears that the His-384 polymorphic variant in the FH gene, as observed in AMD, results in differential binding to glycosaminoglycans (5), CRP, FMOD, DNA, and necrotic cells. As described above, all of these interactions could contribute to AMD disease progression either via increased complement activation and resulting inflammation or through impairment of phagocytosis leading to accumulation of necrotic cell debris. Thus, the presence of the His-384 variant in the retina could have an impact on complement activation, which depending on the spatial and temporal localization of its various ligands could contribute to pathology in AMD. Further work is now required to determine the relative contribution of these FH-ligand interactions to the etiology of AMD.

Acknowledgment—We acknowledge Dr. Fredrik Höök (Lund University) for assistance with affinity constant calculations.

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