Common polymorphisms in C3, factor B, and factor H collaborate to determine systemic complement activity and disease risk

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Common polymorphisms in complement alternative pathway (AP) proteins C3 (C3R102G), factor B (fB32Q), and factor H (fH62V) are associated with age-related macular degeneration (AMD) and other pathologies. Our published work showed that fB32Q influences C3 convertase formation, whereas fH62V affects factor I cofactor activity. Here we show how C3R102G (C5/F) influences AP activity. In hemolysis assays, C3R102G activated AP more efficiently (EC50 C3R102G: 157 nM; C3R102G: 191 nM; P < 0.0001), fB binding kinetics and convertase stability were identical, but native and recombinant fH bound more strongly to C3b (fH C3R102G: 1.0 μM; C3bR102G: 1.4 μM; P < 0.0001). Accelerated decay was unaltered, but fH cofactor activity was reduced for C3bR102G, favoring AP amplification. Combining disease “risk” variants (C3R102G, fB32Q, and fH62V) in add-back assays yielded sixfold higher hemolytic activity compared with “protective” variants (C3102N, fB232, and fH62I; P < 0.0001). These data introduce the concept of a functional complotype (combination of polymorphisms) defining complement activity in an individual, thereby influencing susceptibility to AP-driven disease.

inflammation | infection

Complement plays crucial roles in clearance of pathogens and immune complexes (1), with alternative pathway (AP) “tick-over” in plasma providing a rapid response (2). Assembly of the AP C3-cleaving enzyme (convertase) involves Mg2+-dependent binding of factor B (fB) to C3b, forming the labile proenzyme C3Bb; factor D (fD) then cleaves fB to yield active convertase (C3bBb). Convertase-generated C3b forms more C3bBb, providing exponential AP amplification. C3b clustered around the convertase creates a C5-cleaving enzyme (C3bBbC3b), triggering formation of the cytoplasmic membrane attack complex (MAC).

Nascent C3b binds pathogens and host cells indiscriminately. To prevent damage to self, multiple regulatory proteins limit complement activation by inactivating C3b/C4b, dissociating the C3/C5 convertases, or inhibiting MAC formation. Decay accelerating factor (DAF, CD55) dissociates convertases, whereas membrane cofactor protein (MCP, CD46) is an essential cofactor for factor I (fI) cleavage of C3b (3). In plasma, AP amplification is controlled by factor H (fH), which rapidly dissociates the enzymatic Bb domain from the C3 convertase and catalyses fI cleavage of C3b (4).

Maintenance of complement homeostasis involves equilibrium between activation and control. AP dysregulation is associated with many diseases (5), either due to loss-of-function-expression mutations in regulators or gain-of-function mutations in components; both scenarios cause uncontrolled complement activation and inflammation (6–10). Common polymorphisms in AP components (C3 and fB) and regulators (fH) also link to disease; the fH polymorphism fH402H (rs1061170) is risk for age-related macular degeneration (AMD) (11–14), whereas fH32Q (rs800292) and fB12Q (rs641153) polymorphisms are protective (11, 15, 16).

A common polymorphism in C3, originally identified from electrophoretic mobility and dubbed C3S/F for slow/fast migration (17) (C3R102G, rs2230199; allele frequency: C3R102G [C3]: 0.79, C3R102G [C5]: 0.21), was recently linked with AMD (odds ratio 2.6) (18, 19). Each polymorphism also links to other disorders. fH62V is protective in dense deposit disease (DDD) and atypical hemolytic uremic syndrome (aHUS) (15), whereas fB32Q is risk for infections (20) and overrepresented in autoimmune (21, 22). C3R102G is associated with IgA nephropathy (23), systemic vasculitis (24), kidney allograft dysfunction (25), and DDD (26).

Understanding mechanisms underlying these genetic associations is essential to prove causality, eliminate association through linkage, and aid understanding of disease etiology. The fH402H risk variant had no direct effect on fH AP regulation but influenced binding to sialylated surfaces (27, 28). The AMD-protective variant, fB32Q, formed AP convertase less efficiently, whereas the protective variant fH62I bound C3b more strongly and was a better cofactor for fI inactivation (29). These latter polymorphisms directly influenced AP activity, explaining their link to disease. Effects were additive, “risk” combinations (fB32Q/ fH402H) caused twofold increased AP activation compared with “protective” variants (fB32Q/fH62I) (29). To define mechanisms underlying disease associations of the common C3R102G (C5/F) polymorphism, proteins were purified from homoygote donors and tested for ligand binding, convertase formation, and regulation. The AMD risk variant (C3bR102G) bound fH less well compared with C3b102N, causing decreased fI cofactor activity, extended convertase lifetime, and enhanced AP amplification. By combining risk and protective AP variants, a “complotype” was revealed in which C3, fH, and fB variants collaborate to set levels of AP activity in plasma, thereby influencing risk in complement-dependent diseases.

Results

Differential Activity of C3b102G Variants in Hemolysis Assays. C3, from plasma of healthy individuals homozygous for C3b102N (n = 3) or C3b102G (n = 2) and free of hydrolyzed C3 and aggregates, was added back to normal human serum depleted of C3 (NHSΔC3) and used to deposit C3b and AP convertases on antibody-coated sheep erythrocytes (ShEA); lysis was developed using C3-supplemented methylamine-treated NHS (NHS-MA). NHSΔC3

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supplemented with C3bOGR required 1.3-fold more fB to achieve lysis equivalence with C3bOGR-supplemented NHSAC3, demonstrating that plasma containing C3bOGR (AMD risk) displayed higher AP activity (Fig. 1). EC₅₀ (fB) values were internally consistent for different C3bOGR (158 nM and 156 nM), and C3bOGR (188 nM, 197 nM, and 187 nM) donors, and were significantly different (two-tailed, unpaired t test; P < 0.0001).

**Formation and Decay of C3bOGR and C3bOGR AP Convertases.** To explore molecular mechanisms underlying differential hemolytic activity, formation and natural decay of the AP C3 convertase was analyzed using surface plasmon resonance (SPR) (Biacore). AP C3 convertase formed on Biacore chips replicates native convertase: it is labile, cleaves C3, deposits nascent C3b, and is regulated by decay accelerators (30, 31). Either fB alone, or fB and fD together, were flowed over each C3b variant. Neither proenzyme (C3bB; Fig. S1); KD C3bOGR: 0.85 ± 0.10 μM (n = 5); KD C3bOGR: 0.71 ± 0.15 μM (n = 5); two-tailed, unpaired t test; P = 0.11) nor convertase (C3bbB; Fig. S2); KD C3bOGR: 67 ± 7 nM, n = 10; KD C3bOGR: 71 ± 8 μM, n = 18; two-tailed, unpaired t test; P = 0.23) formation differed significantly between variants. Both enzymes deactivated at the same rate with calculated half-lives at 25°C (ln2/Kd) of 569 ± 35 s for C3bOGR, Bb and 385 ± 33 s for C3bOGR-Bb (two-tailed, unpaired t test; P = 0.06). In multiple experiments using C3bOGR and C3bOGR from different donors, run on different days and chip surfaces, results were consistent. The fB32R variant was used in all analyses.

**Binding of Convertase Regulators to C3bOGR Variants.** After convertase decay, residual C3b is a ligand for cofactors, fH and MCP, which catalyze cleavage/inactivation by fI. C3b interaction with regulators was assessed using SPR. Each variant C3b was coupled to the Biacore chip, and native fH flowed across (Fig. 2 A and B). Binding affinity (KD) of fH for C3bOGR at steady-state equilibrium (Fig. 2 A and B, Inset), was 1.3-fold lower than for C3bOGR (KD = 1.36 ± 0.17 μM, n = 17; KD = 1.03 ± 0.09 μM, n = 16; P < 0.0001; Fig. 2C). Analyses were reproduced with native fH from three different donors and repeated at least three times on different chips and days and with protein from two C3bOGR and three C3bOGR donors. The H32V variant was used throughout these analyses. Binding of fH to immobilized C3 (H2O)OGR and C3(H2O)OGR (hydronized C3) was measured in identical experiments; measured affinities were reduced compared with C3b and were not significantly different for the variants [C3(H2O)OGR, Kd = 8.85 ± 0.64 μM; C3(H2O)OGR, Kd = 9.02 ± 0.13 μM; P = 0.727; Fig. S3].

C3b binding analyses were repeated using recombinant fH comprising short consensus repeats (SCRs) 1–4 (fH1–4) to investigate whether the observed affinity difference was due to differential binding of SCRs 1–4, containing fH decay and cofactor activities (32, 33). fH1–4 bound C3bOGR with 1.3-fold lower affinity than C3bOGR (Kd = 10.2 ± 1.0 μM, n = 5 versus Kd = 7.7 ± 0.7 μM, n = 6; P < 0.0001; Fig. 2 E and F), replicating findings with native fH. Binding affinities of soluble recombinant MCP (sMCP) and soluble recombinant DAF (sDAF) for immobilized C3b variants were measured precisely as described for fH. There was no difference in C3b variant binding affinities of either sMCP (Kd C3bOGR = 1.48 ± 0.24 μM, n = 11; Kd C3bOGR = 1.43 ± 0.23 μM, n = 6; P = 0.68; Fig. S4 A–C) or sDAF (Kd C3bOGR = 1.72 ± 1.7 μM, n = 19; C3bOGR Kd = 12.8 ± 1.2 μM, n = 10; P = 0.39; Fig. S4 D–F).

To test effects of C3R102G polymorphism on convertase accelerated decay, different concentrations of sDAF, native fH and fH1–4 were flowed over C3bOGR-Bb or C3bOGR-Bb-coated surfaces to catalyze rapid decay of Bb (30, 31) (Fig. 3 A–C). The convertases showed identical susceptibility to accelerated decay by each regulator. Cofactor activity for fI cleavage was not tractable using Biacore because mass change from C3b to iC3b (C3f release) was not measurable.

**Hemolysis Assays to Investigate Decay and Cofactor Activities of fH on Variant Convertases.** fH and fH1–4 bound less well to C3bOGR compared with C3bOGR, but reduced binding affinity did not cause decreased decay acceleration. To confirm this finding and test whether affinity differences affected fH cofactor activity, hemolysis assays were performed using C3 variants spiked into NHS depleted of C3, fH, and fB (NHSAC3BB) to deposit C3b on ShEA. AP convertase was then assembled using fB and fD and incubated with different fH concentrations to promote convertase decay before developing lysis (Fig. 3D). No difference in rate of accelerated decay of the variant convertases was seen, in agreement with Biacore data (HIP0 for C3bOGR: 4.4 ± 0.2 nM; C3bOGR: 4.0 ± 0.5 nM; P = 0.31).

Cofactor activity was measured by treating C3b-coated ShEA with fH and different concentrations of fI to inactivate C3b. Convertase was formed on residual C3b and lysis developed. The C3bOGR variant showed 1.5-fold higher sensitivity to inactivation by fI compared with C3bOGR when fI was used as cofactor (HIP0: 1.5 ± 0.2 nM for C3bOGR: 2.3 ± 0.1 nM for C3bOGR; two-tailed unpaired t test; P < 0.001) and 1.7-fold higher sensitivity to inactivation catalyzed by fH2V (HIP0: 3.6 ± 0.1 nM for C3bOGR: 2.2 ± 0.1 nM for C3bOGR; two-tailed unpaired t test; P < 0.001). For C3bOGR, two protective variants (C3bOGR and fH2V) caused a 2.4-fold reduction in ShEA lysis compared with risk combination (C3bOGR and fH2V; two-tailed unpaired t test; P < 0.0001; Fig. 3E).

**Different Combinations of Polymorphic Variants of C3, fH, and fB Regulate AP Activity in Plasma.** Data above show that different combinations of fH and C3 variants yield different plasma AP activities. We previously showed that fB32R forms C3 convertase less efficiently than fB32R, causing decreased AP amplification (34). To test whether C3 and fB variants collaborate to influence AP activity, NHS depleted of C3 and fB (NHSAC3BB) spiked with C3 variants was used to deposit variant C3b on ShEA. Convertase was then formed by incubating with fD and either fB32R or fB32R and fI and lysis developed (Fig. 4A). Complementary effects of C3 (filled/open symbols) and fB (circles/squares) variants were apparent; protective combination requiring 3.2-fold more fB to achieve lysis comparable with risk combination (EC₅₀: C3bOGR fB32R: 46.5 ± 2.8 nM; C3bOGR, fB32R: 149.1 ± 1 nM; P < 0.0001).

Complementarity of the fH2V32P polymorphism was investigated by comparing the variant set promoting most AP amplification (C3bOGR/fB32R/fH2V) with that causing least amplification (C3bOGR/fB32R/fH2D). C3 variants spiked into NHSAC3BB were used to deposit C3b on ShEA (depletion of fH eliminated C3b cleavage by fI), followed by incubation with equivalent concentrations of either fH variant and fI, and finally varying amounts of fB variants and fD to form convertase. Lysis was developed and...
hemolysis measured (Fig. 4B). The C3102G/fB32R/fH62V combination had sixfold greater AP hemolytic activity compared with C3102R/fB32Q/fH62I at equivalent concentrations and in identical depleted serum (EC50 50 ± 1 nM versus 289 ± 14 nM, P < 0.0001). This finding was confirmed in whole serum AP hemolysis by adding back pure C3, fB, and fH variants to hydrazine-treated NHS de-

Fig. 3. Regulatory activities of native fH, rfH1–4, and sDAF on surface-bound convertase formed from C3b102G variants. (A–C) Identical amounts (1,500 RU) of C3b102R (gray dotted line) and C3b102G (black solid line) were deposited on the chip surfaces using thioester coupling. Convertase was formed by flowing (20 μL/min) fB and fD in the presence of Mg2+ and allowed to decay naturally for 150 s. At the arrow, either (A) sDAF (10, 30, 100, and 300 nM; from Upper to Lower curve), (B) fH (30, 100, and 300 nM), or (C) rfH1–4 (30, 100, 300, and 1,000 nM) was injected (90 s) and accelerated decay of the convertase was monitored. Inset shows whole sensorgram; expanded decay curves are illustrated. (D) Decay accelerating activity of fH was quantitated by incubating C3b-coated ShEA with fB and fD to form convertase and then treating with varying concentrations of fH to decay convertase before developing lysis. (E) Cofactor activity was analyzed by treating C3b-coated ShEA with fH and fI before forming convertase and developing lysis. In D and E, curves were fitted using nonlinear regression to calculate the IH50. Data points represent mean ± SD of three determinations. C3b102G-ShEA are shown as circles, C3b102C-ShEA as squares, fH62V as filled symbols, and fH62I as open symbols.
Fig. 4. Polymorphic variations in C3, fB, and fH collaborate to control AP hemolytic activity. (A) C3b was deposited on ShEA using NHSaC3b spiked with either variant of C3 (C3R102G, squares; C3R102A, circles); convertase was formed using fD and either fB32R (filled symbols) or fB32Q (open symbols); lysis was developed using NHSbC3bH4 and used to deposit C3b on cells, which were then treated with fI and a specific variant of fH. Convertase was formed by adding different concentrations of a specific fB variant with fD, and lysis was developed. The complotype predicted to yield highest AP activity (C3R102G, fB32Q, fH62V) is shown by circles, whereas that predicted to yield lowest AP activity (C3R102A, fB32R, fH62I) is represented by squares. Data points represent mean ± SD of three determinations (error bars depicted for each point); nonlinear regression was used to calculate the EC50. Inset shows SDS/PAGE of purified C3, fB, and fH variants.

Discussion

Common polymorphisms in complement proteins alter risk for diverse diseases (11, 15, 16, 19). Polymorphisms in AP proteins C3, fB, and fH strongly influence risk for AMD with quoted odds ratios for homozygotes of 3.51–7.4 for fH402R (11, 14), 2.6 for C3R102G (19), 0.36 for fB32Q (16), and 0.54 for fH62I (11); each also influences risk for other diseases and/or infections, implying that small changes in AP proteins can dramatically affect health. fH402R likely alters capacity of fH to bind surfaces (27, 28), but the reasons why other AP polymorphisms influence disease susceptibility were unknown until our demonstration that fB32Q and fH62I directly impacted on AP activation and amplification (29, 34). Association of the common C3 polymorphism (C3S/F; C3R102G) with disease has been recognized for decades, but the molecular basis was enigmatic. We report here that the C3R102G polymorphism affects AP activation by influencing efficiency of regulation by fH and that different combinations of common variations in C3, fB, and fH dramatically alter AP activity, up to sixfold at the extremes when pure proteins were used in the assay. When variants were added back to whole serum, the C3R102G, fB32Q, fH62I combination of variants had 50% alternative pathway activity (AH50) compared with the most active variants. These data reveal an AP complotype providing clear explanation for association of AP protein polymorphisms with disease.

Given the long association of C3R102G with disease, it is surprising that the functional basis had remained unknown; however, our studies of polymorphisms in fB and fH had demonstrated that functional consequences of the C3R102G polymorphism were likely to be subtle (29, 34). Metabolic attention was therefore given to protein purity and integrity, with replication using protein from several homozygous individuals. Pure protein added back to C3-depleted serum was used to deposit C3b and assemble AP convertase on ShEA, before developing lysis. AP hemolytic activity driven by the C3R102G convertase was consistently less than by C3R102A convertase (Fig. 1). This was not due to differences in kinetics of convertase formation or decay, but to altered susceptibility to regulation. Specifically, fH bound C3bR102G with lower affinity compared with C3bR102A (Fig. 2A–C). This difference was replicated using recombinant fH SCR1–4, locating differential binding in these amino-terminal domains where decay and cofactor activities reside (Fig. 2D–F) (32, 33). Decay accelerating activity of fH was not affected by this change in affinity (Fig. 3 B–D), but fI cofactor activity for C3bR102G inactivation was reduced, decreasing the rate of C3b inactivation and increasing overall AP activity (Fig. 3E). The effect was specific for fH as binding affinities and regulatory control by DAF and MCP were unaffected by the C3bR102G polymorphism. Binding affinity of fH for hydrolyzed C3 was weak and showed no difference for the C3(FiH)R102G variants; thus, the polymorphism affects primarily the amplification convertase rather than the initiation convertase and tickover loop. The observation that a reduction in binding affinity between C3bR102G and fH adversely affected cofactor binding but not decay accelerating activity implies that cofactor activity is more dependent on tight binding, an interpretation supported by our published analyses of fH402R, where lower binding affinity for C3b in fH402R reduced cofactor but not decay activities compared with fH402I (29).

C3R102G polymorphism maps to a positively charged area at the interface between the MG1 domain and TED (thioester-containing domain) (Fig. S5) (35). fH SCR3 binds MG2 and SCR4 contacts TED in close proximity to MG1. Decreasing overall positive charge in C3b by substituting Arg with Gly at position 102 likely alters interdomain associations around the fH binding site, influencing affinity and cofactor activity. In contrast, binding of fB involves different C3b domains; hence, convertase assembly and decay are unaffected by the polymorphism (Fig. S5) (36, 37).

The C3R102G functional effect, modest in isolation, when placed in the context of other AP variants, translates to a large functional effect. C3R102G and fH62I polymorphisms both influence cofactor activity (Fig. 2D–F), as were effects additive (Fig. 3E), as were effects of C3R102G and fB32R Variants (Fig. 4A). Comparison of the protective set (C3R102G, fB32R, fH62I) with risk (C3R102G, fB32Q, fH62Q) revealed a sixfold difference in hemolytic activity, graphically illustrating that different combinations of complement polymorphic variants yield dramatic differences in complement activity (Fig. 4B). We demonstrate that different combinations of activators and regulators (the complotype) set the level of systemic complement activity in an individual, thereby determining risk for various pathologies involving complement. These findings provide functional underpinning to genetic analyses describing altered disease risk depending on the set of complement genes inherited (38, 39). Maintenance of complement homeostasis involves a balance between activation and regulation. Inheritance of a highly active complement system (e.g., homozygous C3R102G, fB32R, fH62I), whereas beneficial in fighting infection by enhanced opsonization, may predispose to pathology in inflammatory diseases such as AMD.

The extreme risk and protective complotypes illustrated here will be rare but they represent the extremities of a continuum in AP activity that define disease risk. Biomarker studies reported complement activation products in AMD plasma, indicative of low-grade, systemic AP activation (39–41). Inheritance of a more active complotype may be detrimental only in the elderly, where chronic inflammation has been sustained long-term, whereas inheritance of a less active complotype, although protective against inflammation, may increase susceptibility to infection, a negative selective pressure in populations where infection is...
prevalent. This is illustrated in a study of a cohort of 347 Dutch immigrants to Surinam in the 19th century that lost 60% of their number soon after arrival due to typhoid and yellow fever. Survivors of these epidemics showed higher frequency of the C3<sub>10G</sub> allele compared with the parent population (P = 0.00001), providing evidence that strong selective pressure in the face of infection selects this C3 variant in a population (42).

We demonstrate here that sets of common polymorphisms in just three complement proteins dramatically alter AP activity; however, the complotype extends beyond these proteins and the AP. Protein levels will influence systemic complement activity, as will polymorphisms in other complement proteins. For example, fH- and fH-related proteins are associated with AMD (43, 44), whereas polymorphisms in C5/TRAF1 are associated with rheumatoid arthritis and systemic lupus erythematosus (45, 46). Complement dysregulation often manifests in inflammatory diseases when there are “multiple hits,” exemplified in aHUS where two or three hits in the complement cascade are required to trigger pathology (47). Frequently, one or more of these hits is a mutation in a complement protein causing overactivation of components or decreased function of regulators; however, our data show that the complotype can also tip the balance toward dysregulation and pathology. An individual’s complotype will therefore influence susceptibility to, or severity of, diseases involving complement dysregulation; knowledge of the complotype will not only aid disease prediction but also dictate avenues of therapy aimed at restoring equilibrium in the complement cascade.

Materials and Methods

Preparation of Complement Components and Activation Fragments. C3, fB, and fH were purified from plasma of consented volunteers, genotyped and homozygous for the relevant protein; hydrolyzed C3 was generated by hydroxylamine treatment of pure C3 (SI Materials and Methods). C3 variants were purified using classical chromatography (Na<sub>2</sub>SO<sub>4</sub> cut; anion and cation exchange), described in SI Materials and Methods. On the day of analysis, C3 samples were dialyzed into 50 mM Na-phosphate, pH 6, fractionated on Mono S (GE Healthcare) to remove hydrolyzed C3, and gel filtered (Superdex200 10/300; GE Healthcare) to remove aggregates and buffer exchange (Hepes-buffered saline (HBS): 10 mM Hepes, 150 mM NaCl; complement fixation diluent (CFD) (Oxoid), AP buffer (5 mM sodium barbitone pH 7.4, 150 mM NaCl, 7 mM MgCl<sub>2</sub>, 10 mM EGTA pH 7.5) as appropriate, and used immediately without concentration. Factor B and fH were affinity purified from EDTA plasma on anti-Bb (mAb 1C1, in-house) or anti-fH (mAb 35H9, in-house) HiTrap columns (GE Healthcare). Eluted protein was gel filtered to remove aggregates and minor contaminants and buffer exchange. Factor D and fI were from Compte.ch. C3b was prepared by fluid phase AP activation using purified proteins as described (31) and gel filtered to remove aggregates. Recombinant fH (rhf1–4) was isolated from Escherichia coli inclusions and refolded as described in SI Materials and Methods.

Hemolysis Assays. NHS depleted of C3 (NHSC3), C3 and fB (NHSC3fB), fB and fH (NHfBH), or all three components (NHfBHfC3) was depleted of C3 and had puriﬁed C3 added-back (SI Materials and Methods). The ability of fH to decay preformed convertase on SHAE-C3b, or catalyze inactivation of C3b to iC3b by fI before convertase formation, was assessed by extent of hemolysis. Complement assays: Combined effect of C3 and fB variants was assessed using the assay described above. C3b was deposited on SHAE using NHSC3 with C3 added-back; different concentrations (6 nM–1.5 μM) of either fB<sub>220</sub> or fB<sub>328</sub> were used to form convertase with either C3B<sub>10G</sub> or C3B<sub>223</sub>. Combined effect of C3 and fH variants was assessed using co-factor assays with either fH<sub>22</sub> or fH<sub>252</sub>. Combined effect of all three variant proteins (C3B<sub>10G</sub>, fB<sub>328</sub>, and fH<sub>252</sub>) was assessed using C3 added-back to depleted C3b on SHAE incubation in AP buffer with fI as described for the co-factor assay, and incubation with 3.4 mM fH (chosen to yield incomplete cleavage of C3b in the assay). Cells were resuspended in AP buffer and lysis developed as described for decay assay, except that varying concentrations of fB variants (1.5–750 nM) were used to form convertase, AHS0 was calculated as previously described using variants added back to NHS-R3ΔBH (SI Materials and Methods).

Surface Plasmon Resonance. All analyses used a Biacore T100 (GE Healthcare), run in HBS, 0.01% surfactant P20 as described and in SI Materials and Methods (34). To assess enzyme kinetics, fB alone (6–788 nM; proenzyme) or fB (3–39 nM) in the presence of 40 nM fD (convertase) was flowed across immobilized C3b in HBS, 0.01% surfactant P20, 1 mM MgCl<sub>2</sub>; proenzyme was regenerated with EDTA and convertase with SDS (0.3 μM) or rhf1–4 (1 μM). Regulator binding to C3b (or C3<sub>H20</sub>) where stated) was analyzed by flowing varying concentrations of fH [45 nM–2.9 μM for C3b; 0.2 μM–28 μM for C3<sub>H20</sub>], rhf1–4 (89 nM–23 μM), sMCP (100 nM–20 μM), or sDAF (0.78–50 μM); for fH interactions, the surface was regenerated with 10 mM sodium acetate pH 4, 1 mM NaCl. Decay of AP C3 convertase was assessed by forming equal amounts of convertase (788 nM fB, 20 nM fD) on each variant C3b surface (1,500 RU) for 150 s. fH, rhf1–4, and sMCP in the range 10–1,000 nM were injected for 15 min at 20°C. Reference curves for each concentration of regulator binding the C3b surface were subtracted to adjust for regulator interaction with C3b alone.

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