Structure Shows That a Glycosaminoglycan and Protein Recognition Site in Factor H Is Perturbed by Age-related Macular Degeneration-linked Single Nucleotide Polymorphism*‡§

Andrew P. Herbert†, Jon A. Deakin†, Christoph Q. Schmidt†, Bärbel S. Blaum†, Claire Egan†, Viviana P. Ferreira‡, Michael K. Pangburn†, Malcolm Lyon†, Dusan Uhrin‡, and Paul N. Barlow††

From the †Edinburgh Biomolecular NMR Unit, School of Chemistry and School of Biological Sciences, University of Edinburgh, West Mains Road, Edinburgh EH9 3JL, Scotland, United Kingdom, ‡Cancer Research UK Glyco-Oncology Group, Paterson Institute for Cancer Research, University of Manchester, Wilmslow Road, Manchester M20 4BX, United Kingdom, and the ¶Department of Biochemistry, University of Texas Health Science Center, Tyler, Texas 75703

A common single nucleotide polymorphism in the factor H gene predisposes to age-related macular degeneration. Factor H blocks the alternative pathway of complement on self-surfaces bearing specific polyanions, including the glycosaminoglycan chains of proteoglycans. Factor H also binds C-reactive protein, potentially contributing to noninflammatory apoptotic processes. The at risk sequence contains His (rather than Tyr) at position 402 (384 in the mature protein), in the seventh of the 20 complement control protein (CCP) modules (CCP7) of factor H. We expressed both His402 and Tyr402 variants of CCP7, CCP7,8, and CCP6–8. We determined structures of His402 and Tyr402 CCP7 and showed them to be nearly identical. The side chains of His/Tyr402 have similar, solvent-exposed orientations far from interfaces with CCP6 and -8. Tyr402 CCP7 bound significantly more tightly than His402 CCP7 to a heparin affinity column as well as to defined-length sulfated heparin oligosaccharides employed in gel mobility shift assays. This observation is consistent with the position of the 402 side chain on the edge of one of two glycosaminoglycan-binding surface patches on CCP7 that we inferred on the basis of chemical shift perturbation studies with a sulfated heparin tetrasaccharide. According to surface plasmon resonance measurements, Tyr402 CCP6–8 binds significantly more tightly than His402 CCP6–8 to immobilized C-reactive protein. The data support a causal link between H402Y and age-related macular degeneration in which variation at position 402 modulates the response of factor H to age-related changes in the glycosaminoglycan composition and apoptotic activity of the macula.

Age-related macular degeneration (AMD) is a leading cause of irreversible visual impairment in the elderly (1). The densely packed photoreceptors in the macula are maintained by the underlying retinal pigment epithelium (RPE) (2). A unique pentalaminar extracellular matrix (ECM), the Bruch’s membrane, separates the RPE from the fenestrated endothelium of the choroidal vasculature. In early AMD, fatty deposits, called drusen, appear between Bruch’s membrane and the RPE (3). Early AMD may progress to severe forms (4), characterized by RPE-cell death and atrophy of the photoreceptor layer, or choroidal neovascularization.

Drusen are rich in cell breakdown products and proteins of the complement system (5). Complement (6, 7) is a potent mediator of inflammation. An association between AMD and the gene (CFH) for the complement regulator factor H (fH) was demonstrated by Hageman et al. (8) and confirmed independently by others (9–12). Hageman et al. (8) additionally reported the RPE to be a source of fH, while drusen components C3a and C5a have been implicated in neovascularization in mouse models of AMD (13), strengthening further the evidence for an AMD-complement link.

The at-risk allele that has received most attention, in CFH exon 9 (rs1061170; 1277T > C), encodes a His rather than a Tyr at position 402 (residue 384 of the mature fH) and is present in ~35% of individuals of European descent. Homozygous individuals have a 6-fold increased risk of developing AMD, whereas heterozygotes are 2.5 times more susceptible (14). Doubts over a causal link between the H402Y variation and the etiology of AMD have been raised by the identification of 20 synonymous or intronic single nucleotide polymorphisms (SNPs) in a 123-kb region overlapping CFH that are even more strongly associated with AMD (15, 16). Moreover, variations in genes encoding complement factor 2 (C2) and complement factor B (Bf) also strongly influence risk, as does an SNP in a gene, LOC387715, of unknown function (16, 17). Other risk factors

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§ To whom correspondence should be addressed: Chemistry Bldg., University of Edinburgh, West Mains Rd., Edinburgh EH9 3JL, Scotland, United Kingdom. Tel.: 44-131-650-4727; Fax: 44-131-650-7155; E-mail: Paul.Barlow@ed.ac.uk.

1 The abbreviations used are: AMD, age-related macular degeneration; AP, alternative pathway; CCP, complement control protein module; CRP, C-reactive protein; CSP, chemical shift perturbation; ECM, extracellular matrix; fH, factor H; GAG, glycosaminoglycan; GMSA, gel mobility shift assay; RPE, retinal pigment epithelium; PBS, phosphate-buffered saline; SNP, single nucleotide polymorphism.
for AMD include smoking, diet, obesity, and underlying vascular disease (18). Detailed investigations of the structural and functional consequences of the Tyr402 variation in fH are thus required to shed light on the putative role of H402Y in the etiology of AMD.

Factor H is a plasma glycoprotein (155 kDa, 500–800 μg/ml) (19) that controls the alternative pathway (AP) of complement activation (20). The AP operates at “tick over” level on any surface, self or foreign, but is normally amplified exclusively on foreign surfaces, thanks in part to the selective action of fH. Factor H recognizes and binds to self-surfaces via sialic acid and glycosaminoglycan (GAG) chains of proteoglycans (21), whereupon its complement-regulating properties are enhanced (20). Factor H destroys the surface-deposited convertases, which are made up from fragments of C3 and factor B, that drive the amplification loop within the AP. Self-cells carry additional, membrane-bound, regulators of the AP. Consequently, fH is particularly important for protecting self-surfaces not enclosed by a cell membrane.

Factor H consists of 20 tandem complement control protein modules (CCPs) (22). The AMD-linked His/Tyr402 variation is in CCP7, which contributes to one of two putative sites that bind C-reactive protein (CRP) (23, 24) and to one of at least three GAG-binding modules in fH (25). Module 7 therefore potentially participates in the ability of fH to selectively act upon convertases formed on self-surfaces. Indeed, a recent report (26) indicated that His402 fH binds less strongly to retinal tissue context and the circumstances.

Expression of Protein and NMR—DNA sequences for the following segments of fH (numbered according to full-length protein, i.e. before removal of secretion signal) were cloned into the Pichia pastoris expression vector pPICZαc: residues 386–444 (CCP7), 386–507 (CCP7,8), and 321–507 (CCP6–8). In each case, DNA encoding both Tyr and His at position 402 was prepared. Expressed proteins were directed to the secretory pathway by placing the coding sequence behind the Saccharomyces cerevisiae α-factor secretion sequence.

Following transformation into P. pastoris strain KM71H, proteins were expressed in a fermentor. Where appropriate, proteins were isotopically labeled in batches of 0.8 liters (initial volume) of cell culture as described (29). Cation exchange chromatography was used to purify all the proteins. Identity of the purified Tyr402 and His402 CCP7 proteins was confirmed using mass spectrometry. Yields were in the region of 1 mg of pure protein/g of wet cells.

NMR spectra were acquired on Bruker AVANCE 600- and 800-MHz spectrometers using 5-mm probes. Data for resonance assignments were acquired at 298 K on a 0.9 mM sample of 13C,15N CCP7 in 20 mM sodium acetate, at pH 5.2. 15N-Edited and 13C-edited NOESY-HSQC experiments were acquired with mixing times of 150 ms.

Resonance Assignment and Structure Calculation—Nearly complete backbone and side chain assignments were achieved using the CCPN (30) program ANALYSIS (Table 1). A series of 15N, 1H HSQC experiments over the pH range 5.0–7.4 allowed assignments of backbone amide 15N and protons at the higher pH. Returning to the pH-5.2 data, resolved peaks in 15N NOESY and 13C NOESY spectra were picked manually and partially assigned where it was possible to do so without ambiguity. Hydrogen bonds were detected using a two-dimensional HNCO experiment (31) and translated into distance restraints (32). All proline residues were deemed trans (33). Chemical shifts, partially assigned NOESY spectra, and hydrogen bond-derived restraints were supplied to the ARIA 2.0 (34) and crystallography and NMR system (CNS) (35) protocols. Eight iterations of filtering, reassignment, and recalibration were performed, each based on the lowest overall energy structures from the previous iteration, followed by a final refinement in explicit water. QUEEN (36) was used to validate structures, restraints, and assignments.

Heparin Affinity Chromatography and Gel Mobility Shift Assay (GMSA)—Protein samples (0.05 mM in 1 ml) in phosphate-buffered saline (PBS) were individually loaded onto either a self-packed heparan sulfate-agarose (Sigma) column (0.5 × 10 cm) or a Poros 20HE (0.46 × 10 cm, Applied Biosystems) heparin affinity chromatography column equilibrated with PBS and eluted with a linear gradient from PBS to PBS supplemented to 1 M sodium chloride. The His402 CCP7 eluted at a lower salt concentration than Tyr402 CCP7. Subsequently the two variants of the single modules were combined prior to resolving them on the heparin column.

Oligosaccharides were prepared from low molecular weight heparin (37) or dermatan sulfate (38), fluorophore-labeled spe-

**EXPERIMENTAL PROCEDURES**

Isolation of His402 and Tyr402 Allotypic Variants of Human Factor H—Factor H was purified as described (28) from normal human plasma (Stewart Regional Blood Bank, Tyler, TX) of different individuals homozygous at the 402 position. The His402 and Tyr402 allotypic variants of the pure factor H were confirmed by mass spectrometry following purification.
cies were produced by attachment of 2-aminoacridone to the oligosaccharide reducing end, and the GMSA was performed, all as described previously (39). Briefly, 2-aminoacridone-tagged oligosaccharides were combined with either Tyr402 or His402 CCP7 (at concentrations indicated in Figs. 1b and 2, a and c) in a total volume of 10 μl of PBS containing 25% (v/v) glycerol for 15 min (room temperature). Samples were then loaded on a 1% agarose gel in 10 mm Tris-HCl, pH 7.4, and 1 mm EDTA. Electrophoresis was performed (200 V, 8–15 min) in a horizontal agarose electrophoresis system, using an electrophoresis buffer comprising 40 mm Tris/acetic acid, 1 mm EDTA, pH 8.0. Immediately thereafter, the 2-aminoacridone-tagged oligosaccharides were visualized.

Surface Plasmon Resonance—This was performed on a Biacore—T100 instrument by immobilizing 717 response units of CRP on a CM5 chip using standard amine coupling. Ligand preconcentration prior to immobilization was performed using 25 μg/ml CRP in 10 mm sodium acetate buffer, pH 5.5. The analysis was performed at 25 °C using 10 mm HEPES, 150 mm NaCl, 0.05% surfactant P20, pH 7.4, as running buffer. Surface consistency and regeneration efficiency tests were performed using 32 μm Tyr402 CCP6–8 and indicated that the surface was stable and regeneration was effective (data not shown).

Locating the Polyanion Binding Site by Chemical Shift Mapping—Initially, 15N-1H HSQC spectra were acquired (600 MHz, cryogenic probe) on 15N-labeled CCP7 samples (20 μm in 20 mm sodium acetate (deuterated), pH 5.0, 25 °C) in the presence of 0–40 μm fully sulfated, heparin-derived tetrasaccharide (purified from a mixed tetrasaccharide fraction by strong anion-exchange high-pressure liquid chromatography and characterized by disaccharide analysis). The titration was repeated at pH 7.4 (20 mm potassium phosphate) plus 140 mm sodium chloride, and again at pH 7.4 (20 mm potassium phosphate, no additional salt) with 0–160 μm tetrasaccharide (saturation not reached). Spectra of the complex were assignabe due to incremental movement of cross-peaks during the titration. The polyanion-binding site was inferred on the basis of amide 15N and 1H resonances that exhibited the largest heparin-induced combined chemical shift perturbation (CSP) (40).

RESULTS

Homing in on Module 7—The His and Tyr allotypic variants of factor H show indistinguishable affinity for a heparin column (Fig. 1a). To achieve a comparison of affinities for more structurally defined GAG ligands, we used the GMSA (39) to explore binding to size-fractionated heparin oligosaccharides. In this assay, the extent of binding of a fluorescently tagged oligosaccharide is judged by the proportional retardation of its normal migration toward the anode. As measured by GMSA, the two variants of full-length human factor H bind equally well to chemically pure, fully sulfated heparin-derived dodecasaccharide (Fig. 1b). A similar result was obtained for a tetrasaccharide (not shown). Because factor H contains multiple GAG-binding sites that may compete or cooperate in such artificial assays, we adopted a dissection approach. We found that P. pastoris-expressed factor H fragments corresponding to modules 6–8 (CCP6–8), with either His or Tyr at position 402, also bound equally well both to a heparin column (not shown) and to a panel of chemically pure, defined-size, fully sulfated heparin fragments (Fig. 2a). We noted, however, that in previous work, Clark et al. (27) had shown that E. coli-expressed Tyr402 and His402 CCP6–8 exhibited differential binding to various GAG preparations. To explore this issue further, we tested the His402 and Tyr402 variants of isolated CCP7 (expressed in P. pastoris).

Strikingly, the two variants of single-module CCP7 were base line-resolved on a heparin affinity column (pH 7.4) (Fig. 2b). Irrespective of oligosaccharide length, Tyr402 CCP7 retards a higher proportion of heparin than His402 CCP7 according to a GMSA (Fig. 2a). The effect is more pronounced with smaller oligosaccharides and particularly obvious for the di- and tetrasaccharide. A dose-response version of the GMSA, conducted using a fixed amount of the tetrasaccharide (Fig. 2c), reinforced this result. Since the Tyr402 version of CCP7 binds more strongly than the His402 version, this appeared not to be merely a charge-related effect. It also appeared to be GAG-specific, since no such distinction was observed between the variants using a panel of size-fractionated dermatan sulfates in the GMSA (Fig. 2a). Our objective was to focus on the role of disease-linked residue 402. We therefore chose to follow up the clear differences in heparin affinity between variants at the level of the single modules using an atomic resolution structural approach.

Module 7 of fH May Be Instructively Studied in Isolation from Its Neighbors—The N and C termini of CCPs lie at opposite ends of their three-dimensional structures, and neighboring CCPs typically adopt an elongated, end-to-end arrangement (41, 42). Indeed, in fH, just three residues separate CCP6 from CCP7, and steric considerations rule out a side-by-side association. On the other hand, the linker between CCP7 and -8 (five residues) might be long enough to allow CCP8 to fold back alongside CCP7. To assess this possibility, a 1H,15N HSQC
spectrum was collected on recombinant Tyr\textsuperscript{402} CCP7,8 (i.e. the double module) for comparison with the equivalent spectrum of the single module, Tyr\textsuperscript{402} CCP7 (Fig. 3). Subsequently (following assignment), it was found that the CSPs in CCP7 due to the presence of CCP8 occur only for residues proximal to the CCP7 C terminus. Equivalent results were obtained in the case of His\textsuperscript{402} CCP7. Thus, the three fH CCPs, CCP6 to -8, must be arranged in an end-to-end, not a side-by-side, fashion, exposing a large surface area of CCP7 for binding to ligands. The structure of CCP7 (see below) further revealed that the Tyr/His\textsuperscript{402} side chain is located far from either end of the seventh module and is unable to affect intermodular orientations. Therefore, it is reasonable to compare structures of the His/Tyr\textsuperscript{402} variants of fH by focusing on isolated module CCP7.

Structure Determination of Tyr\textsuperscript{402} and His\textsuperscript{402} CCP7—The \textsuperscript{1}H-\textsuperscript{15}N HSQC spectra of the CCP7 variants did not change drastically between pH 5.0 and 7.4 (supplemental Fig. 1), legitimizing structure determination at the experimentally convenient lower pH. There were no significant chemical shift changes in HSQC spectra over the range 20–600 \textmu M CCP7 and no nuclear Overhauser effects symptomatic of nontransient self-association, and line widths were inconsistent with oligomerization in the 600 \textmu M NMR samples. Therefore, CCP7 is predominantly monomeric under the conditions used for structure calculations. Nearly complete assignment of nuclei in Tyr\textsuperscript{402} and His\textsuperscript{402} CCP7 (see Table 1 and supplemental Fig. 2) was achieved.

The two nearly identical structures (Fig. 4) resemble previously solved CCP structures (43–45). The 402 side chain is solvent-exposed and lies between two exposed nonconserved Tyr side chains (Tyr\textsuperscript{390} and Tyr\textsuperscript{393}) (Fig. 4b). This face also features His\textsuperscript{417}, Tyr\textsuperscript{398}, and Tyr\textsuperscript{420} side chains. The arrangement of these and nearby residues is conserved between the two variants so that the only difference between them is localized to position 402. The side chains of Arg\textsuperscript{404}, Lys\textsuperscript{405}, and Lys\textsuperscript{410} contribute to the main patch of positive charge on CCP7 (Fig. 4c); another positive patch near the module’s C terminus arises from Arg\textsuperscript{443} and Lys\textsuperscript{446}. We next opted to map the binding site on CCP7 for a pure, fully sulfated heparin tetrasaccharide.

Locating the Heparin-binding Site on CCP7 Tyr\textsuperscript{402} and His\textsuperscript{402}—Initial titrations with the tetrasaccharide, conducted under conditions used for structure determination (low salt, pH 5.2), yielded widespread CSPs (supplemental Fig. 3). Perturbations were similar for both variants, although for His\textsuperscript{402} CCP7, more heparin was required to produce an equivalent effect. The residues involved are not confined to a single face of CCP7, and these results suggest that a conformational rearrangement, or dimerization, occurs in CCP7 upon binding to tetrasaccharide at nonphysiological pH 5.2. It was therefore impossible to distinguish CSPs caused by direct contacts from those due to structural adjustments.
Structure of a GAG and Protein Recognition Site in Factor H

At approximately physiological ionic strength and pH (140 mm NaCl, 10 mm phosphate buffer, pH 7.4), binding of the tetrasaccharide by Tyr402 CCP7 was undetectable using NMR. At pH 7.4 but with no NaCl (20 mM potassium phosphate), on than its Tyr402 variant for immobilized CRP (Fig. 6). The participation of His/Tyr402 in a face of the module that contains all Tyr and His residues of CCP7 is noteworthy, since aromatic stacking interactions commonly occur in protein-carbohydrate (51, 52) interactions.

Unlike the isolated module 7, the His402 and Tyr402 variants of the triple module, CCP6–8 (and of intact fH), have similar affinities for heparin. Thus, neighboring modules appear to mask the differential recognition properties exhibited by the two variants of CCP7, at least for the model forms of GAG used in the current study. Indeed, heparin titration of CCP7,8, monitored by a series of HSQC spectra (data not shown), suggests that its side chain very likely contributes directly to recognition by CCP7 of GAGs. The participation of His/Tyr402 in a face of the module contains all Tyr and His residues of CCP7 is noteworthy, since aromatic stacking interactions commonly occur in protein-carbohydrate (51, 52) interactions.

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DISCUSSION

Detailed structural and functional comparisons between His402 and Tyr402 allotypic variants of fH are required to investigate the possibility of a causal link between this common SNP and AMD. The multiple polyanion-binding sites of fH have the capacity to cooperate or interfere with one another under physiological circumstances. We therefore adopted a “dissection-based” approach based on recombinant expression of specific CCPs. This allowed us to focus on the single GAG-binding site that encompasses the disease-associated SNP. Recombinant CCP7, which harbors His/Tyr402, adopts an elongated arrangement with respect to neighboring modules. The 402 side chain is exposed and distant from the module’s N or C termini. Focusing on individual CCP7, we found that replacement of Tyr402 with His has structural consequences only for that individual side chain. This SNP thus corresponds to a small and localized change in a large, extended, and flexible protein. It follows that the His/Tyr402 substitution can influence directly only a limited number of the many binding sites distributed along the fH molecule (46, 47). Such a corollary is consistent with the His402 (at risk) variant of fH, present in 35% of Western populations (8), functioning adequately until at least old age.
of CCP7; this region was also implicated by heparin-titration experiments on the CCP7,8 module pair (not shown), so this result is not an artifact of working with the individual module. Thus, at pH 7.4 and low salt, tetrasaccharide molecules bind in two distinct places on CCP7 (a similar result was obtained with disaccharide; data not shown). Longer GAGs, present on biological surfaces, have the potential to occupy both regions simultaneously (see below) and could bind more tightly and discriminately.

Coincidence between the two sets of 15 residues whose resonances shifted the most at pH 5.2 and 7.4 is good (12 of 15). At neither pH do the array of Tyr side chains that lie on the same face of CCP7 as residue 402 appear to be involved (apart from Tyr402), despite their potential to participate in stacking interactions with pyranose rings. It remains possible that this tyrosine-rich region could accommodate a less sulfated structure within a longer, more complex physiological ligand, such as heparan sulfate. The two positively charged regions on CCP7 would then bind, as inferred from CSP, to neighboring sulfated structures. Such a pattern of sulfation is found, for example, where the highly sulfated “S domains” terminate in the less sulfated “transition zones” that separate them from the unmodified “NAc domains” (54) of heparan sulfate. According to this model, replacement of Tyr402 by His provides additional potential for electrostatic interactions with sulfate groups but compromises the putative aromatic stacking interactions of the Tyr402 variant. In that case, His402 and Tyr402 CCP7 would bind optimally to GAGs bearing different patterns of sulfation, in agreement with the differences in the affinity of these two protein variants for various forms of heparin measured in this and previous studies (27).

Factor H has two further polyanion-binding sites. The site

**FIGURE 4.** *Three-dimensional solution structures of Tyr402 CCP7 and His402 CCP7.* (a), backbone traces of lowest energy Tyr402 (blue) and His402 (red) structures (stereo view); each 30-member ensemble is superimposed on its nearest-to-mean structure. (b), schematic (PyMol; DeLano Scientific, LLC, San Carlos, CA) of Tyr402 (cyan) and His402 (magenta) CCP7, with ball-and-stick representations of all Tyr and His residues and only His/Tyr402 labeled. (c) electrostatic surface of Tyr402 (top) and His402 (bottom) CCP7 (GRASP (62)). Red, negative charge; blue, positive charge. Electrostatic surface potential ranged from $-10$ to $+10 \ kT$ (where $k$ represents Boltzmann’s constant and $T$ is temperature in Kelvin).
located in CCP20 additionally mediates cell binding (55) and is critical for many fH functions (56). In one model, CCPs 7 and 20 bind to the same GAG stabilizing a bent back conformation of fH bringing previously distant sites into critical juxtaposition (29). Thus, the Tyr/His variation at 402, via variable affinity for different GAGs, could modulate strongly the ability of fH to act with full effectiveness at specific self-surfaces.

Disturbance of GAG binding in fH CCP20 was linked to cases of the microthrombotic kidney disease, atypical hemolytic uremic syndrome (29). Many individuals with membranoproliferative glomerulonephritis type II (dense deposit disease), also associated with Tyr/His402 fH (57), have ocular drusen (5). Brüch’s membrane and the glomerular basement membrane are both regions of the ECM that form a selective barrier between fenestrated vasculature with high blood flow and a layer of specialized and metabolically active cells (the RPE or podocytes). In both cases, fH probably plays a crucial role in avoiding complement activation, and consequent inflammation, by the accumulating debris trapped in the ECM, or by the ECM itself; the ECM has no other form of protection from the AP of complement. Significant age-related changes in activity of lysosomal GAG-metabolizing enzymes occur in the macula, manifested in shifts in relative levels of various GAGs within the RPE and Brüch’s membrane (58). Thus, the abilities of the His/Tyr402 variants of fH to recognize and thereby protect the Brüch’s membrane may fluctuate as the eye ages.
Structure of a GAG and Protein Recognition Site in Factor H

Binding sites for CRP and for GAGs overlap on CCP7, so it was not unexpected to observe that CRP binding is also affected by the His/Tyr<sup>402</sup> variation. Furthermore, weaker binding to CRP of the His<sup>402</sup> variant of intact fH, compared with the Tyr<sup>402</sup> version, has been reported recently (59, 60). This acute phase pentraxin recognizes outer membranes of apoptotic cells, whereupon it promotes opsonization and phagocytosis by activating the classical pathway of complement. The role of fH in this process of apoptotic cell clearance could be to block the AP and thereby diminish inflammatory side effects (23). Accumulating environmental insults, such as oxidative stress, could increase the number of apoptotic cells arising from either the RPE or the overlying photoreceptors, placing extra demands on the machinery needed to clear apoptotic cells in a noninflammatory fashion. This is thought to be the principal role of the CRP-fH interaction (23). Why CRP- and GAG-binding sites on fH for its two ligands, CRP and GAG, in the mechanism would entail an imbalance in the relative affinities of fH for these two ligands could be crucial. A plausible disease other according to circumstances (61). Hence, relative affinities be undesirable, with one ligand needing to out-compete the CCP7 overlap is unknown, but simultaneous occupation may the CRP-fH interaction (23). Why CRP- and GAG-binding sites on inflammatory fashion. This is thought to be the principal role of the machinery needed to clear apoptotic cells in a noninflammatory

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