Disease-linked mutations in factor H reveal pivotal role of cofactor activity in self-surface–selective regulation of complement activation

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Spontaneous activation enables the complement system to respond very rapidly to diverse threats. This activation is efficiently suppressed by complement factor H (CFH) on self-surfaces but not on foreign surfaces. The surface selectivity of CFH, a soluble protein containing 20 complement-control protein modules (CCPs 1–20), may be compromised by disease-linked mutations. However, which of the several functions of CFH drives this self-surface selectivity remains unknown. To address this, we expressed human CFH mutants in Pichia pastoris. We found that recombinant 162-CFH (protective against age-related macular degeneration) and V62-CFH functioned equivalently, matching or outperforming plasma-derived CFH, whereas R53H-CFH, linked to atypical hemolytic uremic syndrome (aHUS), was defective in C3bBb decay-accelerating activity (DAA) and factor I cofactor activity (CA). The aHUS-linked CCP 19 mutant D1119G-CFH had virtually no CA on (self-like) sheep erythrocytes (Es) but retained DAA. The aHUS-linked CCP 20 mutant S1191L/V1197A-CFH (LA-CFH) had dramatically reduced CA on Es but was less compromised in DAA. D1119G-CFH and LA-CFH both performed poorly at preventing complement-mediated hemolysis of Es. PspCN, a CFH-binding Streptococcus pneumoniae protein domain, binds CFH tightly and increases accessibility of CCPs 19 and 20. PspCN did not improve the DAA of any CFH variant on Es. Conversely, PspCN boosted the CA, on Es, of I62-CFH, R53H-CFH, and LA-CFH and also enhanced hemolysis protection by I62-CFH and LA-CFH. We conclude that CCPs 19 and 20 are critical for efficient CA on self-surfaces but less important for DAA. Exposing CCPs 19 and 20 with PspCN and thus enhancing CA on self-surfaces may reverse deficiencies of some CFH variants.

The complement system provides antibody-independent first-line immune defense. It also augments antibody-mediated and cellular immunity (1). In addition, complement contributes to clearance of by-products of cellular metabolism and cell turnover (2). The cleavage of C3, a thioester-containing serum protein, into C3a and C3b, is pivotal to all three pathways of complement activation. In the alternative pathway, C3 is cleaved by C3bBb, a short-lived complex of C3b and Bb, a serine protease. In a Mg2+–dependent process, C3bBb–generated C3b binds to complement factor B (CFB),3 a proenzyme, whereupon CFB is cleaved by complement factor D (CFD) to yield more C3bBb. Thus, a C3b amplification loop is generated (3) (Fig. 1A). Transiently, nascent C3b can utilize its freshly activated thioester to covalently attach to surfaces (4). Here, unless regulated, C3b is amplified, forms clusters, functions as an opsonin, and triggers further steps in the complement cascade, including C5 cleavage, C5a release, and formation of membrane attack complexes.

A set of plasma proteins calibrates C3b amplification. Properdin (5) stabilizes C3bBb, whereas complement factor H (CFH) (6–8) (Fig. 1B) destabilizes it; this is called decay-accelerating activity (DAA). CFH also has cofactor activity (CA) for cleavage of C3b by complement factor I (CFI). The product, iC3b, cannot bind CFB but is an opsonin in its own right (9). iC3b may be further degraded to C3dg and, ultimately, to C3d. C3d(g) remains covalently bound to the surface for an extended period of time and thereby marks sites of historical complement activation and regulation. As a soluble circulating complement regulator, CFH suppresses spontaneous complement activation in fluid phase that is considered “futile” and that consumes C3 and factor B. Critically, CFH efficiently suppresses C3b amplification on self-surfaces (10) but not on foreign ones. Other host complement regulators (11), which also exhibit DAA and/or CA, are permanently attached to cell surfaces. Together, the complement regulators ensure that the opsonizing, inflammatory, and cytolytic consequences of complement

3 The abbreviations used are: CFB, CFD, CFH, and CFI, complement factor B, D, H, and I, respectively; aHUS, atypical hemolytic uremic syndrome; AMD, age-related macular degeneration; C3G, C3 glomerulopathy; CA, cofactor activity; CCP, complement-control protein module; C-FxD, complement-fixation diluent; DAA, decay-accelerating activity; EEs, rabbit erythrocytes; EEs, sheep erythrocytes; EEs, sensitized sheep erythrocytes; GAG, glycosaminoglycan; LA-CFH, S1191L/V1197A-CFH; NHS, normal human serum; SA, sialic acid; PspCN, S. pneumoniae choline-binding protein, N-terminal domain; SPR, surface plasmon resonance; CFHR, CFH-related protein; RU, resonance units.
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activation manifest on targets rather than on healthy self-surfaces. CFH-related proteins 1–5 (CFHR-1 to -5) provide additional regulatory sophistication. Some of them antagonize CFH by competing with it for binding to targets including C3b (13, 14).

Inherited variations in membrane cofactor protein (MCP, CD46), CFb, C3, CFIs, CFHs, and, in particular, CFH are linked to diseases (15, 16). Numerous disease-related sequence variations were identified in CFH, which consists exclusively of 20 complement-control protein modules (CCPs). The SNP, H402Y, within the seventh CCP (CCP 7) is linked to age-related macular degeneration (AMD) (17–20) and the kidney disorder, C3 glomerulopathy (C3G) (21); H402 is the at-risk allele. The SNP, I62V, in CFH CCP 1 is also linked to AMD (20, 22); Ile-62 is protective. Deletion of the CFHR-1 and CFHR-3 genes decreases the risk of immunoglobulin A nephropathy and AMD (23–26) but predisposes to systemic lupus erythematosus and atypical hemolytic uremic syndrome (aHUS), a form of thrombotic microangiopathy leading to end-stage renal failure (27). Most mutations found in aHUS patients cluster in the CFH C-terminal CCPs 19 and 20 (28). Other potentially disease-linked sequence variations occur elsewhere in CFH (29–31).

Penetrance is low for many of the CFH mutations linked to diseases. Variation in the genes for other complement proteins and environmental factors may also contribute to disease risk (32). The complexity of complement modulation underlines the need to explore functional effects of each potentially disease-causing CFH variant. However, the scarcity of pure CFH samples is limiting (22, 33). Most patients are heterozygous for mutations that occur on a polymorphic background. Recombinant production of full-length CFH, a single polypeptide containing 40 disulfides (two per CCP), is challenging (34–36). In only one case (36) were yields sufficient for rigorous sample authentication.

Many studies have tried to recapitulate disease-linked mutations in more easily produced CFH truncations containing up to five CCPs (37–42). Indeed, most disease-linked variations occur within subsets of CCPs, namely CCPs 1–4, CCP 7, or CCPs 19 and 20, which coincide with functional sites (43) (Fig. 1B). For example, a fragment consisting of CFH CCPs 1–4 (CFH 1–4) binds C3b and has CA (44) and DAA (45). CFH 19-20 has neither CA nor DAA but binds C3b, iC3b, and C3d(g) (46). Recombinant CFH 6–8 and CFH 19-20 bind glycosaminoglycans (GAGs) and sialic acids (SAs) found on self-surfaces (47). Recombinant CFH 19-20 binds well to C3d and thereby competes with full-length CFH for binding to C3b-decorated sheep erythrocytes (Eγ) (37), whereas disease-related mutations in CCPs 19 and 20 perturbed this ability (39).

Crucially, the use of small CFH truncations cannot capture the collaboration between multiple binding sites that characterize CFH function in its biological setting. CFH probably undergoes a conformational adjustment (48–50) in a concerted process that involves engagement, via GAG-binding and SA-binding sites in CCPs 7 and 20, with self-surface markers (43). This is thought to allow the C3b/C3d(g)-binding sites located in CCPs 1–4 and CCPs 19 and 20 (47) to engage simultaneously with C3b molecules bound on a host surface (50). Crucially, the resultant CFH–C3b–GAG/SA complex is better at complement suppression than the CFH–C3b complex formed on a surface lacking GAGs and SAs. Whether this enhanced efficacy on self-surfaces is driven by higher DAA, higher CA, or both is unknown. Recombinant full-length CFH is needed to investigate these key self versus non-self discriminatory properties and to establish the phenotypic consequences of disease-related mutations.

Herein, we illustrate the value of producing recombinant full-length CFH for characterizing disease-linked sequence variations. After benchmark work on substitutions in N-terminal CCPs, we focused on potentially more revealing mutations in C-terminal CCPs (Fig. 1B). We investigated the activities of these CFH mutants on the man-made surfaces of surface plasmon resonance (SPR) chips and the foreign surface of rabbit erythrocytes (Eγ) as well as on the more hostlike surface of Eκ. We additionally investigated the functions of our mutants when in complex with PspCN. PspCN is a domain from a bacterial complement evasion protein that binds very tightly to CFH (51) and causes it to adopt a different conformation with enhanced regulatory efficacy (50). Binding to CFH of PspCN increases the accessibility of CCPs 19 and 20, as also probably occurs when CFH binds to a self-surface. Our studies suggest that CA, more than DAA, is boosted by interactions between the C-terminal region of CFH and self-surfaces.

Results

High-yield production of CFH variants in P. pastoris

We produced the two common allelotype CFH variants with Ile or Val at position 62 of the mature protein (I62-CFH or V62-CFH, respectively) in recombinant P. pastoris. We also produced the disease-related mutants, R53H-CFH (on Ile-62 background) and H402C-CFH (on Val-62 background). We also investigated the activities of these CFH mutants on the man-made surfaces of surface plasmon resonance (SPR) chips and the foreign surface of rabbit erythrocytes (Eγ) as well as on the more hostlike surface of Eκ. We additionally investigated the functions of our mutants when in complex with PspCN. PspCN is a domain from a bacterial complement evasion protein that binds very tightly to CFH (51) and causes it to adopt a different conformation with enhanced regulatory efficacy (50). Binding to CFH of PspCN increases the accessibility of CCPs 19 and 20, as also probably occurs when CFH binds to a self-surface. Our studies suggest that CA, more than DAA, is boosted by interactions between the C-terminal region of CFH and self-surfaces.

V62-CFH and I62-CFH have similar biological activities

We compared binding to C3b of I62-CFH, V62-CFH, and CFH purified from human plasma (plasma CFH). Both recombinant protein samples were freshly produced, purified, and assayed in parallel. The plasma CFH sample was purified from pooled human plasma and contained no CFH-related proteins detectable by Coomassie Blue-stained SDS-PAGE (Fig. 1C). Aliquots were stored at −20 °C. An aliquot was freshly thawed for each experiment. The C3b sample (Complement Technology, Tyler, TX) had been stored at −80 °C and was also thawed immediately before use. The C3b was immobilized on a Biacore C1-sensor chip, using amine coupling. By flowing CFH solu-
Indeed, premixing 0.6 μM I62-CFH or V62-CFH with PspCN was previously (13, 49, 50), too weak to measure (Fig. 2C). Conversely, binding of plasma CFH to C3d was, as observed previously (50), enhanced their binding to both C3b and C3d (Fig. 2D). Premixing I62-CFH or V62-CFH with PspCN transformed it into a C3d binder (Fig. 2C, Table 1), as seen previously (50). Premixing I62-CFH or V62-CFH with PspCN also enhanced their binding to both C3b and C3d (Fig. 2C and Table 1). For confirmation, two additional SPR-based experiments were performed on a different batch of I62-CFH, each on a different day with a different chip. In both experiments (Table 1), the KD of I62-CFH for C3b was approximately halved following preincubation (of I62-CFH) with a 2-fold molar excess of PspCN. Thus, both plasma CFH and recombinant CFH bind PspCN, via their central CCPs (50), and undergo a conformational change that renders the C-terminal modules more accessible to C3d.

In SPR-based assays on a CM5-sensor chip (Biacore), we compared the abilities of recombinant and plasma CFH to accelerate decay of the preassembled C3bBb, the alternative pathway C3 convertase. Both I62-CFH and V62-CFH, at 20 nM, had identical DAA in this assay, whereas 20 nM plasma CFH was less active (Fig. 3A). The presence of PspCN (at a 2:1 ratio to CFH) enhanced the DAA of I62-CFH and V62-CFH to identical extents (Fig. 3B). As expected, PspCN also enhanced the DAA of plasma CFH in this assay.

Functional similarities between I62-CFH and V62-CFH extended to hemolysis protection assays. Upon exposure to increasing percentages of CFH-depleted normal human serum (NHS), E5 are susceptible to lysis by complement unless protected by added CFH. E5, in contrast, lack human-like SAs and are not effectively protected by human CFH. Both I62-CFH and V62-CFH proved equally effective (e.g. at 250 nM) in preventing E5 hemolysis (Fig. 4A) in this assay, whereas both were, as expected, equally ineffective, at 1 μM, in protecting E8 (Fig. 4B). Both I62-CFH and V62-CFH showed more ability to prevent E5 hemolysis when in complex with PspCN.

In a more specific E5-surface assay (22), C3bBb was preassembled on E5, and the resultant C3bBb-decorated cells were exposed to a CFH concentration series. This was followed by an assay of surviving C3bBb, thus allowing estimation of an IC50 value for DAA on a “hostlike” cell surface. Both I62-CFH and V62-CFH exhibited an IC50 of ~1.3 nM in this assay (Fig. 5A and Table 2). In a related study, E8 surfaces were preloaded with C3b and then treated with CFH and a CFH concentration series. The remaining C3b was estimated in a hemolysis assay, as before. This provided an IC50 for the CA of CFH on a surrogate self-surface. In this assay, both I62-CFH (IC50 = 8.0 nM) and V62-CFH (IC50 = 9.5 nM) had similar potencies (Fig. 5B and Table 2). The effect of PspCN in both of these assays was explored for I62-CFH. Unexpectedly, I62-CFH alone and I62-CFH-PspCN complex had indistinguishable DAA on E5-bound C3bBb (Fig. 5C). Conversely, and interestingly, PspCN improved the CA of I62-CFH on E5-bound C3b by a factor of 2 (Fig. 5D and Table 2).

In summary, I62-CFH and V62-CFH bound with similar affinities to C3b and to C3d on SPR chips. They had nearly identical DAA on this man-made “foreign” surface. In hemolysis protection assays, both I62-CFH and V62-CFH protected self-like E5, but neither protected the less self-like E8 unless PspCN was present. PspCN enhanced binding of both I62-CFH and V62-CFH to C3b and C3d on SPR chips, where it also enhanced DAA. PspCN increased the CA, but not the DAA, of CFH on E8.

A disease-linked CCP 1 mutation has similar effects on self-surfaces and foreign surfaces

The disease-linked R53H mutation in CFH CCP 1 was previously studied within recombinant CFH 1–4 (41). Although R53H did not perturb binding of C3b by CFH 1–4, it did disrupt both the CA and DAA of CFH 1–4 on E8. Here, we prepared full-length R53H-CFH. In side-by-side comparisons, R53H-CFH bound about as tightly as I62-CFH to amine-coupled C3b; SPR-derived KD values of 1.2 μM for I62-CFH and 1.8 μM for R53H-CFH (Fig. 6A and Table 1) in a first experiment and 1.0 and 1.2 μM for I62-CFH and R53H-CFH, respectively, in a second experiment (Table 1). In these two experiments, PspCN...
Despite their similar affinities for C3b, R53H-CFH (at 40 nM) exhibited poorer DAA on a CM5-sensor chip (Fig. 6B) in comparison with 40 nM I62-CFH. Similarly, in assays of DAA performed on C3bBb-decorated $E_\text{so}$, R53H-CFH (IC$_{50}$ = 6.6 nM) performed less well than I62-CFH (IC$_{50}$ = 1.4 nM) (Fig. 6C and Table 2). In an assay on C3b-decorated $E_\text{so}$, R53H-CFH (IC$_{50}$ = 43 nM) was likewise less effective in CA than I62-CFH (IC$_{50}$ = 18 nM) (Fig. 6D). PspCN (2:1 molar ratio to CFH) did not noticeably improve the DAA of R53H-CFH measured on $E_\text{so}$ (Fig. 6E). A 2-fold ratio of PspCN to R53H-CFH did improve its CA on $E_\text{so}$ (Fig. 6F and Table 2).

These functional deficiencies of R53H-CFH were not immediately evident in hemolysis protection assays. Although R53H-CFH, with or without PspCN, did not protect foreign $E_R$ (Fig. 4D), it was able to protect the more “self-like” $E_S$ (Fig. 4C).
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Asp-1119 of CCP 19 is a key interface residue in the CFH C-terminal C3b/d-binding site (53). In recombinant CFH 19-20, D1119G abrogated binding to C3b and C3d (39, 53) and destroyed the ability to compete with plasma CFH for binding to C3b-decoy receptors. As described below, LA-CFH prevents C3 depletion in fluid phase, although it exhibits impaired binding to SA-decorated surfaces. The R53H-CFH/LA-CFH mixture was less able to prevent $E_S$ hemolysis than R53H-CFH alone (Fig. 4E). This is consistent with non-depletion of C3, an expected consequence of the fluid-phase regulatory action of LA-CFH. Importantly, it confirms the lack of ability of either LA-CFH or R53-CFH, for different reasons, to fully control C3b amplification on the $E_S$ surface.

To summarize, R53H-CFH is functionally deficient despite retaining good affinity for C3b. The inability of R53H-CFH to regulate C3b amplification on $E_S$ is consistent with its poor $E_S$ hemolysis protection properties under circumstances where fluid-phase C3 levels are not depleted.

Perturbation of the C-terminal C3b/d-binding site

Asp-1119 of CCP 19 is a key interface residue in the CFH 19-20–C3d complex (53). In recombinant CFH 19-20, D1119G abrogated binding to C3b and C3d (39, 53) and destroyed the ability to compete with plasma CFH for binding to C3b-decoy receptors (37, 39). In full-length CFH, D1119G was previously shown to degrade performance in $E_S$ hemolysis protection assays (50). To explore further the functional consequences of D1119G in full-length CFH and allow comparisons with the other CFH mutants reported herein, we measured separately the DAA and CA of D1119G-CFH on $E_S$. As shown in Fig. 7A and Table 2, the ability of D1119G-CFH to accelerate decay of C3b bound to $E_S$ was diminished compared with wild-type CFH ($IC_{50} = 1.3$ nM for I62-CFH versus $IC_{50} = 11$ nM for D1119G-CFH). The negative effect of D1119G on the CA of CFH for cleavage of C3b bound to $E_S$ is consistent with its poor $E_S$ hemolysis protection properties under circumstances where fluid-phase C3 levels are not depleted.

Perturbation of the sialic acid-binding site in CCP 20

Ser-1191 and Val-1197 in CCP 20 are critical for recognition of SA according to a crystal structure of a C3d–CFH 19-20–SA complex (54). In previous work, the disease-related double
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A

Figure 4. Erythrocyte-protection assays. E₅ or E₆ were incubated with CFH-depleted NHS at various percentages (v/v) that had been reconstituted with our recombinant CFH variants. A, reconstitution with 250 nM I62-CFH or 250 nM V62-CFH afforded protection of E₅, with or without 500 nM PspCN. B, neither 1 μM I62-CFH nor 1 μM V62-CFH protected E₆. PspCN (2 μM) enhances CFH protection in both cases. C, LA-CFH (500 nM), unlike 250 nM I62-CFH, offered little protection against E₅ lysis. No lysis occurred in the presence of 250 nM R53-CFH. The addition of 500 nM PspCN to 250 nM LA-CFH (final concentrations) improved its protective properties. D, neither 1 μM LA-CFH (alone) nor 1 μM R53-CFH (with or without 2 μM PspCN) protected E₆, but a mixture of 1 μM LA-CFH + 2 μM PspCN was more protective than LA-CFH alone. E, LA-CFH (500 nM) (unlike 500 nM I62-CFH) did not protect E₆ from lysis; nor did it mix a mixture of 250 nM LA-CFH + 250 nM I62-CFH. A mixture of 250 nM R53-CFH + 250 nM I62-CFH was protective. A mixture of 250 nM R53-CFH + 250 nM LA-CFH was more protective than 500 nM LA-CFH alone but less protective than R53-CFH alone (see C). Six independent measurements were made in B, whereas A and C–E are representative of two independent experiments. The mean and S.D. (error bars) for each measurement were calculated for all data sets (in most cases, error bars are obscured by the symbol).

mutation S1191L/V1197A perturbed the ability of CFH 19-20 to compete with full-length CFH for binding to C3b-decorated E₅ (39, 53) but did not perturb the structure of CFH 19-20 nor interfere with its binding, measured by SPR, to C3b or C3d (42). Here, we found that the SPR-measured affinities of full-length LA-CFH and I62-CFH for amine-coupled C3b are indeed similar: $K_D = 0.8 \mu M$ for LA-CFH versus $1.2 \mu M$ for I62-CFH in one experiment (Fig. 8A) and $0.9 \mu M$ for LA-CFH versus $1.0 \mu M$ for I62-CFH in a second experiment (Table 1). In these two experiments, preincubation of LA-CFH with PspCN in a 2-fold molar excess produced little if any gain in affinity for C3b ($K_D = 0.70 \mu M$ versus $0.83 \mu M$ and $K_D = 0.60 \mu M$ versus $0.86 \mu M$, respectively; Table 1). No effect of these mutations on the DAA of full-length CFH, assayed on the SPR chip surface, could be detected (Fig. 8B). Taken together, these results suggest that the N-terminal region of CFH dominates its properties on the foreign surface of a SPR chip such that C-terminal mutations have undetectable effects.

When assayed on hostlike E₅ cells, S1191L/V1197A had a smaller effect on DAA than on CA (Fig. 8, compare C and D). The effect on DAA was measurable but minor (IC₅₀ = 16 nM for I62-CFH versus IC₅₀ = 2.9 nM for LA-CFH) (Fig. 8C and Table 2), whereas in the CA assays, a larger effect was evident (IC₅₀ = 18 nM for IYD-CFH versus IC₅₀ = 63 nM for S1191L/V1197A-CFH) (Fig. 8D and Table 2). Thus, SA binding matters less for DAA than it does for CA. Furthermore, we found that LA-CFH affords significantly less protection than wild-type CFH to E₅ in the hemolysis protection assay (Fig. 4C). As is apparent from the results of further experiments on E₅, PspCN did not measurably increase the DAA of LA-CFH, but it clearly enhanced the CA of LA-CFH (Fig. 8, compare E and F), although the wild-type IC₅₀ was not restored (Table 2). In E₅ hemolysis protection assays, the addition of PspCN was, notably, able to partially rescue LA-CFH (Fig. 4C), just as it had previously been found to partially rescue another C-terminal mutant, D1119G-CFH (50). Moreover, PspCN
also boosted the ability of LA-CFH to protect the foreign surface of ES (Fig. 4D).

Discussion

Nearly 100 variations in the human CFH gene potentially predispose to aHUS or other diseases (16, 31). Most encode versions of CFH that differ by a single amino acid. Wild-type CFH both inhibits fluid-phase C3 consumption and prevents formation of clusters of deposited C3b molecules on selected surfaces. Only a small fraction of the putatively disease-linked versions of CFH have been functionally characterized in any detail, and 75–80% have not been functionally characterized at all (31). It is often impractical to purify sufficient CFH for rigorous functional analysis from a heterozygous patient. Aiming at a fast and efficient means of obtaining tens of milligrams of protein, we expressed CFH variants in P. pastoris.

A previously reported careful comparison between plasma-purified versions of V62-CFH and I62-CFH provided a benchmarking opportunity (22). Our yields of recombinant V62-CFH and I62-CFH exceeded those of previous attempts to produce CFH in mammalian (33), insect (34), and moss cells (35, 55). Our versions of V62-CFH and I62-CFH differ in sequence at position 936 as well as at position 62 (i.e. we compared V62,E936-CFH with I62,D936-CFH). The E936D SNP is not unique to at-risk or protective haplotypes (20) nor located near binding sites (43) for C3b, C3d(g), or carbohydrates. Although Asp-936 protects against susceptibility to meningococcal disease (56), this SNP is unlikely to impact our assays.
Our recombinant V62-CFH and I62-CFH matched functionally our plasma CFH in our assays of C3b binding, DAA, and CA (Figs. 2–5). Remarkably, however, recombinant V62-CFH and I62-CFH bound very much better than plasma CFH to immobilized C3d (Fig. 2B). Our pooled plasma-derived CFH contains a mixture of common haplotypic variants, but these are located in CCPs 1, 7, and 15, so are unlikely to impact directly on C3d binding that involves only CCPs 19 and 20. Contamination with CFH-related proteins in the plasma CFH sample is unlikely because these were removed during purification. Whereas chemical modifications may be present in aging plasma proteins (57), these would be unlikely to have such a drastic and specific effect. The most likely explanation for the difference in C3d binding between recombinant CFH and plasma CFH is the lack of N-glycans in recombinant CFH. Natural CFH (36, 58) has eight of these. Some might contribute to the hidden or cryptic nature of the C3d-binding site in CCPs 19 and 20 discussed previously (50).

I62-CFH bound marginally better than V62-CFH to C3b in our SPR-based comparison using identically prepared samples (Table 1), but more replicates would be required to afford significance to this. In further SPR measurements, PspCN enhanced C3b binding and the DAA of I62-CFH and of V62-CFH to nearly identical extents. A slightly tighter complex with C3b formed by I62-CFH was reported in an SPR study conducted on plasma-derived CFH in reverse mode (22). The ratio of either IC50 for the variant/IC50 for I62-CFH or (in parentheses) IC50 for CFH with PspCN/IC50 for CFH without PspCN.

Table 2

| Experiment            | CA IC50 (nM) | Effect of the variation (or of PspCN) | DAA IC50 (nM) | Effect of the variation (or of PspCN) |
|-----------------------|--------------|---------------------------------------|---------------|---------------------------------------|
| I62-CFH versus        | 8.00 ± 0.33  | 1.19 ± 0.12                           | 1.35 ± 0.10   | 0.94 ± 0.11                           |
| V62-CFH               | 9.50 ± 0.57  |                                       | 1.27 ± 0.05   |                                       |
| I62-CFH versus        | 8.00 ± 0.33  | (0.48 ± 0.03)                         | 1.77 ± 0.05   | (0.86 ± 0.05)                         |
| I62-CFH + PspCN       | 3.82 ± 0.06  |                                       | 1.52 ± 0.05   |                                       |
| I62-CFH versus        | 17.5 ± 0.8   | 2.44 ± 0.26                           | 1.41 ± 0.44   | 4.72 ± 0.32                           |
| R53H-CFH              | 42.7 ± 2.5   |                                       | 6.65 ± 0.25   |                                       |
| R53H-CFH versus       | 42.7 ± 2.5   | (0.40 ± 0.04)                         | 6.68 ± 0.6    | (0.91 ± 0.15)                         |
| R53H-CFH + PspCN      | 17.2 ± 0.6   |                                       | 6.06 ± 0.05   |                                       |
| I62-CFH versus        | 12.0 ± 0.8   | >40                                   | 1.33 ± 0.09   | 8.06 ± 1.31                           |
| D1119G-CFH versus     | >500         |                                       | 10.7 ± 1.05   |                                       |
| D1119G-CFH + PspCN    | >500         |                                       | 11.5 ± 0.7    | (1.24 ± 0.15)                         |
| I62-CFH versus        | 17.5 ± 2.6   | 3.63 ± 1.35                           | 1.59 ± 0.06   | 1.83 ± 0.14                           |
| LA-CFH                | 63.4 ± 14    |                                       | 2.92 ± 0.12   |                                       |
| LA-CFH versus         | 63.4 ± 14    | (0.75 ± 0.38)                         | 2.23 ± 0.16   | (0.87 ± 0.14)                         |
| LA-CFH + PspCN        | 47.5 ± 13    |                                       | 1.94 ± 0.17   |                                       |

a Shown are the S.E. values for the fits of the data from each experiment.

b The ratio of either IC50 for the variant/IC50 for I62-CFH or (in parentheses) IC50 for CFH with PSPCN/IC50 for CFH without PspCN.

Such functional deficiencies are consistent with R53H causing aHUS (29), although it should be borne in mind that patients are heterozygous, and serum concentrations of CFH are 1–2 μM (61). Moreover, 250 nM R53H-CFH had wild-type-like protective activity in the CFH-reconstituted E3 hemolysis assay (Fig. 4). To circumvent a potential anomaly of this assay, that fluid-phase C3 depletion arises from lack of control by R53H, we included along with R53H-CFH equimolar amounts of I62-CFH or LA-CFH. I62-CFH regulates complement in fluid phase and on surfaces; LA-CFH regulates complement in fluid-phase only (see below). The 1:1 I62-CFH/R53H-CFH combination, crudely emulating the scenario in heterozygous patients, was also protective in our assay because adequate I62-CFH was present to achieve both fluid-phase and on-surface regulation. Conversely, the 1:1 LA-CFH/R53-CFH was not protective, demonstrating that 250 nx R53-CFH could not protect E3 from hemolysis if C3 is present in the fluid phase.

Whereas easily prepared N-terminal truncation fragments, such as CFH 1–4, serve as a good model for full-length CFH, the equivalent claim cannot be made for C-terminal fragments. More than 30 substitutions in CCPs 19 and 20 have been linked
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**A**
- R53H-CFH vs C3b
- I62-CFH vs C3b
- 1.8 ± 0.3 μM
- 1.2 ± 0.2 μM

**B**
- CFH
- Buffer
- R53H
- I62

**C**
- Protection from lysis (%)
- DAA
- Log [CFH] (nM)
- I62-CFH
- R53H-CFH

**D**
- Protection from lysis (%)
- CA
- Log [CFH] (nM)
- I62-CFH
- R53H-CFH

**E**
- Protection from lysis (%)
- DAA
- Log [CFH] (nM)
- R53-CFH
- R53H-CFH:P

**F**
- Protection from lysis (%)
- CA
- Log [CFH] (nM)
- R53-CFH
- R53H-CFH:P
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Figure 6. Effects of R53H on CFH. A, an SPR experiment was performed in a manner similar to that shown in Fig. 2B to compare the affinity (for immobilized C3b) of R53H-CFH (left) with that of I62-CFH (right; see experiment 1 in Table 1). This experiment was repeated with similar results (see experiment 2 in Table 1). B, an SPR-based assay of DAA was performed in a manner similar to those shown in Fig. 3 except that 40 nM R53H-CFH or I62-CFH solutions were used. The data shown are representative of two experiments. C, an E₅₇₅-based assay of DAA was carried out in a manner similar to that shown in Fig. 5A. D, an E₅₇₅-based assay of CA was carried out in a manner similar to that shown in Fig. 5B. Triplicate measurements were made for each data point shown in C and D, and each experiment was performed once. E, in an E₅₇₅-surface assay (see Fig. 5A), PspCN (mixed 2:1 with R53-CFH) does not restore DAA to R53-CFH. F, in an E₅₇₅-surface assay (see Fig. 5B), PspCN at a 2:1 ratio with R53-CFH enhances its CA. For C–F, the mean and S.D. (error bars) of triplicate measurements are plotted, and sigmoidal curves were fitted using SigmaPlot. Each graph is representative of two experiments.

to aHUS (31). Cross-linking and other evidence suggests that the C-terminal CCPs of CFH modulate the activities carried out by the N-terminal modules of the same molecule (8, 50). Thus, the use of full-length CFH is essential to characterize functional consequences of mutations in CCPs 19 and 20. Aspartate 1119, in CCP 19, is a key residue in the C-terminal C3d and C3b-binding site of CFH (53, 62). In the CFH 19-20 context, an aHUS-linked mutation, D1119G (29), severely reduced binding to C3d and C3b (39, 53). Moreover, D1119G-CFH 19-20 was 40-fold less able than native-sequence CFH 19-20 to compete with CFH for binding to C3b-coated E₅₇₅ (39). We previously reported that full-length D1119G-CFH, despite having wild type–like C3b-binding affinity and DAA activity on a foreign SPR chip surface, did not protect E₅₇₅ from hemolysis (50). Herein, we have taken this further by showing that D1119G diminishes 10-fold the ability of CFH to accelerate decay of...
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A. RU vs CFH concentration for LA-CFH vs C3b and I62-CFH vs C3b.

B. Normalized response over time for CFH, buffer, LA, and I62.

C. Protection from lysis for DAA.

D. Protection from lysis for CA.

E. Protection from lysis for DAA with LA-CFH and LA-CFH:P.

F. Protection from lysis for CA with LA-CFH and LA-CFH:P.
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C3bBb assembled on $E_5$. The effect of D1119G on the CA of CFH, in the $E_5$ context, is still greater.

We further explored this theme through studies of the aHUS-linked double mutation in CCP 20 of CFH corresponding to a gene conversion with CFHR-1 (i.e. S1191L/V1197A) (63). The crystal structure of S1191L-CFH 19-20 bound to C3d is barely distinguishable from that of the wild-type CFH-19-20-C3d complex (42). Conversely, LA-CFH 19-20 failed to bind C3b-decorated $E_5$ (39). Subsequently, Ser-1191 and Val-1197 were shown to contribute to the primary SA-binding site in the CFH molecule (54). Here, we found that LA-CFH was significantly poorer than wild-type CFH at protecting $E_5$ from hemolysis by CFH-depleted normal human serum (Fig. 4). A similar result (see above) was obtained using a 1:1 mixture of LA-CFH and R53-CFH, yet in SPR experiments, the S1191L/V1197A substitutions had little impact on binding of CFH to C3b. Moreover, these mutations had only a small effect on C3bBb-DAA whether C3bBb was assembled on an SPR chip surface or, surprisingly, on the surrogate host surface of $E_5$ (Fig. 8).

PspCN binds plasma CFH tightly, enhancing its binding to C3b and strongly increasing its affinity for C3d. The C3b/ C3d(g)-binding and self-recognition site in CCPs 19 and 20 of CFH becomes exposed upon binding PspCN (50), perhaps emulating what happens when CFH binds to a self-surface. Herein, PspCN enhanced the affinities for C3b on an SPR chip, of recombinant I62-CFH, V62-CFH, R53H-CFH, and, to a lesser degree, LA-CFH (Table 1). PspCN also increased, to roughly equal extents, the DAA of I62-CFH and V62-CFH on an SPR chip (Fig. 3). In contrast, PspCN did not increase the DAA of I62-CFH on C3bBb-decorated $E_5$ and there was a similar lack of responsiveness to PspCN from R53H-CFH, D1119G-CFH, and LA-CFH DAA in the $E_5$ context (Table 2).

The discrepancy between the two assays probably reflects the different surfaces employed: SA-bearing and self-like in the case of $E_5$ and foreign in the case of SPR. In contrast with its effects on $E_5$-surface DAA, PspCN significantly enhanced the CA of I62-CFH on C3b-loaded $E_5$. It likewise had a positive effect, in this context, on the CA of R53H-CFH, and it even partially rescued the CA of LA-CFH (Fig. 8). This difference between the responsiveness to PspCN of DAA and CA on $E_5$ echoes differences between the impacts, on DAA (small) and CA (larger), of the C-terminal mutations S1191L/V1197A, and, to a less pronounced extent, D1119G (Table 2).

Both DAA and CA reside in CFH CCPs 1–4 and can act in a concerted manner, but each involves a distinct sequence of events (Fig. 1). To accelerate C3bBb decay, CFH probably binds both the surface-attached C3b and the Bb (64) components of C3bBb, whereupon it acts catalytically to dissociate C3bBb into C3b and Bb. Following release of Bb, surface-tethered C3b retains affinity for CFH because both binding sites on C3b, engaged by CFH CCPs 1–4 and CCPs 19 and 20, remain intact, as do interactions between CCP 20 and SA on the surface. The CFH molecule must subsequently dissociate from C3b to catalyze another C3bBb decay event. As a cofactor, CFH binds to surface-attached C3b and recruits CFI (65). Subsequent cleavage of C3b to iC3b destroys its binding site for CFH CCPs 1–4 (66). Importantly, the binding site on iC3b used by CCPs 19 and 20, along with the interaction between CCP 20 and SA, is not affected. The presence of the product, iC3b or C3d(g), on an SA-decorated surface thus creates the possibility of retaining or enriching CFH on that surface by binding to the CFH C terminus. Thus retained, these CFH molecules could utilize their CCPs 1–4 to act as a cofactor on nearby C3b molecules. In short, complete detachment and reattachment of CFH from the surface is likely between cycles of DAA, but in the case of CA, CFH can cycle between iC3b(C3d)-bound, iC3b(Cd)-plus-C3b-bound, and C3b-bound forms, without leaving the surface.

The results obtained here for disease-linked C-terminal mutants on $E_5$ surfaces support this line of reasoning because they show that retention of CFH on a surface via its CCPs 19 and 20 is more important for CA than for DAA. Our results with PspCN likewise support this reasoning because they confirm that the PspCN-induced increase in access to the surface-engaging C-terminal modules has little effect on DAA but a positive effect on CA.

To summarize, our results show that acceleration of C3bBb decay on a hostlike surface is not very responsive to the presence or the absence (as tested by mutagenesis (Fig. 8C)) or the availability or unavailability (tested by the inclusion or not of PspCN (Fig. 8E)) of the self-recognition site in CCPs 19 and 20. On the other hand, CA (for CFI on C3b bound to $E_5$) is sensitive to the presence or absence (mutagenesis) of the self-recognition site (Fig. 8D) and its availability as enhanced by PspCN (Fig. 8F).

Soluble PspCN has a protective effect on $E_5$ in hemolysis assays (Fig. 4). This was observed previously and is consistent with bacterially displayed CspC preventing C3b amplification on the microbial surface. PspCN also partially rescued functionally deficient LA-CFH in an $E_5$ hemolysis protection assay. These are more complex assays (compared with the individual CAA and DAA assays) and less straightforward to interpret. Nonetheless, we infer that PspCN can work as a protector of self-surfaces mainly by enhancing CFH CA on these surfaces, and this suggests new therapeutic strategies based on CFH activators.

Experimental procedures

Preparation of proteins

Human CFH was either purchased from Complement Technologies or purified from pooled plasma (TCS Biosciences...
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Measurement of CFH and CFH–PspCN binding to C3b by SPR

Experiments were performed on a Biacore T200 at 25 °C as described previously (43). Briefly, C3b was attached using standard amine coupling. Subsequently, C3bBb was assembled on the chip by a 180-s 10-μl/min injection of a solution containing 500 nM CFB and 50 nM CFD. Formation of C3bBb (~150 RU) was monitored, and then an initial dissociation phase (of 240-s duration) allowed observation of intrinsic convertase decay (i.e., a decline in RU attributable to loss of Bb). Subsequently, CFH or CFH–PspCN was injected (10 μl/min for 180 s), allowing observation of a further dissociation phase. The chip was regenerated between cycles by a 30-s injection of 0.1 μM CFH and three 30-s injections of 1 mM NaCl.

Preparation of cells for complement regulation assays

According to Tortajada et al. (22), EA5 in Alsever’s solution (TCS Biosciences) were pelleted and resuspended three times in complement-fixation diluent (CFxD) (Oxoid, Thermo Fisher Scientific). The washed cells, at 2% (w/v) in C-FxD, were sensitized by incubation with anti-EA5 stroma antibody from rabbit (Sigma) (diluted 1:4000 (v/v) in the C-FxD) for 30 min at 37 °C. Sensitized sheep erythrocytes (EA5) were washed three times in C-FxD and then resuspended in C-FxD to a final cell density of 2% (w/v). C3b was then deposited on the EA5 surface by adding one volume of 4% (v/v) CFB-deficient and CFH-deficient normal human serum (ΔBAH-NHS) (22) containing 6 μg/ml Ornithodoros moubata complement inhibitor (a gift from Varleigh Immuno Pharmaceuticals) in C-FxD, followed by a 20-min incubation at 37 °C. The C3b-coated EA5 (EA5–C3b) were washed three times in AP buffer (5 mM sodium barbitone, pH 7.4, 150 mM NaCl, 7 mM MgCl2, 10 mM EGTA). Finally, EA5–C3b cells were resuspended at 2% (v/v) in AP buffer.

Cofactor assays on erythrocytes

Cofactor activity was determined by adding (in triplicate) 50 μl from a dilution series of wild-type or mutant CFH solutions, with or without PspCN at a 2:1 PspCN/CFH molar ratio, to 50 μl of a 2% (w/v) suspension of EA5–C3b in AP buffer containing 2.5 μg/ml CFI. After 15 min (25 °C) of C3b proteolysis, cells were washed three times in 200 μl of AP buffer and pelleted. Washed cells were resuspended in 50 μl of AP buffer in preparation for C3bBb formation as an indirect measure of surviving C3b (22). To this end, C3bBb was formed by adding 50 μl of a solution containing 40 μg/ml CFB and 0.2 μg/ml CFD to 50 μl of the CFH-treated and CFI-treated EA5–C3b and incubating for 15 min at 37 °C. Lysis was then used as a read-out of C3bBb formed on cells. This part of the assay was performed by adding ΔBAH-NHS to a final concentration of 4% (v/v) to the suspended cells and incubating for 20 min at 37 °C. To quantify lysis, cells were pelleted, and the absorbance (A420 nm) of the supernatant was measured on a Multiskan Ascent Plate Reader (Agilent). The no-lysis controls, for blank subtraction, contained no CFB or CFD. Maximal lysis was determined by an A420 measurement following the addition of PBS, instead of CFH solution, to the EA5–C3b. Mean readings from experiments performed in triplicate were calculated. Total protection from lysis for each concentration of CFH was determined by subtracting, from 1, the following: (mean A420 for a particular CFH sample) – (mean A420 for a no-lysis control sample)/(A420 for a sample where PBS was used rather than CFH).
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was then multiplied by 100. A sigmoidal curve was fitted using SigmaPlot and employed to determine the concentration at which CFH gave 50% protection from lysis (IC$_{50}$).

**Decay-accelerating activities on erythrocytes**

For these assays, C3bBb was formed on EA$_5$-C3b by adding 50 μl of AP buffer containing 40 μg/ml CFB and 0.2 μg/ml CFD to 50 μl of a cell suspension (22). After 15 min (37 °C), C3bBb formation was stopped by the addition of PBS containing 250 μM EDTA, pH 7.4, at a 1:20 dilution. Following the addition of 50 μl from a dilution series of (wild-type or mutant) CFH solutions (with or without PspCN at a 2:1 ratio) in PBS, C3bBb was allowed to decay for 15 min (25 °C). The extent of lysis then served as a measure of remaining C3bBb, as described above. As above, mean readings of experiments in triplicate were calculated. Total protection from lysis for each concentration of CFH was determined as above, and a sigmoidal curve was fitted using SigmaPlot to determine the concentration at which CFH gave 50% protection from lysis (IC$_{50}$).

**Hemolysis protection assays**

For this 96-well plate assay (46), $E_s$ and $E_r$ (TCS Biosciences) were prepared by washing three times with buffer E1 (20 mM HEPES, 145 mM NaCl, 0.1% (w/v) gelatin (porcine skin; Fluka), 10 mM EDTA, pH 7.3) and then buffer E2 (20 mM HEPES, 145 mM NaCl, 0.1% (w/v) gelatin, 10 μM EDTA, pH 7.3). A stock of E was suspended in E2 at a density such that an A$_{412}$ nm = 1.0–1.2 resulted following complete lysis in water. CFH-depleted serum (ΔH-NHS) was reconstituted with a stated, final concentration (250 nm to 1.0 μM) CFH, with or without PspCN (at a 2:1 PspCN/CFH ratio), and then aliquots of varying size were combined, to give various percentages of ΔH-NHS/CFH, with 5 μl of E stock and buffer E2 to a final volume of 45 μl. Lysis was initiated by adding 5 μl of 50 mM MgEGTA, pH 7.3. The reaction was incubated (37 °C) for 30 min and then quenched with 200 μl of buffer E1. The plate was spun (1500 x g, 10 min, 4 °C) to pellet non-lysed cells, and then 100 μl of supernatant from each well was transferred to another 96-well plate for A$_{412}$ measurements.

**Author contributions**—H. K. produced the CFH mutants and carried out some of the assays; E. W. performed the erythrocyte-based assays of CA and DAA; E. M. contributed to the production, and carried out the SPR-based comparisons, of V62-CFH and I62-CFH; Y. Y. carried out the hemolysis protection assays; K. M. designed experiments and contributed to writing the manuscript; D. K. and A. R. helped to conceive the study and contributed to the experimental design; A. P. H. contributed to the experimental design and to the production and purification of proteins; P. N. B. helped to conceive the study, contributed to the experimental design, and wrote the manuscript.

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