His-384 Allotypic Variant of Factor H Associated with Age-related Macular Degeneration Has Different Heparin Binding Properties from the Non-disease-associated Form*§

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A polymorphism in complement factor H has recently been associated with age-related macular degeneration (AMD), the leading cause of blindness in the elderly. A histidine rather than a tyrosine at residue position 384 in the mature protein increases the risk of AMD. Here, using a recombinant construct, we show that amino acid 384 is adjacent to a heparin-binding site in CCP7 of factor H and demonstrate that the allotypic variants differentially recognize heparin. This functional alteration may affect binding of factor H to polyanionic patterns on host surfaces, potentially influencing complement activation, immune complex clearance, and inflammation in the macula of AMD patients.

Age-related macular degeneration (AMD)4 is the leading cause of natural blindness in the Western world, and its prevalence may become greater with an increasing elderly population (1, 2). AMD manifests itself by the progressive destruction of the macula, causing central vision loss. The dry form of AMD, which accounts for 90% of cases, is associated with the presence of small yellow “drusen” deposits between the choroid and the retinal pigment epithelium that result in gradual vision loss; about 10–20% of patients with dry AMD go on to develop the more severe wet form. Recently, a common allelic variant of human complement factor H (3) has been linked to an increased risk of developing dry AMD (4–6). This variant arises from a tyrosine/histidine polymorphism at amino acid 384 in the mature protein (referred to as residue 402 in Refs. 4–6), where ~35% of individuals of European descent carry the disease-associated His-384 allele. This increases the likelihood of developing AMD by 2.7-fold and may account for 50% of the attributable risk of AMD (4). In individuals who are homozygous for the risk allele, the likelihood of AMD is increased by a factor of 7.4 (5). Recently, the His-384 allele has also been associated with an increased risk of myocardial infarction, where it has been suggested that atherosclerosis could contribute to macular degeneration (7).

Factor H is a 155-kDa plasma protein that acts as a cofactor for the breakdown of complement C3b by factor I (8). It is composed of 20 complement control protein (CCP; also termed short consensus repeats) modules (9), each of ~60 amino acids with a compact structure (10). The Y384H polymorphism is located within CCP7 (3). Factor H is believed to discriminate self from non-self by recognizing polyanionic structures on the former, such as sialic acid and the glycosaminoglycan (GAG) chains of proteoglycans (e.g. heparan sulfate (HS) and dermatan sulfate (DS)) and thus inhibits complement activation on host surfaces (11, 12). Factor H has been shown to be present in retinal blood vessels in the choroid (5) and is associated with the drusen of AMD patients (2, 13). In addition, markers of complement activation (e.g. C5b-9 and C3 fragments, including iC3b) have been detected in the Bruch’s membrane and drusen of AMD patients, leading to the hypothesis that AMD results from an aberrant inflammatory process that includes inappropriate complement activation (2, 5, 13–15). Furthermore, it has been reported that the glycosaminoglycan HS is present in the macula from AMD patients but not detectable in controls (16).

**EXPERIMENTAL PROCEDURES**

**Purification of Full-length Factor H from Human Serum—**Factor H was purified from 400 ml of plasminogen/plasmin-depleted pooled human plasma (HD Supplies, High Wycombe, UK) (17) on a 25-ml column of Sepharose to which was coupled a mouse anti-human factor H monoclonal antibody (MRC OX23) (18).
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**Heparin-binding Site Predictions**—Previously, coordinates for four models of intact factor H were created from x-ray and neutron scattering data for purified factor H along with homology modeling for 17 of the 20 CCPs based on known NMR structures for factor H (CCP5, CCP15, CCP16) and vaccinia coat protein CCP3 and CCP4 (19). We used three of these models (termed B, C, and D; Protein Data Bank accession code 1haq) to predict heparin-binding sites on factor H in conjunction with a heparin pentasaccharide model of heparin (20) using the program AutoDock, essentially as described before (21). All possible pairs of CCPs (e.g. CCP1–2, CCP2–3, etc.) were extracted from each model, and XPLOR version 3.8 (22) was used to add hydrogen atoms, build in the disulfide bonds, and energy-minimize the structures; three rounds of energy minimization were conducted, the first using a repulsive energy term only, the second also including a Lennard-Jones potential, and the third with added electrostatics. Autogrid version 3 was used to create the docking grid with a box size of 120 × 120 × 120 points spaced at 0.7 Å intervals, a dielectric constant of 1.0, in which the grid center was positioned at the center of the CCP pair. Autodock version 2.4 (23) was used for docking predictions using a simulated annealing protocol with 300 steps, where 128 runs were performed for each CCP pair with a heparin pentasaccharide model created previously (20). In addition, an Autodock prediction was performed for the CCP6–8 following the procedures described above.

**Expression and Refolding of FHCCP6–8**—Factor H cDNA corresponding to CCP6, CCP7, and CCP8 was amplified from factor H clone PE3 (9), which encodes the His-384 variant, and was modified by PCR to include NcoI and BamHI restriction sites allowing ligation into a pET14b vector (Merck, Nottingham, UK); primers, which are shown in supplemental table S1, were synthesized by Applied Biosystems (Warrington, UK). Analysis of the construct on an ABI 3730xl Prism DNA sequencer using the T7 promoter and terminator primers (see supplemental figure Fig. S1) determined that there were no changes to the expected sequence. This construct was transformed into BL21(DE3)pLysS competent cells (Merck) using the manufacturer’s protocol, expressed in high perfomance liquid chromatography column (Phenomenex, Macclesfield, UK), equilibrated in 0.1% (v/v) trifluoroacetic acid, and the biotinylated protein was eluted with an 10–80% acetonitrile gradient over 35 min and collected manually. The protein was recovered on a centrifugal evaporator (Holbrook, NY) and reconstituted in phosphate-buffered saline (137 mM NaCl, 2.6 mM KCl, 8.2 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.3; Oxoid, Basingstoke, UK).

**Heparin Affinity Chromatography**—The heparin binding properties of His-384 and Tyr-384 variants of FHCCP6–8 were compared with full-length human factor H by affinity chromatography on a HiTrap Heparin column (Amersham Biosciences) or a “home-made” column in which 20 mg of 4IS was coupled to 1.5 ml of CNBr-activated Sepharose (Sigma) in 0.1 M NaHCO₃, 0.5 M NaCl, pH 8.3, using the manufacturer’s protocol. The 1-ml HiTrap column and ~1-ml 4IS-column were equilibrated in 20 mM HEPES, 130 mM NaCl, 1 mM EDTA, pH 7.3. Protein (200 µg of recombinant proteins; 100 µg of full-length factor H) was loaded onto the columns (in 1 ml of equilibration buffer), and any unbound material was removed with 5 column volumes of buffer and collected in 1-ml fractions. Protein was eluted from the columns using a linear salt gradient of 130 mM to 1 M NaCl over 20 min by mixing 20 mM HEPES, 1 M NaCl, 1 mM EDTA, pH 7.3, with the equilibration buffer at a flow rate of 1 ml/min. One-ml fractions were collected and analyzed by SDS-PAGE, which demonstrated that the species eluting between 340 and 460 or between 170 and 290 mM NaCl, for the HiTrap and 4IS columns, respectively, corresponded to FHCCP6–8/factor H proteins. This approach was also used to determine the relative heparin binding activities of K370A, R386A, and K387A mutants, in which 200 µg of protein was loaded individually onto the HiTrap column.
Microtiter Plate Assays—The heparin/HS/DS binding activities of the FHCCP6–8(His-384) and FHCCP6–8(Tyr-384) variants (using biotinylated FHCCP6–8 constructs), and in some cases, full-length factor H (detected with an antibody) were compared using an assay in which the GAGs were immobilized on allylamine-coated EpranEx plates (28), kindly supplied by Plasso Technology Ltd., Sheffield, UK. Previously, we have shown that heparin/HS can bind non-covalently to surfaces plasma-polymerized with allylamine in such a way that these GAGs can still associate with a wide range of proteins (21, 28). The following GAG preparations were analyzed: unfractionated heparins corresponding to 2IS, 4IS, and 5IS (27); enoxaparin, dalteparin, and low molecular weight heparin from Sigma (catalog number H3400); selectively desulfated heparins prepared from 2IS heparin as described in Refs. 29 and 30; HSI and HSII preparations of HS (see Ref. 21); and DS purified from porcine mucosa and characterized by NMR as described previously (31) on a Varian Inova 500 MHz spectrometer at 60 °C in D2O. The GAGs (200 μl/well; 1 μg) were coated overnight at room temperature onto EpranEx plates in phosphate-buffered saline. Plates were blocked with 1% (w/v) bovine serum albumin (Sigma, catalog number A-4503) in standard assay buffer (20 mM HEPES, 130 mM NaCl, 0.05% (v/v) Tween 20, pH 7.3) for 90 min at 37 °C. The standard assay buffer was used for all subsequent incubations, dilutions, and washes at room temperature. The proteins were incubated at various concentrations with the immobilized glycosaminoglycans for 4 h. In the case of the biotinylated FHCCP6–8(His-384) and FHCCP6–8(Tyr-384) constructs, bound material was detected by adding 200 μl/well of a 1:10,000 dilution of ExtrAvidin alkaline phosphatase (Sigma, catalog number E-2636) for 30 min. Plates were then developed with 200 μl/well of 1 mg/ml disodium p-nitrophenylphosphate (Sigma) in 0.05 M Tris-HCl, 0.1 M NaCl, pH 9.3, and developed for 40 min, except for the DS-coated plates that were developed for 50 min. For the full-length protein, the level of binding (to 4IS, HIS, and HSII) was determined by incubation of the plates for 30 min with MRC OX23 (a monoclonal antibody against factor H (18); 1 μg/well) followed by a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, catalog number E-2636) for another 30 min. The plates were then developed for 10 min as described above. The absorbance values at 405 nm were determined and corrected against blank wells (i.e. those that contained no GAGs).

Alternatively, the unlabeled FHCCP6–8(His-384), FHCCP6–8(Tyr-384), and mutant proteins (i.e. K370A, R386A, K387A in the context of the FHCCP6–8(His-384) construct) were adsorbed at 1 μg/ml in phosphate-buffered saline onto microtiter plates (Nunc Maxisorb, Kastrup, Denmark), and their ligand binding activities at pH 7.3 were determined using biotinylated 4IS heparin essentially as described in Ref. 21. Plate assays were carried out in 20 mM HEPES, 130 mM NaCl, 0.05%
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![Graph](image)

**FIGURE 2. Comparison of heparin-binding properties for histidine and tyrosine variants of factor H.** A and C, FHCCP6–8(His-384), FHCCP6–8(Tyr-384), and full-length factor H were analyzed by heparin affinity chromatography at pH 7.3 on either a HiTrap heparin column (A) or a home-made column in which heparin (4IS) had been coupled to CNBr-activated Sepharose (C). The proteins were eluted with a linear NaCl gradient, in which dashed and solid lines show the conductivity of eluent and absorbance, respectively. In the case of the FHCCP6–8His/Tyr constructs, all the protein adhered to the columns, whereas with full-length factor H, a small amount of a 155 kDa-species was present in the flow-through (3–5% of protein loaded), as determined by SDS-PAGE. B and D, analysis of FHCCP6–8(His-384) and FHCCP6–8(Tyr-384) proteins by microtiter plate assays (circles and squares, respectively). B, binding of biotinylated heparin (4IS) to immobilized FHCCP6–8(His-384) and FHCCP6–8(Tyr-384) proteins. D, interaction of biotinylated FHCCP6–8His-384/Tyr-384) proteins with immobilized heparin (4IS). In B and D, values are plotted as mean absorbance (n = 8) ± S.E. All data are representative of at least two independent experiments.

(v/v) Tween 20, pH 7.3, and the level of bound heparin was determined as described above for the biotinylated proteins. In these plate assays, all data points were determined in quadruplicate from each of two independent experiments.

**RESULTS AND DISCUSSION**

Here we have investigated whether the Y384H polymorphism affects the interaction of factor H with the GAG heparin, as a model both for HS and for polyanions in general.

**Prediction of Heparin-binding Sites on Factor H**—Initially, coordinates for a theoretical model of factor H (19) and a heparin pentasaccharide model (20) were used in systematic docking calculations to predict the positions of potential heparin-binding sites using the program Autodock (21, 23). This analysis indicated that two CCP modules of factor H (7 and 20) harbor interaction sites for heparin, consistent with previous biochemical studies (32–34). In CCP7, docking calculations consistently placed the bound pentasaccharide model in close proximity to three basic amino acids (Lys-370, Arg-386, and Lys-387), the latter two of which are adjacent to residue 384 (Fig. 1).

Determinant of Heparin-binding Residues in CCP7—To investigate the role of the amino acid residues in CCP7 predicted to bind heparin, a recombinant protein composed of CCP6, CCP7, and CCP8 was expressed in E. coli and then used as the basis for site-directed mutagenesis. The wild-type protein (His-384 variant, denoted FHCCP6–8(His-384)) was demonstrated to be correctly folded, having the expected disulfide bond arrangement (data not shown), and to bind to heparin in a variety of assays (Fig. 2), providing evidence that this region of factor H is involved in the recognition of polyanions. Mutants were produced in the context of this construct (see supplemental Fig. S1) so that Lys-370, Arg-386, and Lys-387 were individually altered to alanine. One-dimensional NMR spectroscopy demonstrated that the mutants all had essentially identical spectra to the wild-type protein (data not shown), indicating that these alterations do not perturb the tertiary structure of the protein. Furthermore, these proteins all had identical disulfide bond patterns. The effect of mutation on heparin binding function was assessed using affinity chromatography or using a microtiter plate assay, in which the proteins were immobilized and the binding of biotinylated heparin was determined essentially as described in Ref. 21. As can be seen from the chromatograph in Fig. 3A, the R386A and K387A mutants have substantially reduced binding activity when compared with wild-type FHCCP6–8(His-384), eluting from the heparin affinity column at lower NaCl concentrations, whereas mutation of Lys-370 to alanine had no affect. Results from the plate assay were consistent with this (Fig. 3B); however, the K370A protein showed a small reduction in binding. These data indicate that Arg-386 and Lys-387 are both likely to play an important role in heparin binding. This is consistent with an earlier study in which these amino acids were replaced with alanine in a double mutant expressed in the context of CCP1–7, which was shown to have reduced binding to heparin-bovine serum albumin (35); in this case, no structural studies were undertaken to assess the effect of mutagenesis on the protein fold. Furthermore, Giannakis et al. (35) reported that a R369A/K370A double mutant had greatly reduced heparin binding function. Our data indicate that Lys-370 has a minor contribution, if any, to the association with heparin.
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Determining the Role of His-384/Tyr-384 in Heparin Binding—Given that His-384 is in close proximity to the heparin-binding residues on CCP7 (Fig. 1), it seemed plausible that this amino acid may participate in the interaction with heparin and that the histidine and tyrosine variants could have different functional activities. Therefore, FHCCP6–8(His-384) was mutated to tyrosine to generate the FHCCP6–8(Tyr-384) protein, and their heparin binding properties were compared; FHCCP6–8(Tyr-384) was shown to be folded, with correct disulfides, as before. From Fig. 2A, it is apparent that the His-384 variant elutes at lower salt strength than Tyr-384 from the HiTrap heparin affinity column (340 and 390 mM NaCl, respectively), whereas a higher salt strength is required to recover intact factor H. It is not surprising that the full-length protein binds more tightly to the affinity column given that it has at least two sites of heparin binding (i.e., CCP7 and CCP20; see Refs. 33, 34, and 36). However, the finding that it can be eluted at 460 mM NaCl, which is not substantially higher than for the FHCCP6–8(Tyr-384) protein, suggests that the heparin-interaction site in CCP7 makes a major contribution to this activity.

The relative heparin binding activities of the allotypic variants were further analyzed by microtiter plate assays. Either the proteins were immobilized and their interaction with biotinylated heparin determined (Fig. 2B), or heparin was bound to an allylamine-coated surface and used to assess the binding of the Tyr-384/His-384 proteins, in which these had been biotinylated (Fig. 2D). For these experiments, a well characterized unfractionated heparin (4IS (26, 27)), was utilized. In both cases, FHCCP6–8(Tyr-384) was shown to bind less well to the 4IS heparin than the FHCCP6–8(His-384) variant. This is the inverse of the result using affinity chromatography (Fig. 2A), which was performed on a commercial HiTrap column in which the exact source and composition of heparin has not been made known. Therefore, to rule out that this was an artifact resulting from the different assays used, we analyzed the binding of these proteins to a column to which 4IS heparin had been coupled. As can be seen from Fig. 2C, the relative binding affinities of FHCCP6–8(Tyr-384) and FHCCP6–8(His-384) are consistent with the plate assay data, in which an identical source of heparin was employed. It seems possible, therefore, that not only do the His-384/Tyr-384 allotypic variants have distinct heparin binding activities but that they can also differentiate between heparins of different structure.

Characterization of the His-384/Tyr-384-Heparin Interaction—To investigate this further, we analyzed the binding of the FHCCP6–8(Tyr-384) and FHCCP6–8(His-384) proteins to a wide range of heparin preparations. For the three unfractionated heparins tested (i.e., 2IS, 4IS, and 5IS), more of the FHCCP6–8(His-384) protein bound to the immobilized glycosaminoglycans when compared with the FHCCP6–8(Tyr-384); where the 4IS and 5IS preparations gave essentially identical results (Fig. 2D and supplemental Fig. S2A). The difference was less marked for the 2IS (Fig. 4A) due to an increased level of binding for the FHCCP6–8(Tyr-384) protein. In the case of the low molecular weight heparins, there was no difference in the binding of the two constructs to enoxaparin (Fig. S2C), and only a small difference was observed for their binding to dalteparin (Fig. S2D) and the LMw heparin obtained from Sigma (Fig. S2B). These data indicate that the results obtained for FHCCP6–8(His-384) and FHCCP6–8(Tyr-384), including their differential binding activities, are dependent on the type of heparin used and, thus, may correlate with differences in the structure/composition of these preparations. That said, it is difficult to attribute the differences in binding seen for the His-384 and Tyr-384 proteins to specific structural features. Therefore, to provide some insight into this, we investigated the effect of selective desulfation of IS2 heparin on its binding to FHCCP6–8(His-384) and FHCCP6–8(Tyr-384).

As can be seen from Fig. 4, the desulfation of heparin has a much larger effect on its interaction with the FHCCP6–8(His-384) protein when compared with FHCCP6–8(Tyr-384). In this regard, the removal of either the 2-O-sulfate (2-O-deS) or the N-sulfate (N-deS) leads to a large reduction in the binding of the His-384 variant (Fig. 4, B and E), where re-N-acetylation of the N-desulfated preparation (N-deS/re-N-Ac) has no additional/compensating effect (Fig. 4F). Interestingly, the binding
of the Tyr-384 variant appears to be less sensitive to loss of these functional groups, in that its binding to the 6-O-desulfated and N-desulfated/re-N-acetylated preparations appears to be indistinguishable from the parental 2IS material. Furthermore, removal of 2-O-, 2,6-O-, or N-sulfates only has a small effect on the FHCCP6–8(Tyr-384) interaction (as determined from a comparison of the shapes of the binding curves relative to unmodified 2IS). From the above experiments, it is apparent that the FHCCP6–8(Tyr-384) protein can bind more strongly to particular heparin preparations than the FHCCP6–8(His-384) form, consistent with the data from the HiTrap affinity column (Fig. 2A). However, in other cases (i.e. 2IS, 4IS, and 5IS), it is the histidine form of the protein that exhibits greater binding activity. This analysis provides compelling evidence that the His-384/Tyr-384 allotypic variants of factor H recognize different structural features within heparin.

**Determining Whether His-384/Tyr-384 Bind HS and DS**—Unexpectedly, neither the FHCCP6–8(His-384/Tyr-384) constructs nor the full-length factor H bound to the HS preparations tested (Fig. 5). HSI and HSII have different levels of sulfation, where the latter is more highly sulfated than the former (see Ref. 21). It seems unlikely that this lack of activity is a consequence of the assay used because we have previously seen binding of another protein (TSG-6) to HSI and HSII preparations immobilized on EpranEx plates (21). However, we cannot rule out the possibility that the passive adsorption of these HS molecules to the allylamine surface masks their factor H-binding sites.

If factor H does interact with HS, as has been suggested (36), then it is likely to be through its recognition of a distinct structural feature (e.g. sulfation pattern), which is clearly present within heparin (e.g. 3-O-sulfation, which is an essential component of anti-coagulant heparan sulfate (37)) rather than being dependent on the absolute level of sulfation. In this regard, the complete desulfation of heparin abolished its binding to both the His-384 and the Tyr-384 proteins in assays in which the heparin was either immobilized or present in the solution (data not shown), indicating that at least some sulfation is required for the interaction with factor H.

The binding of the FHCCP6–8(His-384) and FHCCP6–8(Tyr-384) proteins to DS was also investigated since factor H has been previously reported to bind to this GAG (41). From Fig. 6, it can be seen that these constructs do interact with DS, albeit to a lesser degree than heparin, and appear to have somewhat different binding properties. The DS used here is of high purity, where the 1H NMR spectrum did not contain resonances attributable to heparin impurities (data not shown); signals from the H1 of N-sulfate or N-acetylated glucosamine, which do not coincide with any resonances in the DS spectrum, were absent. Furthermore, almost all the intensity in the spectrum could be assigned to signals from the repeating unit [α-L-IdoA-(1–4)-β-D-
GalNAc4SO₃(1–4)], containing a few percentages of \([\alpha-L-\text{IdoA2SO}_3\text{O}_{\text{H}}(1–4)-\beta-D-\text{GalNAc4SO}_3\text{O}_{\text{H}}(1–4)]\). This makes it likely, therefore, that factor H is a \textit{bona fide} DS-binding protein and that the His-384/Tyr-384 allotypic forms may differentially recognize DS-containing proteoglycans \textit{in vivo}. However, further analysis is clearly required to fully characterize the interaction between factor H and DS.

The Consequences of Factor H-GAG Interactions for AMD—The lack of HS binding observed here notwithstanding, it is clear that the His-384 and Tyr-384 variants have differential heparin binding properties, which is likely to reflect a difference in their association with polyanionic structures on complement activator surfaces. However, age- or disease-related changes in HS structure and expression could also promote binding of one or another of the allotypic forms. Age-linked changes in GAG structure have been seen in various tissues (38–40), including DS (40), to which factor H has also been shown to bind (41), and as noted above, HS has been reported to be present only in the macula from patients with AMD (16). Furthermore, age-related changes in GAG content and sulfation, including an increase in the level of HS, occur in the trabecular meshwork, in which this is likely to be a general phenomenon in other regions of the human eye (40).

In a recent study describing the characterization of the heparin-binding site in CCP20 of human factor H (34), it has been suggested that a GAG molecule may be able to bind simultaneously to CCP7 and CCP20, stabilizing the “bent-back” structure of the protein inferred from solution studies (19). This is an attractive hypothesis given that heparin/HS often supports protein-protein interactions (\textit{i.e.} in which the GAG chain is sandwiched between the two proteins). If this does indeed occur, then it is possible that the His-384/Tyr-384 allotypic variants of factor H may differ in this property depending on the nature of the GAG chain involved. Clearly, such alterations in the structural organization of factor H could have a significant effect on its functional activities (34).
Enhanced binding of factor H to developing drusen bodies (which contain cell debris and immune complexes (14, 15)) could lead to reduced complement-mediated opsonization, giving rise to impaired phagocytosis. In this regard, the cells of the retinal pigment epithelium have macrophage-like activity and are believed to have a role in the clearance of drusen (42). Macrophages also accumulate in the choroids of AMD patients (43) and may be involved in drusen uptake (44). Therefore, it seems plausible that AMD results from impaired immune complex clearance caused by enhanced binding of the disease-associated variant of factor H to polyanionic structures present in drusen, leading to reduced complement activation. The findings we report here are the first insights into the effect of the H384Y polymorphism on factor H function, providing new possibilities for therapeutic intervention in AMD.

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