

Mechanistic insights into complement inhibition

by

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B.S., Dalian Medical University, 2018

M.S., Emporia State University, 2020

AN ABSTRACT OF A DISSERTATION

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Department of Biochemistry and Molecular Biophysics
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Abstract

The complement system is a fundamental component of innate immunity, acting as a first line of defense against pathogens, maintaining immune homeostasis, and bridging the gap between initial pathogen recognition and the adaptive immune response. It is tightly regulated to ensure efficient pathogen elimination while preventing host tissue damage. However, dysregulation of this system can lead to a range of pathologies driven by excessive or misdirected complement activation. Existing treatments have brought substantial benefits to patients with complement-mediated diseases such as paroxysmal nocturnal hemoglobinuria (PNH) and atypical hemolytic uremic syndrome (aHUS), yet limitations regarding efficiency and specificity underscore the need for novel complement inhibitors. This study explores four distinct strategies of complement inhibition that collectively aim to expand our understanding of complement modulation and provide insights into precise complement regulation and inhibitor development.

The first strategy involves engineered variants of the Complement Receptor of the Immunoglobulin Superfamily (CRIg). Contrary to earlier reports of only micromolar binding affinities, we found that CRIg binds C3b and iC3b with nanomolar affinity, offering potent inhibition of the alternative pathway (AP). By depleting lysine residues and applying crosslinking modifications, we further enhanced their inhibitory efficacy, suggesting that CRIg can serve as a versatile scaffold for designing next-generation biologics with improved potency and pathway specificity. The second approach centers on synthetic DNA aptamers targeting factor B (FB), a critical protease for AP activation. These aptamers successfully neutralize multiple aHUS-associated FB mutants by disrupting their interaction with C3b, underscoring the aptamers' capacity to address disease-causing complement genetic variants. Third, we discovered that the sandfly salivary protein SALO, a classical pathway (CP) inhibitor, binds the zymogen form of C1r

with nanomolar affinity. By shielding the activation loop, SALO blocks C1r zymogen autoactivation and thereby prevents downstream C1r and C1s functions. This “zymogen-shielding” mechanism distinguishes SALO from other immune evasion proteins or therapeutic inhibitors. Lastly, we expanded our understanding of the *Staphylococcus aureus* virulence factor EfbC by identifying human complement factor H-related protein 2 (FHR2) as a novel target. We found that EfbC forms a ternary interaction with FHR2 and C3b, resulting in stronger inhibition of the terminal complement pathway. Collectively, these four strategies—spanning engineered human complement receptor, synthetic aptamers, arthropod-derived inhibitors, and bacterial immune evasion protein—underscore the immense therapeutic potential of fine-tuning complement at multiple points along its activation pathways. Such targeted approaches not only advance our understanding of complement biology but also offer valuable insights for developing innovative, more precise treatments for a range of complement-driven diseases.

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Brian V. Geisbrecht, Ph.D.

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Abstract

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Chapter 1 - Introduction

Complement System Overview

The complement system is a key component of innate immunity, acting as a first line of defense against pathogens and maintaining immune homeostasis (1, 2). It includes more than 30 soluble and membrane-bound proteins, most of which are produced in the liver and circulate in the blood in an inactive state (3). Upon activation, these proteins engage in a tightly regulated cascade that eliminates invading pathogens, clears immune complexes, and modulates the immune response (4).

The complement system fulfills several essential immunological functions. Opsonization involves complement fragments like C3b and C4b binding to microbial surfaces, marking them for phagocytosis by immune cells such as macrophages and neutrophils. Cytolysis occurs when the membrane attack complex (MAC) creates pores in the membranes of pathogens or infected cells, resulting in cell destruction. Smaller complement fragments (C3a, C4a, and C5a), termed anaphylatoxins, promote inflammation by recruiting immune cells to infection sites, increasing vascular permeability, and stimulating the release of inflammatory mediators (2, 4). In addition, the complement system helps remove immune complexes from the bloodstream, preventing their accumulation in tissues and mitigating the risk of autoimmune disease (3, 5, 6). Beyond its innate functions, the complement system bridges to adaptive immunity. It enhances antigen presentation by facilitating the display of complement-tagged antigens to B and T cells via antigen-presenting cells (APCs) such as dendritic cells and macrophages. Complement fragments like C3d bind to complement receptors on B cells, lowering the activation threshold and promoting antibody production. Furthermore, complement components interact with T cells, influencing their differentiation and function, ultimately shaping the adaptive immune response (5, 7, 8).

Complement Activation Pathways

The complement system is initiated through three activation pathways: the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP) (Fig.1.1) (1). Each pathway has distinct activation triggers yet converges at a shared endpoint, resulting in MAC formation. The CP is typically initiated by antigen–antibody complexes. It starts when the C1 complex—comprising C1q and two serine proteases (C1r and C1s)—binds to the Fc region of IgG or IgM attached to antigens. This binding induces conformational changes, activating C1r, which then activates C1s (1, 9). The activated C1s cleaves C4 into C4a and C4b; C4b binds to the pathogen surface. C1s also cleaves C2 into C2a and C2b, forming the C4b2a complex, which serves as the CP C3 convertase. The C3 convertase subsequently cleaves C3 into C3a and C3b, promoting opsonization and amplifying the complement cascade (1, 10). The LP is triggered by specific carbohydrate patterns on microbial surfaces. Mannose-binding lectin (MBL) or ficolins recognize sugar residues like mannose or N-acetylglucosamine, activating MBL-associated serine proteases (MASP-1 and MASP-2). These proteases cleave C4 and C2, yielding the C4bC2a complex, which functions as the C3 convertase for the LP (1, 11). The AP differs by being constitutively activated at low levels through spontaneous hydrolysis of C3, known as “tick-over.” Hydrolyzed C3, or C3(H₂O), binds factor B (FB), which is then cleaved by factor D (FD) to form the fluid-phase C3(H₂O)Bb complex. This complex cleaves C3 into C3a and C3b. On pathogen surfaces devoid of complement regulators, C3b binds FB and properdin, generating the C3bBb complex (the AP C3 convertase) (1, 12). This process establishes a self-amplifying loop in which C3bBb continually produces additional C3b, accelerating complement deposition and intensifying the response. Consequently, the AP amplifies overall complement activation, regardless of the

initiating pathway. All three pathways converge at the stage of C3b generation. The C3 convertase recruits a second copy of C3b to form the C5 convertase (C4b2a3b or C3bBb3b). The C5 convertase then cleaves C5 into C5a—a potent chemoattractant and anaphylatoxin—and C5b, which initiates the sequential recruitment of C6, C7, C8, and C9 to assemble the membrane attack complex (MAC), ultimately leading to cell lysis (1, 10).

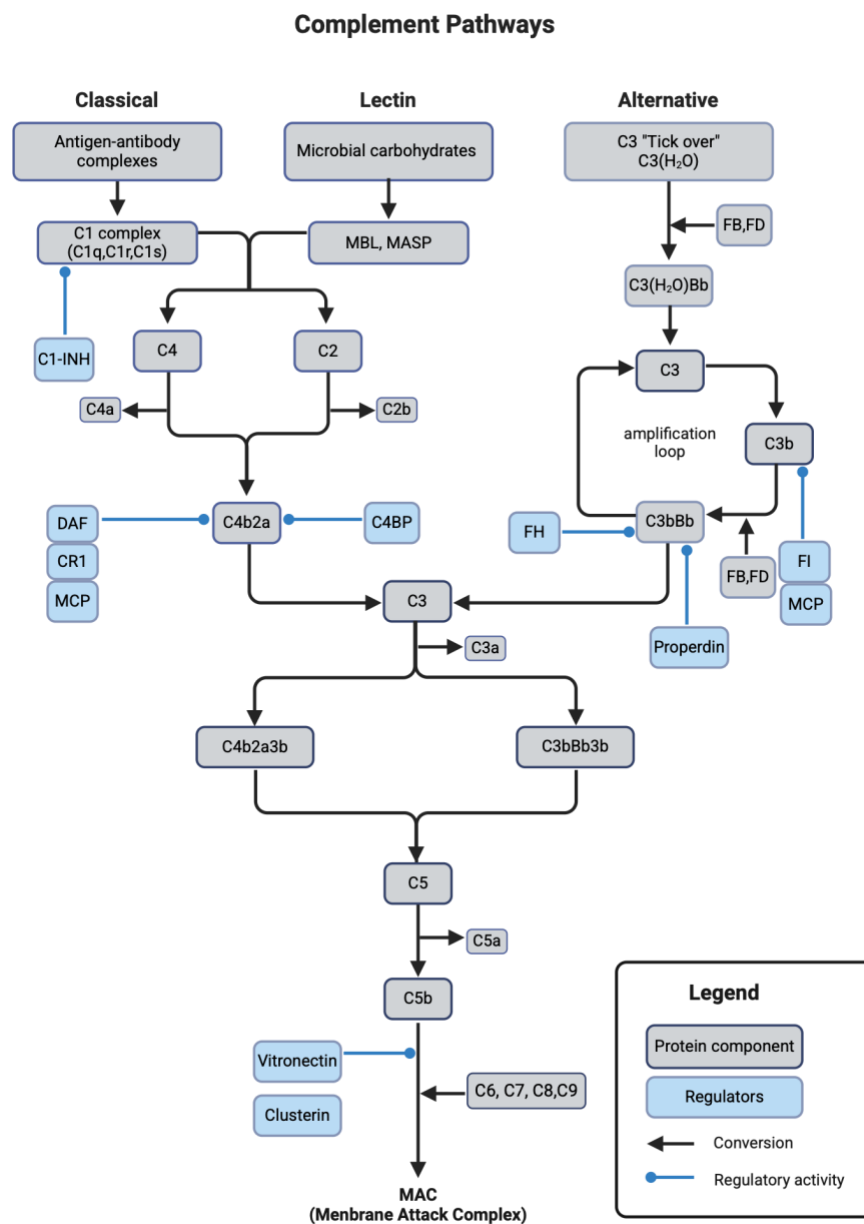


Figure 1.1 Complement activation and regulation

The complement system can be activated through the CP, LP, or AP. In the CP, C1 complex (composed of C1q, C1r, and C1s) recognizes the antigen–antibody complexes on cell surfaces which cleaves C4 and C2 to form the C3 convertase C4b2a. The LP is activated when MBL binds to carbohydrate ligands on pathogen surfaces, then activated MASPs cleave C4 and C2, similarly generating a C3 convertase. The AP is initiated by the spontaneous “tick-over” of C3 to C3(H₂O), which binds FB and is cleaved by FD to form the fluid-phase C3(H₂O)Bb. This complex produces C3a and C3b, with C3b depositing on pathogen surfaces and forming the AP C3 convertase (C3bBb) after binding FB and FD. A self-amplification loop arises as the AP C3 convertase cleaves additional C3. All three pathways converge at the formation of C5 convertases (C4b2a3b or C3bBb3b), which cleave C5 to C5a and C5b; C5b sequentially recruits C6, C7, C8, and C9 to assemble the MAC, lead in cell lysis. Strict regulation prevents host damage: C1-INH inactivates C1r, C1s, and MASPs in the CP and LP; FI cleaves C3b and C4b, assisted by MCP, FH, C4BP and CR1. DAF and FH accelerate the convertase decay, while C4BP binds C4b to prevent C3 convertase formation. Protectin, vitronectin, and clusterin inhibit MAC formation, whereas properdin stabilizes the AP C3 convertase.

Complement Regulation

The complement system is tightly regulated to ensure efficient pathogen elimination while preventing host tissue damage (Fig.1.1). Surface-bound regulators protect host cells from complement-mediated damage. Decay-Accelerating Factor (DAF, CD55) impedes C3 convertases by displacing Bb from C3bBb in the AP and C2a from C4bC2a in the CP and LP (13, 14). Membrane Cofactor Protein (MCP, CD46) serves as a cofactor for factor I, facilitating cleavage of C3b and C4b into inactive fragments (15). Protectin (CD59) blocks the final step of MAC assembly by binding C8 and C9 to insertion and polymerization of MAC (16). Complement Receptor 1 (CR1, CD35) promotes the decay of C3 and C5 convertases and assists factor I in cleaving C3b and C4b. CR1 is predominantly expressed on erythrocytes, leukocytes, and phagocytes, thus aiding in the clearance of complement-coated materials (17).

Fluid-phase regulators circulate in the blood and extracellular fluids, systemically controlling complement activity. Factor H (FH) is a major negative regulator that competes with factor B for C3b binding, accelerating the decay of the C3bBb complex in the AP. It also acts as a cofactor for factor I, limiting excessive complement activation (10, 18). Factor I (FI), a serine

protease, cleaves C3b and C4b in the presence of cofactors (e.g., FH, MCP, CR1), preventing overamplification of complement (19, 20). C1 Inhibitor (C1-INH) blocks the CP and LP by binding and inactivating C1r, C1s, and MASPs, thereby preventing complement initiation (21). Additional fluid-phase regulators include C4b-Binding Protein (C4BP), which binds C4b to impede the formation of the C4bC2a convertase and acts as a factor I cofactor, as well as Vitronectin (S-Protein) and Clusterin (Apolipoprotein J), which inhibit MAC assembly by preventing C9 polymerization (22-24).

Although fewer in number, positive regulators play crucial roles under specific conditions. Properdin (Factor P, FP), for instance, stabilizes the C3bBb convertase in the AP, extending its half-life and reinforcing complement activation (25). Maintaining the interplay between positive and negative regulators is essential for balanced complement function, ensuring efficient pathogen clearance while minimizing collateral damage to host tissues.

Complement-Mediated Diseases and Therapeutic Strategies

Although the complement system is critical for immune defense, its dysregulation can lead to a range of pathologies driven by excessive or misdirected complement activation. Disruptions may arise from genetic mutations, autoantibodies, or unregulated activation during infections or tissue injury, resulting in inflammation, tissue damage, and other pathological conditions. Overactivation often involves the accumulation of complement components such as C3b or the membrane attack complex (MAC), ultimately harming host cells. Mutations in regulatory proteins (e.g., FH, FI, MCP) may impair their capacity to control complement, whereas autoantibodies can stabilize convertases, prolonging activation (26, 27).

Complement dysregulation underlies multiple diseases. In Paroxysmal Nocturnal Hemoglobinuria (PNH), deficiencies in CD55 and CD59 on erythrocytes trigger unrestrained

complement-mediated lysis, causing hemolysis, anemia, and thrombosis (28). Atypical Hemolytic Uremic Syndrome (aHUS) arises from loss-of-function mutations in regulators like factor H and factor I, or gain-of-function mutations in components like C3b and factor B, leading to excessive alternative pathway activation, endothelial damage, thrombocytopenia, hemolytic anemia, and acute kidney injury (29, 30). C3 Glomerulopathy (C3G) stems from persistent alternative pathway activity, characterized by C3 deposition in the glomeruli, proteinuria, hematuria, and progressive renal failure (31). Other example include Age-Related Macular Degeneration (AMD), where dysregulated alternative pathway activity results in chronic retinal inflammation and vision impairment (31, 32).

Targeting complement is considered an attractive therapeutic strategy because it allows for precise modulation of disease-driving pathways, often sparing other immune processes. The well-characterized involvement of complement in various diseases provides clear targets for therapeutic intervention (33). Monoclonal antibodies such as Eculizumab and Ravulizumab, which target complement protein C5, are approved for conditions like PNH and aHUS (34, 35). Pegcetacoplan, a compstatin derivative that inhibits C3, is used to treat PNH and AMD (36). Sutimlimab selectively blocks C1s to reduce hemolysis in adults with cold agglutinin disease (37). Avacincaptad pegol, an aptamer-based inhibitor of C5, is administered via intravitreal injection for geographic atrophy (GA) in AMD (38, 39). Plasma-derived and recombinant C1 inhibitors also target C1-esterase to treat or prevent hereditary angioedema attacks (40). Despite these therapeutic advances, many complement-mediated disorders remain inadequately addressed. Current therapies can be limited by incomplete pathway specificity, high costs, risks of infection, and burdensome dosing regimens. Certain disease-causing mutations may also reduce treatment efficacy (41-43). Consequently, there is a growing need for novel anti-complement approaches that can optimize

efficacy, safety, patient convenience, and pathway selectivity. The following sections will detail my research on various complement inhibitors, offering new insights into complement inhibition and expanding the landscape of complement therapeutics.

Complement Receptor of the Immunoglobulin Superfamily (CRIg)

The Complement Receptor of the Immunoglobulin Superfamily (CRIg) is a transmembrane protein predominantly expressed on tissue-resident macrophages, including Kupffer cells in the liver (44). CRIg binds directly to C3 cleavage products, such as C3b and its inactivated fragment iC3b, deposited on pathogen surfaces during complement activation (45). By recognizing these opsonins, CRIg promotes macrophage-driven immune clearance. Soluble forms of CRIg—particularly its isolated extracellular domain—have been shown to inhibit complement activity in a pathway-selective manner, substantially inhibiting the AP while sparing the classical and lectin pathways (45). Studies indicate that by binding to C3b, CRIg blocks C5 binding at the AP C5 convertase, thereby preventing C5 activation via the AP, yet leaving the classical and lectin pathway C5 convertases unaffected (45). This selectivity helps minimize the systemic immunosuppression often associated with broad-spectrum complement inhibitors, highlighting CRIg’s potential as a therapeutic agent in diseases driven by AP dysregulation. In Chapter 2, we investigate CRIg’s binding affinity to various C3 fragments, assess the potential of CRIg mutants and chemical modifications to optimize AP inhibition, and explore strategies for CRIg’s specificity in complement-targeted therapies.

Chemically modified DNA Aptamers target disease-causing gain-of-function FB Mutants

Aptamers are short, single-stranded oligonucleotides with high specificity and affinity for target biomolecules—often termed “chemical antibodies” (46). They have emerged as powerful

therapeutic agents due to their capacity to bind proteins, peptides, and other biomolecules. To improve intrinsic binding affinity and stability, researchers often introduce chemically modified nucleotides (e.g., 2'-O-methyl ribose) into aptamer sequences (47). These modifications significantly enhance binding strength and serum stability, thereby expanding the therapeutic potential of aptamers.

Through a collaboration between scientists at SomaLogic (Boulder, CO) and our lab, a class of chemically modified DNA aptamers targeting FB was characterized. By incorporating non-natural nucleotides, these aptamers achieve elevated binding affinity and serum stability. Structural and functional studies show that these aptamers bind FB and disrupt its interaction with C3b, thereby blocking proconvertase formation (47). This mechanism could be especially valuable for aHUS patients harboring FB variants that over-activate the complement system by increasing C3b affinity or reducing factor H-mediated decay. In Chapter 3, we present a comprehensive evaluation of multiple aHUS-associated gain-of-function FB mutants and examine the inhibitory capacity of these engineered aptamers, underscoring their promise as targeted therapies for AP-driven disease.

Salivary Anti-complement from *Lutzomyia longipalpis* (SALO)

During blood feeding, hematophagous insects must evade or suppress components of the host immune system to secure a blood meal and protect their own tissues (48). The complement system is a primary target—an innate immune pathway capable of detecting and eliminating pathogens and foreign bodies, including insect midgut tissue. Accordingly, many blood-feeding arthropods (e.g., sand flies, mosquitoes, ticks) have evolved salivary anti-complement proteins to neutralize these host responses (49). One particularly noteworthy example is SALO (Salivary Anti-complement from *Lutzomyia longipalpis*). First identified in the sand fly *L. longipalpis*, SALO is

a potent, dose-dependent inhibitor specific to the CP, acting at a stage before C4 cleavage (50). Despite its pronounced inhibitory effect, SALO bears no clear sequence similarity to known complement regulators and is not a conventional protease inhibitor, leaving its precise mechanism poorly defined. Moreover, its small (~11 kDa), four-helix structure shares little similarity with other complement inhibitors, suggesting a novel mode of complement inhibition (51). Understanding this inhibition mechanism could broaden our understanding of how blood-feeding insects evade host defenses and may inform new therapeutic strategies for modulating complement in human disease. In Chapter 4, we clarify SALO's inhibitory mechanism and uncover a unique mode to complement inhibition, extending our understanding of how hematophagous insects circumvent host immunity and suggesting potential therapeutic avenues for diseases involving complement dysregulation.

Complement evasion by *Staphylococcus aureus* virulence factor EfbC

Bacterial pathogens such as *Staphylococcus aureus* have evolved diverse methods to evade the complement system (52). By interfering with various complement pathways, these bacteria can reduce immune recognition and clearance, thereby enhancing their viability within the host. One evasion strategy involves secreting extracellular fibrinogen-binding protein (Efb), a virulence factor that manipulates complement components to interfere with host defenses. The C-terminal region of Efb, referred to as EfbC, specifically targets C3b, a key opsonin in the complement cascade. By blocking FB binding to C3b, EfbC disrupts formation of the alternative AP C3 convertase (C3bBb), effectively blocking the cascade's amplification loop (53). Our findings suggest that EfbC's immune-evasive capabilities extend beyond simply inhibiting convertase assembly. We discovered that EfbC can also recruit factor H-related protein 2 (FHR2), a member of the factor H family, although its role in regulating complement activation is not well defined. In Chapter 5, we

explored in depth how EfbC engages FHR2 and the functional consequences of this interaction for complement activation and bacterial pathogenesis. Unraveling this mechanism not only advances our understanding of *S. aureus* immune evasion but could also inform the importance of FHR proteins in complement regulations.

Chapter 2 - New Insights into the Complement Receptor of the Ig Superfamily (CRIg) Obtained from Structural and Functional Studies on Two Mutants[#]

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Abstract

The extracellular region of the complement receptor of the immunoglobulin superfamily (CRIg) binds to certain C3 cleavage products (C3b, iC3b, C3c) and inhibits the complement alternative pathway (AP). Here, we provide further insight into the CRIg protein and describe two CRIg mutants that lack multiple lysine residues as a means of facilitating selective chemical modifications of the protein. Structural analyses confirmed preservation of the native CRIg architecture in both mutants. In contrast to earlier reports suggesting that CRIg binds to C3b with an affinity of $\sim 1 \mu\text{M}$, we found that wild-type CRIg binds to C3b and iC3b with affinities below 100 nM, but to C3c with an affinity closer to $1 \mu\text{M}$. We observed this same trend for both lysine substitution mutants, albeit with an apparent ~ 2 - 3 -fold loss of affinity when compared to wild-type CRIg. Using flow cytometry, we confirmed binding to C3 fragment-opsonized *S. aureus* by each mutant, again with an ~ 2 - 3 -fold decrease when compared to wild-type. Whereas wild-type CRIg inhibits AP-driven hemolysis with an IC_{50} of $1.6 \mu\text{M}$, we observed an ~ 3 -fold reduction in inhibition for both mutants. Interestingly, we found that amine-reactive crosslinking of the CRIg mutant containing only a single lysine results in a significant improvement in inhibitory potency across all concentrations examined when compared to the unmodified mutant, but in a manner sensitive to the length of the crosslinker. Collectively, our findings provide new insights into the CRIg protein and suggest an approach for engineering increasingly potent CRIg-based inhibitors of the AP.

Introduction

The extracellular complement system consists of dozens of soluble and cell-surface associated proteins and serves critical roles in many processes related to the innate immune response (3, 4). There are three canonical routes for initiation of complement activity known as the classical (CP), lectin (LP) and alternative (AP) pathways, which differ largely in the biochemical stimuli that trigger them. However, they all converge upon cleavage of the central complement component (i.e. C3) into its bioactive fragments (i.e. C3a and C3b) via two multi-subunit proteases known as C3 convertases. The CP/LP C3 convertase (i.e. C4b2a) assembles following initiation of either the CP or LP. It consists of the activated form of the protease C2 bound in a Ca^{2+} -dependent complex to the platform molecule C4b. By contrast, the AP C3 convertase (i.e. C3bBb) assembles following initiation of the AP or in response to ongoing CP/LP activity. It consists of the activated form of the protease FB bound in a Mg^{2+} -dependent complex to the platform molecule C3b. Since the AP C3 convertase generates the platform molecule upon which new convertases assemble, it serves as a powerful means of self-amplification especially when localized to complement-reactive cells, surfaces, and materials.

One of the best understood aspects of complement function lies in the recognition, opsonization, and lysis of microorganisms, including bacteria (3, 4). In this setting, C3b deposition on bacterial cells promotes several effector mechanisms of complement. Increasing levels of cell-surface C3b promotes association of a second copy of C3b with preformed C3 convertases and thereby changes their substrate preference toward cleavage and activation of C5 into C5a and C5b (54-57). Whereas formation of C5b triggers assembly of the terminal complement complex (TCC) (i.e. C5b-9) that can directly lyse Gram-negative bacteria, the C5a generated serves as a potent chemoattractant to phagocytic cells. This latter effector mechanism is especially important in the

case of Gram-positive bacteria (e.g. *Staphylococcus aureus*), which are resistant to attack by the TCC. Phagocytic cells typically express one or more classes of transmembrane complement receptors, which serve as selective ligands for the C3 fragments that serve as opsonins, namely C3b, iC3b, and C3d (3, 4, 58). These receptors facilitate the effector mechanism of opsonophagocytosis, whereby complement-opsonized bacteria are engulfed by phagocytes and subsequently killed within the phagosome by oxidative, proteolytic, or various other means (59).

The Complement Receptor of the Ig Superfamily (CRIg) is expressed primarily by tissue-resident macrophages, whereby it promotes phagocytosis of C3-opsonized bacteria and particles (44, 60). Like other complement receptors, CRIg does not bind to native C3; however, it does bind to activated C3 fragments including C3b, iC3b, and C3c (44, 45). The C3b-binding region of CRIg lies within its N-terminal Ig-V domain (44) (Fig. 2.1A-B), which recognizes a discontinuous site within the so-called “key ring” core of C3 that is comprised primarily of its β -chain(45) (Fig. 2.1C-D). This binding site is unique from other complement receptors and regulators (45, 58, 61). Moreover, unlike other complement receptors (e.g. CR1) or regulatory proteins (e.g. FH), CRIg lacks the canonical complement regulatory activities of accelerating decay of the AP C3 convertase or serving as a cofactor for FI cleavage of C3b (45). The isolated C3b-binding domain of CRIg inhibits the AP, however, as it blocks activation of both C3 and C5 by their respective convertases (45). Further mechanistic studies showed that CRIg binding to C3b interferes with binding of C5 to the AP C5 convertase, but not to the CP/LP C5 convertase (45). These features make CRIg an intriguing template molecule for developing AP-selective inhibitors.

The last two decades have resulted in an increasingly sophisticated appreciation of the roles played by complement in either causing or promoting human inflammatory diseases (62, 63). This has led to a widespread interest in developing potent and specific inhibitors for therapeutic

manipulation of the complement system (62, 63). The first FDA-approved complement targeted therapeutic, eculizumab, is a humanized monoclonal antibody that binds to an epitope accessible in native C5 and prevents its activation by C5 convertases (34, 64). CRIg likewise blocks C5 activation, albeit through the AP C5 convertase alone (45); however, CRIg has the additional activity of blocking upstream activation of C3 (45). Taking advantage of these properties, Katschke and coworkers showed that a soluble form of murine CRIg blocked AP-driven inflammation in two different animal models of experimental arthritis (65). Subsequent work employed structure-based affinity maturation to enhance the potency of CRIg, since the affinity of this molecule for C3b was judged too weak ($K_D=1.1 \mu\text{M}$) on its own to achieve full inhibition of the AP (66). Although a gain-of-function variant of CRIg was described (66), further development of CRIg for therapeutic purposes seems to have stalled.

Here, we undertook a study with two objectives. First, we sought to provide more detailed information on CRIg binding to activated C3 fragments including C3b, iC3b, and C3c. Our results show that CRIg binds to C3b and iC3b with affinities below 100 nM, which is at least an order of magnitude tighter than originally reported. Second, we explored whether chemically crosslinked forms of CRIg could be prepared with enhanced inhibitory properties. To overcome heterogeneity resulting from amine-reactive crosslinking, we prepared two CRIg mutants where only a single lysine residue remained in the protein. We found that replacement of multiple lysine residues does not affect the overall structure of CRIg, and the two mutants exhibited only mild (i.e. <3-fold) loss of function when compared to wild-type. Interestingly, we observed enhanced AP inhibition using a chemically-modified CRIg mutant when compared to the unmodified protein. Together, these results provide new insights into CRIg and suggest that modified forms of CRIg may be useful tools for basic studies of complement or for manipulating the AP therapeutically.

Materials and Methods

Protein samples

Samples of C3b, iC3b, and C3c that had been purified from pooled human serum were obtained from Complement Technology. DNA fragments encoding wild-type and site-directed mutant forms of the CRIg Ig-V domain (NCBI Reference Sequence NP_009199.1 residues 20-137) were synthesized individually and were subcloned into the prokaryotic expression vector pT7HMT (67). The corresponding plasmids were transformed into *Escherichia coli* BL21(DE3) cells. Cells were grown in 1 L of Terrific Broth (IBI Scientific) until an OD₆₀₀ of 0.6-0.8 was reached, induced with 1 mM IPTG, and incubated at 37 °C for an additional 16 hours. Following harvest by centrifugation, the induced cells were resuspended and lysed using a solution of 100 mM Tris-HCl (pH 8.0), 10 mM Imidazole, and 6M Guanidine HCl. After preparing a clarified extract via high-speed centrifugation, recombinant CRIg proteins were purified by NiNTA affinity chromatography under denaturing conditions using previously published methods (67, 68). Following elution, each purified protein was reduced with 1 mM TCEP at 37 °C for 30 min then dialyzed overnight against 2 L of 100 mM Tris-HCl (pH 8.6), 20 mM glycine, 2 mM L-cysteine, 0.5 mM EDTA, and 2.5 M urea. Each sample was then dialyzed overnight against 4 L of PBS (pH 7.4). When necessary, the affinity tag was digested using Tobacco Etch Virus protease as previously described (67, 68). Briefly, TEV was added to the target protein at a 100:1 mass ratio and incubated overnight at room temperature. The digested sample was reapplied to a NiNTA affinity column, and the unbound fraction was collected. Final purification was achieved by gel-filtration chromatography using a Superdex 75 26/60 column (Cytiva Life Sciences) with PBS (pH 7.4) as the running buffer. Fractions were analyzed by SDS-PAGE and those containing purified

CRlg proteins were pooled, concentrated, quantitated, and stored at either 4 °C or -80 °C for later use.

Samples of CRlg-Mut-1 that had been crosslinked by reactive PEG derivatives were prepared by incubating CRlg-Mut-1 with a corresponding Bis-PEG-NHS ester. Purified CRlg-Mut-1 was concentrated to 10 mg/ml and incubated with 2-fold molar excess of Bis-PEG12-NHS-ester or Bis-PEG25-NHS-ester (BroadPharm) for 1 hour at room temperature according to the manufacturer's suggestions. The reaction was terminated by gel-filtration chromatography using a Superdex 75 26/60 column (Cytiva Life Sciences) with PBS (pH 7.4) as the running buffer. The column eluate was analyzed by SDS-PAGE and fractions containing crosslinked CRlg-Mut-1 were pooled, concentrated, quantitated, and stored at either 4°C or -80°C for later use.

Crystallization, X-ray Diffraction, Structure Solution, and Refinement

Samples of CRlg-Mut-1 and CRlg-Mut-2 were prepared at 10 mg/ml in ddH₂O. Initial crystallization conditions were identified by vapor diffusion of sitting drops that were prepared by a Crystal Gryphon liquid handling system (Art Robbins Instruments, LLC) using the Crystal Screen HT and Index HT screening kits (Hampton Research Corp). Individual crystals of each protein were prepared by vapor diffusion of hanging drops at 20 °C that were established by mixing 1 µL of protein solution with 1 µL of precipitant solution consisting of 0.1 M sodium acetate (pH 6.5), 0.2 M ammonium acetate, 30% (w/v) PEG-4000 and equilibrating over 500 µL of the same precipitant solution. Crystals were cryopreserved by a brief incubation in a buffer consisting of precipitant solution supplemented with 5% (v/v) PEG-400 followed by flash cooling in liquid N₂.

X-ray diffraction data were collected at beamline 22-ID of the Advanced Photon Source at Argonne National Laboratory. Diffraction data were processed using the HKL-2000 software package (69). PHASER was used to solve each structure by molecular replacement (70) with the

reduced and scaled reflection file as an input and the structure of the wild-type CRlg Ig-V domain (PDB entry 2ICC) as a search model (45). Structure solutions were improved through a combination of automated and manual rebuilding and refined by reciprocal space positional and B-factor refinement implemented within the PHENIX software suite (71, 72). The refined models of CRlg-Mut-1 and CRlg-Mut-2 have been deposited in the PDB using accession codes 8TE6 and 8TE5, respectively. Additional information on X-ray diffraction data collection statistics and the properties of each model can be found in Table 2.1.

Surface Plasmon Resonance Binding Studies

SPR experiments were performed on a Biacore T200 instrument at 25 °C using a flow rate of 30 $\mu\text{L min}^{-1}$ and a running buffer of HBS-T (20 mM HEPES (pH 7.4), 140 mM NaCl, and 0.005% (v/v Tween-20)). A ligand capture strategy using NiD200M Ni^{2+} chelator-modified sensor chips (XanTec Bioanalytics GmbH) was employed as follows. First, NiD200M surfaces were conditioned for 1 min with 350 mM EDTA (pH 8.0). Second, a solution of 0.5 mM nickel chloride was then injected for 1 min followed by a 1 min wash with 3 mM EDTA (pH 8.0). Third, His-tagged CRlg proteins were captured on individual experimental flow cells (i.e. fc2, fc3, and fc4) at densities between 1000-1600 RU, while a single flow cell was reserved for reference subtraction (i.e. fc1); an additional set of experiments were performed with His-tagged CRlg-WT, where a lower density of 130-200 RU was captured. Finally, following surface stabilization by equilibration in running buffer, single cycle kinetic series were collected using five concentrations of C3b, iC3b, and C3c (i.e. 0.8, 4, 20, 100, and 500 nM). Kinetic evaluation was performed on each reference-subtracted injection series using either a 1:1 (Langmuir) or two-state binding model as implemented in the Biacore T200 Evaluation Software (Cytiva). Observed association rate (k_a), dissociation rate (k_d) and apparent equilibrium dissociation constants (K_D) were determined from

the resulting fits and presented as the mean plus or minus the standard deviation from four technical replicates as shown in Table 2.2.

Binding of CRlg Proteins to C3-opsonized *S. aureus*

mAmetrine-labeled *S. aureus* Newman $\Delta spa/sbi$ cells were constructed as previously described (73). Bacteria were grown overnight in Todd Hewitt broth (THB) plus 10 $\mu\text{g/mL}$ chloramphenicol, diluted to an $\text{OD}_{600} = 0.05$ in fresh THB plus chloramphenicol, and cultured until midlog phase ($\text{OD}_{600} = 0.5$). Bacteria were then washed and resuspended in RPMI-H medium (RPMI + 0.05% (w/v) human serum albumin [HSA]) and stored until use at -20°C . Newman $\Delta spa/sbi$ (7.5×10^5 CFU) were incubated with threefold serial dilutions (starting from 10% (v/v)) of AS or HIS, or with 3% (v/v) AS/HIS in RPMI-H. Unless stated otherwise, all incubation steps were performed under shaking conditions (± 700 rpm) for 30 min at 37°C , followed by a single wash with RPMI-H and separation by centrifugation (3600 rpm for 7 min). C3-opsonized bacteria were incubated with 25 μL of 5 $\mu\text{g/mL}$, or with a threefold dilution series (starting from 15 $\mu\text{g/mL}$) of his-tagged forms of CRlg-WT, CRlg-Mut-1, CRlg-Mut-2, or untagged CRlg-WT in RPMI-H. To detect CRlg, bacteria were incubated for 30 min at 4°C with shaking with a FITC-conjugated mouse anti-His tag antibody (clone AD1.1.10, LifeSpan Biosciences) that had been diluted 1/80 in RPMI-H. After a final wash with RPMI-H by centrifugation, samples were fixed with 1% (w/v) paraformaldehyde in RPMI-H and the fluorescent signal was detected using flow cytometry (BD FACSVerse). Data were analyzed using FlowJo software.

Hemolysis assay

Assays that quantified AP-mediated lysis of rabbit erythrocytes (Er) were used to monitor the complement inhibitory properties of CRlg proteins. Briefly, all assays were conducted in a 100 μL reaction mixture consisting of 12 μL of AS (Creative Biolabs), 50 μL of 0.1% (w/v) gelatin

veronal buffer (GVBo without Mg^{2+} and Ca^{2+} ; Complement Technologies), 10 μ L of 0.1 M MgEGTA (Complement Technologies), 8 μ L of each protein sample in GVBo needed to achieve the indicated final concentration, and 20 μ L of rabbit erythrocytes (5×10^8 /ml exchanged into GVBo) that were resuspended immediately prior to beginning each assay. After 30 min at 37 °C, all samples were centrifuged for 10 min at 200 $\times g$ and the supernatant was collected. The supernatants were diluted 4-fold in water and the absorbance was determined at 412 nm. Per cent inhibition was determined by the following equation: $100 - 100 \times (OD_{412, \text{sample}} - OD_{412, \text{buffer}}) / (OD_{412, \text{no inhibitor}} - OD_{412, \text{buffer}})$. Apparent IC_{50} values and associated confidence intervals were determined by a four-parameter fit using GraphPad Prism software.

Results

Design of Lysine-substitution Mutants of CRIg

Human CRIg exists as two isoforms that are generated via alternative splicing of transcripts (44). The longer isoform is expressed as a pre-protein of 399 amino acids and differs from the shorter isoform primarily by the presence of a C2-class Ig domain (Fig. 2.1A). The C3b-binding activity of CRIg arises from its N-terminal Ig-V domain, however, which allows both the long and short isoforms to act as cell surface-retained complement receptors (44). The Ig-V domain itself consists of 118 residues (~13.4 kDa) that adopts a canonical β -sandwich fold (45), where the N- and C-termini are located at opposite poles of the protein (Fig. 2.1B). Structures of this Ig-V domain bound to human C3b (Fig. 2.1C) and C3c (*Data Not Shown*) reveal a large and discontinuous binding interface between CRIg and its C3-derived ligands (45). The CRIg binding site on C3b is comprised of macroglobulin-like domains three through six (MG3-MG6), as well as the so-called linker domain (LNK) of the C3b β -chain. Although this binding site is accessible

in the structures of C3b (74), iC3b (75) and C3c (76), it remains masked in native C3 owing to a slight rotation of the MG3 domain relative to the remainder of the β -chain (76). The CRIg binding site is also absent from C3d since this fragment is proteolytically released from the remainder of the C3-derived polypeptide. Together, these features allow CRIg to bind selectively those forms of activated C3 that also contain a β -chain (Fig. 2.1D).

Primary amines, such as those found at the N-terminus of polypeptides or on the sidechains of lysines, represent an abundant functional group of proteins. In that regard, the CRIg Ig-V domain contains six lysine residues (K15, K36, K62, K72, K110, and K118) in addition to its N-terminus (Fig. 2.1A, B). Aside from K118, which appears to form a distant salt-bridge with the sidechain of E277 in C3b and C3c (45), only the sidechain of K62 appears to be significantly buried at the interface of C3b. Therefore, we hypothesized that it would be possible to prepare multi lysine-to-arginine substitution mutants in the CRIg Ig-V domain. By mutating multiple lysine residues to arginine simultaneously, a reduced number of free amines would be present on the variants that result. Furthermore, with a limited number of primary amines present, those amines that remained could be utilized in protein chemistry approaches to generate crosslinked or otherwise modified forms of the protein that might have enhanced functional properties.

Subsequently, we designed two different multi lysine-to-arginine substitution mutants in the CRIg Ig-V domain. These mutants are hereafter referred to as CRIg-Mut-1 and CRIg-Mut-2. CRIg-Mut-1 consists of five simultaneous lysine-to-arginine substitutions, K15R/K36R/K62R/K72R/K110R, but retains the K118 as in the wild-type protein. CRIg-Mut-2 also retains K118, but consists of four lysine-to-arginine substitutions, K15R/K36R/K72R/K110R, as well as a K62Q mutation. This latter mutation was made to avoid unexpected loss of C3-

fragment binding that might result from insertion of the comparatively large arginine sidechain at a potentially sensitive area of the protein-protein interface.

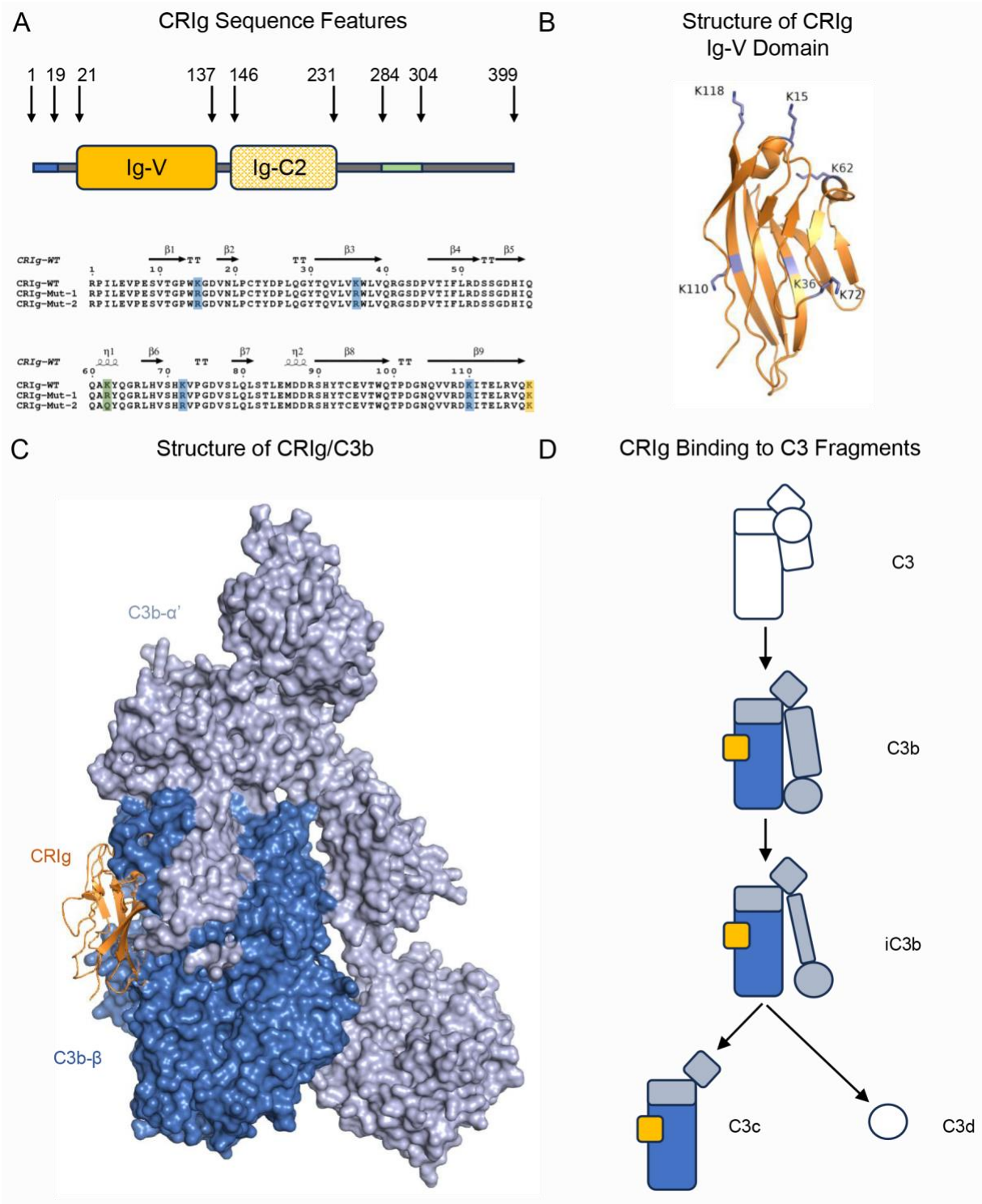
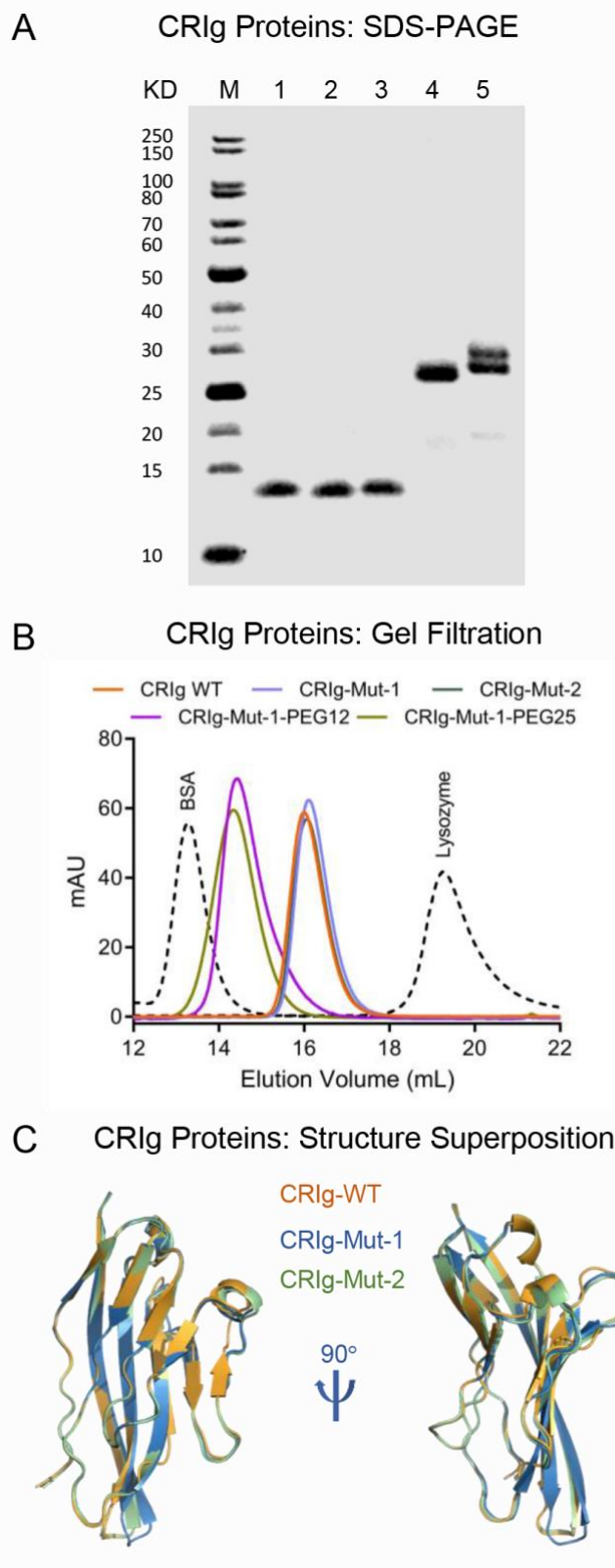


Figure 2.1 Sequence and Structure Features of the Complement Receptor of the Ig Superfamily, CRiG.

(A) *Top Panel*, Schematic diagram of the CRiG pre-protein is shown for its long isoform indicating the presence of the signal peptide (blue), Ig-V (solid orange), Ig-C2 (patterned orange), and transmembrane (green) domains. Amino acid positions defining approximate domain boundaries are numbered above the diagram. *Bottom Panel*, Sequence comparison of the Ig-V domains for CRiG-WT, CRiG-Mut-1, and CRiG-Mut-2 is shown along with the secondary structure elements seen in the crystal structure of CRiG-WT (45). The positions of lysines mutated to arginines in both CRiG-Mut-1 and CRiG-Mut-2 are highlighted blue, while the lysine mutated to either arginine or glutamine is highlighted green. The C-terminal lysine is highlighted orange. (B) Depiction of the CRiG Ig-V domain (orange ribbon) with its lysine residues in blue. Structure drawn from PDB entry 2ICC (45). (C) Depiction of the CRiG Ig-V domain (orange ribbon) bound to C3b (molecular surface), where its β -chain is dark blue and its α' -chain is light blue. Structure drawn from PDB entry 2ICF (45). (D) Schematic representing CRiG (orange rectangle) binding to various forms of C3. C3 fragments that CRiG does not bind are depicted as hollow shapes, while those that it does bind are depicted in solid colors. The C3 β -chain is drawn dark blue, while the α' -chain is drawn light blue for CRiG-binding fragments of C3.

Structural Analysis of Lysine-Substitution Mutants of CRiG

We expressed CRiG-Mut-1 and CRiG-Mut-2, as well as a wild-type control, as his-tagged fusion proteins in *E. coli* (Fig. 2.2A). Following purification of the recombinant proteins under denaturing conditions, we refolded each protein using a previously published procedure and characterized the renatured molecules ((68); please see Materials & Methods). Using analytical scale gel-filtration chromatography, we found that the CRiG-Mut-1 and CRiG-Mut-2 eluted as a single species at an apparent molecular weight comparable to that of CRiG-WT but intermediate to that of BSA (66 kDa) and chicken lysozyme (14.3 kDa) (Fig. 2.2B). To gain further insight into the structural properties of these proteins, we crystallized both mutants and determined their structures by X-ray diffraction methods (Table 2.1). We solved the structure of CRiG-Mut-1 to 1.25 Å limiting resolution and refined the model to R_{work} and R_{free} values of 16.4% and 19.6%, while we solved the structure of CRiG-Mut-2 to 1.5 Å limiting resolution and refined the model to R_{work} and R_{free} values of 16.6% and 22.3%. We found that CRiG-Mut-1 and CRiG-Mut-2 adopted structures essentially identical to CRiG-WT (Fig. 2.2C & Fig. S2.1), as the final models



superimpose upon that of the wild-type protein with R.M.S.D. values of 0.175 Å and 0.200 Å, respectively. These observations were consistent with our earlier observations using gel-filtration chromatography, and together confirmed that both CRlg-Mut-1 and CRlg-Mut -2 were well-folded proteins even though each contained multiple site-directed mutations.

Figure 2.2 Physical Characterization of Lysine-Substitution Mutants of CRlg.

(A) Samples of recombinant CRlg proteins and their cross-linked derivatives were processed under non-reducing conditions, separated by SDS-PAGE, and visualized by Coomassie blue staining. Lane M, molecular weight marker; Lane 1, CRlg-WT; lane 2, CRlg-Mut-1; lane 3, CRlg-Mut-2; lane 4, CRlg-Mut-1-PEG12; lane 5, CRlg-Mut-1-PEG25. (B) Samples of recombinant CRlg proteins and their cross-linked derivatives were separated by analytical size exclusion chromatography. A legend identifying the chromatograms corresponding to individual proteins is inset. Chromatograms corresponding to the control proteins BSA and lysozyme are shown as a dashed line. (C) Comparison

of the X-ray crystal structures for CRlg-WT, CRlg-Mut-1, and CRlg-Mut-2. The final models of

each polypeptide were superimposed and represented as ribbon diagrams from two orthogonal views. A legend identifying each model and the axis of rotation is inset.

Table 2.1 X-ray Diffraction Data, Structure Solution, and Refinement Statistics

X-ray Diffraction Data, Structure Solution, and Refinement Statistics^a		
PDB Accession Code	CRlg-Mut-1 8TE6	CRlg-Mut-2 8TE5
<i>Data Collection</i>		
Space Group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Wavelength (Å)	1.000	1.000
Cell Dimensions		
a, b, c (Å)	31.83, 50.28, 60.98	31.01, 49.78, 60.03
Resolution (Å)	50.00-1.25	50.00-1.50
Wilson B-Factor (Å ²)	13.9	22.6
Completeness (%)	94.0 (90.5)	99.1 (99.5)
I / σ I	12.3 (7.2)	8.9 (3.0)
R _{pim}	0.039 (0.129)	0.061 (0.376)
CC _{1/2}	0.986 (0.946)	0.996 (0.702)
Redundancy	7.8 (7.8)	7.0 (5.4)
<i>Refinement</i>		
Resolution (Å)	30.49-1.25	30.02-1.50
Number of Reflections	25,744	14,849
R _{work} / R _{free} (%)	16.4 / 19.6	16.6 / 22.3
Atoms Modeled		
Total	1,114	1,062
Amino Acid Atoms	962	960
Water	152	102
Ramachandran Plot		
Favored/Allowed (%)	94.02 / 5.98	94.02 / 5.98
Average B-Factors (Å ²)	20.2	30.4
R.M.S. Deviations		
Bond Lengths (Å)	0.004	0.005
Bond Angles (°)	0.79	0.80

Values in parentheses are for the highest-resolution shell. CC_{1/2}, correlation coefficient between intensity estimates from half datasets.

Interactions of Lysine-Substitution CRlg Proteins with C3 Activation Products

Previous studies have suggested that wild-type CRlg binds C3b and C3c with comparable affinity and that the affinity of the CRlg extracellular domain for C3b is on the order of ~1 μ M

(44, 45, 66). However, most of these studies made use of dimeric ligands or receptors (e.g. Fc-fusion proteins) that may have influenced somewhat the results obtained. To investigate this issue using an alternative approach, as well as to gain insight into the functional properties of CRIG-Mut-1 and CRIG-Mut-2, we utilized surface plasmon resonance to characterize the interaction of surface-bound CRIG proteins with various C3 fragments. We devised a ligand-capture strategy where a His-tagged CRIG protein was captured on all three experimental flow cells of a Ni²⁺-loaded biosensor surface followed by assessment of C3 fragment binding through single-cycle kinetics (Fig. 2.3A-C). We then performed each experimental series in quadruplicate to enable statistical evaluation of the reproducibility of our observations (Fig. 2.3D, Figs. S2.2-2.3, & Table 2.2).

We first measured binding of immobilized CRIG-WT, CRIG-Mut-1, and CRIG-Mut-2 to C3b (Fig. 2.3A-C, left panels) using surfaces of an intermediate ligand capture density between 1000-1600 RU of His-tagged protein. In these experiments, we found that wild-type CRIG binds C3b with low-nanomolar affinity (apparent $K_D=7.3\pm0.2$ nM), which is nearly 100-fold tighter than what has been reported previously ($K_D \sim 1$ μ M) (45, 66). Although we found that both CRIG-Mut-1 and CRIG-Mut-2 have lower affinities for C3b than the wild-type protein (apparent $K_D=25.2\pm5.1$ nM and apparent $K_D=16.2\pm0.5$ nM, respectively), these values remain in the low nanomolar range. We next measured binding of immobilized CRIG proteins to iC3b using a similar approach (Fig. 2.3A-C, center panels). We determined that the interaction with iC3b was not well fit by a simple one to one model, but instead required a two-state model to describe the experimental data (Fig. S2.2). Nevertheless, we found that CRIG-WT binds to iC3b with an affinity that is approximately 10-fold diminished when compared to C3b under these conditions (apparent $K_D=68.0\pm9.2$ nM). This result was surprising, considering that the CUB domain that is cleaved during generation of

iC3b from C3b is far removed from the CRiG-binding site (Fig. 2.1C) (45, 58). As was the case for C3b, we also found that both CRiG-Mut-1 and CRiG-Mut-2 have lower affinities for iC3b than CRiG-WT (apparent $K_D=115\pm23.2$ nM and apparent $K_D=84.6\pm12.3$ nM). Finally, we measured binding of the same three immobilized proteins to C3c using a similar approach (Fig. 2.3A-C, right panels). As with iC3b, the interaction with C3c also required a two-state model to describe the experimental data (Fig. S2.2). Interestingly, we found that CRiG-WT binds to C3c with an affinity similar to the value that has been previously reported (apparent $K_D=623\pm4.1$ nM) (45, 66). We also noted that both CRiG-Mut-1 (apparent $K_D=2780\pm971$ nM) and CRiG-Mut-2 (apparent $K_D=1130\pm193$ nM) bind C3c weaker than CRiG-WT. Considering these observations as a whole, both CRiG-Mut-1 and CRiG-Mut-2 display diminished binding of ~ 3 -fold and ~ 1.7 -fold, respectively, when compared to CRiG-WT across all three C3-activation products we examined here (Fig. 2.3D, Fig. S2.3, & Table 2.2).

We also remeasured the binding of immobilized CRiG-WT to the same C3 activation fragments using surfaces of lower ligand capture density between 130-200 RU of His-tagged protein (Fig. S2.3 & Table 2.2). In these experiments, we observed a somewhat weaker affinity for C3b (apparent $K_D=47.2\pm6.5$ nM) but an enhanced affinity iC3b (apparent $K_D=10.5\pm4.3$ nM). However, we found an affinity for C3c consistent with the value that we determined earlier using the higher density surface (apparent $K_D=623\pm4.1$ nM). Although the reasons for the observed differences in affinity of CRiG-WT for C3b and iC3b between these two surface densities are not clear, these two sets of experiments together strongly suggest that the affinity of CRiG-WT for C3b and iC3b is at least an order of magnitude greater than it is for C3c.

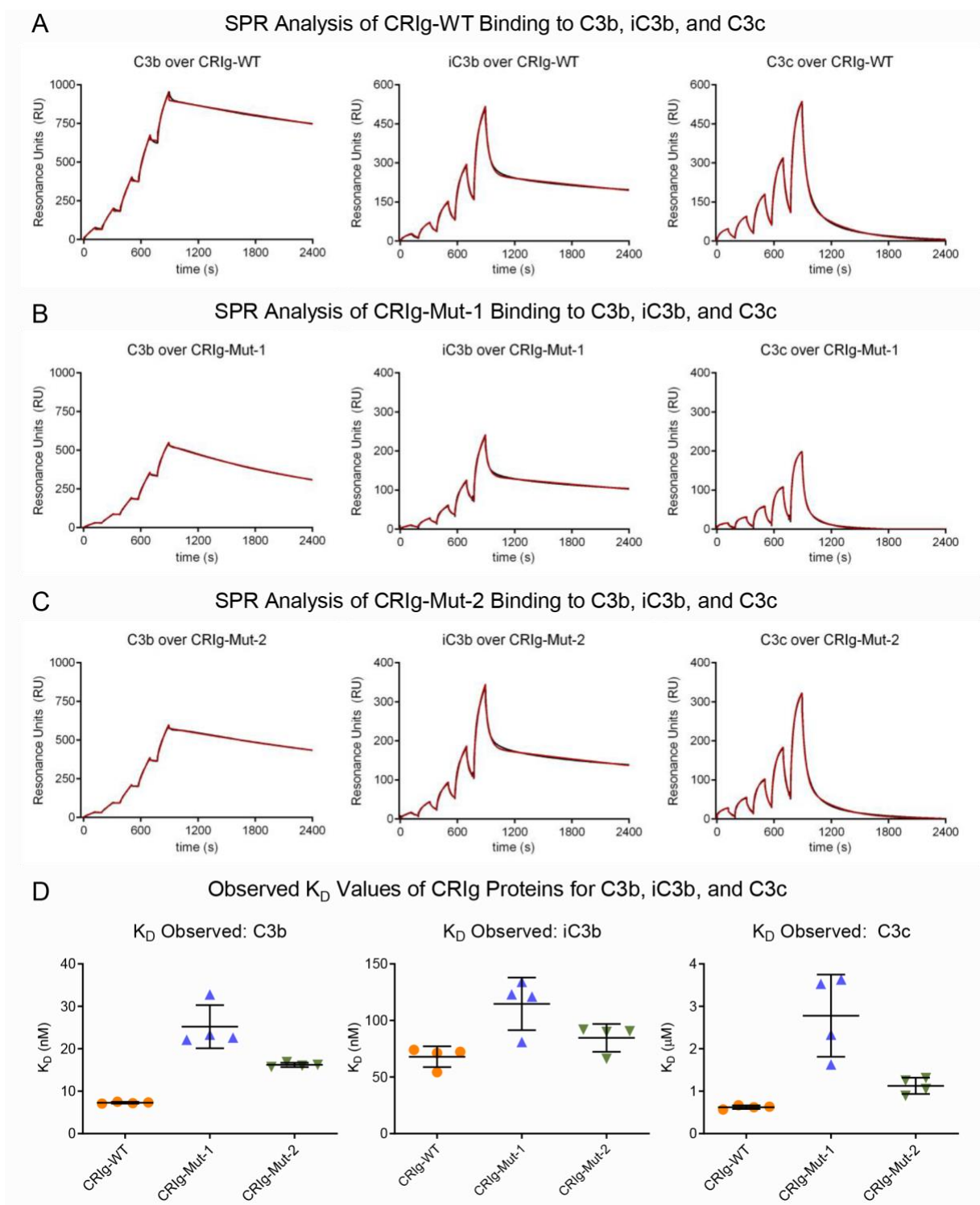


Figure 2.3 Direct Binding Studies of Lysine-Substitution Mutants of CR1g with Various C3 Activation Fragments.

His-tagged forms of CR1g proteins were captured on three experimental flow cells of a Ni-loaded biosensor while leaving one flow cell unmodified for reference subtraction. Protein-protein interactions were then monitored by injecting increasing concentrations of C3 activation fragments

in single-cycle mode. Sensorgrams were analyzed using standard procedures (*please see Materials & Methods*). (A) Binding of C3b, iC3b, and C3c to CRiG-WT. (B) Binding of C3b, iC3b, and C3c to CRiG-Mut-1. (C) Binding of C3b, iC3b, and C3c to CRiG-Mut-2. (D) Comparison of the apparent K_D values from four replicate injections of C3b, iC3b, and C3c across all three forms of CRiG. Note the roughly differences in affinity between C3b, iC3b, and C3c observed independently of the CRiG protein on the surface.

Table 2.2 Interaction Parameters of C3-Fragments with Immobilized CRiG Proteins^a

Analyte	Ligand	k_{a-1} ($10^4 \text{ M}^{-1} \text{ s}^{-1}$)	k_{d-1} (10^{-4} s^{-1})	k_{a-2} (10^{-3} s^{-1})	k_{d-2} (10^{-4} s^{-1})	K_D (nM)	R_{max} (RU)
C3b	CRiG-WT ^b	1.71±0.03	1.25±0.03	-	-	7.29±0.20	1070±26
	CRiG-Mut-1 ^b	1.82±0.50	4.41±0.58	-	-	25.2±5.07	721±47
	CRiG-Mut-2 ^b	1.26±0.89	2.03±0.12	-	-	16.2±0.50	878±58
	CRiG-WT (LD) ^c	2.51±0.05	71.4±3.9	1.66±0.04	3.31±0.49	47.2±6.5	205±42
iC3b	CRiG-WT ^c	1.60±0.25	207±8.2	3.76±0.15	2.06±0.05	68.0±9.20	1260±94
	CRiG-Mut-1 ^c	0.71±0.14	209±4.5	5.89±0.20	2.31±0.09	115±23.2	955±144
	CRiG-Mut-2 ^c	1.06±0.17	191±2.0	4.69±0.13	2.27±0.05	84.6±12.3	979±82
	CRiG-WT (LD) ^c	3.58±0.28	168±7.0	4.63±0.22	1.05±0.40	10.5±4.3	148±35
C3c	CRiG-WT ^c	2.07±0.07	217±13	2.01±0.15	29.4±2.26	623±4.1	1380±88
	CRiG-Mut-1 ^c	1.20±0.24	491±89	5.20±1.43	92.8±24.4	2780±971	1356±410
	CRiG-Mut-2 ^c	1.48±0.09	271±34	2.62±0.51	41.4±7.95	1130±193	1160±160
	CRiG-WT (LD) ^c	2.58±1.78	288±162	0.37±0.03	3.88±1.45	598±148	873±67

^aAll values are presented as their mean ± standard deviation obtained for four replicate injections over the biosensor surface, except for the low-density surface which used three replicate injections.

^bModel accounts for a single binding site between the analyte and ligand described by one association and dissociation rate constant.

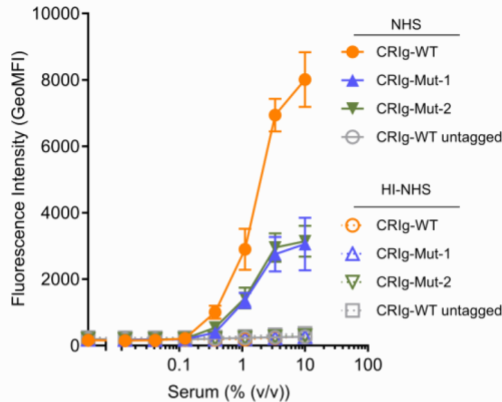
^cModel accounts for a single binding site between the analyte and the ligand described by two sets of association and dissociation rate constants that are linked to one another.

Lysine-Substitution CRiG Proteins Retain Binding to C3-opsonized *S. aureus*

CRiG was originally characterized as a previously unknown receptor for C3 activation products expressed by various subsets of tissue-resident macrophages (44, 60). This work established a role for CRiG in phagocytic clearance of C3-fragment opsonized materials. We therefore used a flow cytometry approach to compare the ability of CRiG-WT, CRiG-Mut-1 and CRiG-Mut-2 to recognize C3 fragment opsonized particles (Fig. 2.4 & Fig. S2.4). In these experiments, *S. aureus* cells that are devoid of the two Ig-binding proteins SpA and Sbi (Newman Δ spa/sbi) were incubated with various concentrations of active human serum (AS) or heat-inactivated serum (HIS) as a control, probed with His-tagged forms of CRiG, and quantified by

staining using a fluorescent anti-His-tag antibody. When we incubated *S. aureus* cells with a dilution series of AS followed by probing with a fixed concentration of his-tagged CRlg-WT, we observed a dose-dependent increase in fluorescence (Fig. 2.4A & Fig. S2.4). This signal was abolished when either untagged CRlg-WT was used as a probe or when HIS was used as a source of complement components. When the initial experiment was performed with either His-tagged CRlg-Mut-1 or CRlg-Mut-2, we observed a dose-dependent increase in fluorescence but with roughly half the signal intensity seen for CRlg-WT. Thereafter, we performed a titration study wherein we incubated *S. aureus* cells with a fixed concentration of AS, probed with increasing concentrations of His-tagged CRlg proteins, and stained using a fluorescent anti-His-tag antibody. When we performed this experiment and probed with an increasing concentration of His-tagged CRlg-WT, we observed a dose-dependent increase in fluorescence as before (Fig. 2.4B & Fig. S2.4). This signal was abolished when either untagged protein was used as a probe or when HIS was employed. However, when the titration experiment was performed with either His-tagged CRlg-Mut-1 or CRlg-Mut-2, we again observed a dose-dependent increase in fluorescence that was approximately half the intensity of CRlg-WT. Thus, the mild loss-of-function (~2-3-fold) seen for CRlg-Mut-1 and CRlg-Mut-2 in the SPR studies was also observed in cytometry experiments using C3 fragment-opsonized bacteria.

A CRlg Binding to Serum-Treated *S. aureus* Cells



B CRlg Binding to Serum-Treated *S. aureus* Cells

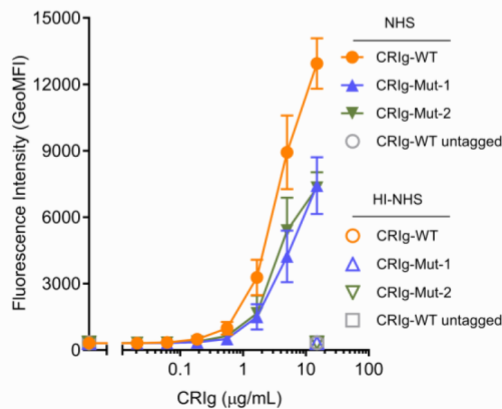


Figure 2.4 Assessment of Binding to C3-Opsonized *S. aureus* Cells by Lysine-Substitution CRlg Mutants.

Cells of an *S. aureus* strain lacking Ig-binding activity were incubated with either active (AS) or heat-inactivated (HIS) human serum. CRlg binding was assessed by flow cytometry following incubation of various samples with His-tagged CRlg proteins (*please see Materials & Methods*). (A) *S. aureus* cells were treated with a dilution series of either AS or HIS, then probed with His-tagged forms of either CRlg-WT, CRlg-Mut-1, or CRlg-Mut-2. The fluorescence intensity observed in three technical replicates is plotted as a function of serum dilution, with a legend inset. (B) *S. aureus* cells were treated with 3% (v/v) of either AS or HIS, then probed with a dilution series of His-tagged forms of either CRlg-WT, CRlg-Mut-1, or CRlg-Mut-2. The fluorescence intensity observed in three technical replicates is plotted as a function of CRlg concentration, with a legend inset.

Inhibition of Complement Mediated Hemolysis by Lysine-Substitution CRlg Mutants

Although CRlg acts as a cell-surface receptor of C3b/iC3b opsonized materials (44, 60), its Ig-V domain in isolation also inhibits the complement alternative pathway (AP) by virtue of its interaction with C3b (45, 66). Previous work has revealed that CRlg binding to C3b blocks an important substrate binding site of the AP C3 and C5 convertases (of which C3b is an integral part), since CRlg appears to inhibit binding and cleavage of C3 and C5 by these convertases (45). Under normal physiological circumstances, assembly of the terminal complement complex (TCC) requires proteolytic cleavage of C5 regardless of the initiation mechanism. Thus, any molecules that interfere with C5 cleavage will block TCC formation and inhibit complement-dependent lysis of erythrocytes. We therefore measured the efficiency of complement-driven lysis of rabbit

erythrocytes in the presence of increasing concentrations of CRiG proteins under AP-specific conditions using AS as a source of complement components. We found that CRiG-WT inhibits hemolysis ($IC_{50}=1.6 \mu M$) more potently than either CRiG-Mut-1 ($IC_{50}=4.9 \mu M$) or CRiG-Mut-2 ($IC_{50}= 5.0 \mu M$) (Fig. 2.5A). Nevertheless, the relative change in IC_{50} we observed between these proteins is comparable to that we found previously in both the SPR (Fig. 2.3 & Table 2.2) and cytometry studies (Fig. 2.4). It should be noted that although the observed IC_{50} values are considerably higher than the apparent K_D values observed by SPR (Table 2.2), they are consistent with the expected C3b levels present under these assay conditions (i.e. 12% (v/v) AS is $\sim 0.78 \mu M$ C3).

Our previous experiment showed that CRiG-Mut-1 displays a ~ 3 -fold decrease in AP inhibition when compared to CRiG-WT. However, this relatively small loss-of-function suggested that CRiG-Mut-1 might still be amenable for further study including chemical modifications that enhance its activity. Since linking of functional units into a single species can contribute to increased activity through avidity-like effects, we wondered if the same might also be possible for CRiG-Mut-1. CRiG-Mut-1 has only a single lysine residue (i.e. K118) in addition to its N-terminus, and so we predicted that reaction of this protein with bifunctional, amine-reactive crosslinkers could be used to generate relatively homogenous species for functional studies. Bis-PEG-NHS esters are symmetric, water-soluble derivatization reagents that react with primary amines and are available in various chain lengths. Therefore, we modified CRiG-Mut-1 with either a Bis-PEG₁₂-NHS ester or a Bis-PEG₂₅-NHS ester and purified the crosslinked species via size exclusion chromatography (Fig. 2.2A). The expected molecular weights of CRiG-Mut-1-PEG₁₂ and CRiG-Mut-1-PEG₂₅ are 27.6 kDa and 28.2 kDa, respectively, which corresponds well to the behavior of both molecules in size-exclusion chromatography and non-reducing SDS-PAGE experiments (Fig.

2.2A, B). Thus, we proceeded to evaluate both PEG-modified forms of CRiG-Mut-1 in hemolysis experiments.

Due to the limited amounts of material available, we could not obtain full dose-response inhibition curves on both crosslinked forms of CRiG-Mut-1. Instead, we opted to investigate AP-mediated lysis of rabbit erythrocytes at three concentrations of unmodified CRiG-Mut-1 that gave suboptimal inhibition in our earlier experiment. We pre-incubated rabbit erythrocytes with CRiG-Mut-1, CRiG-Mut-1-PEG₁₂, or CRiG-Mut-1-PEG₂₅ at 1.5, 3, and 6 μ M of protein (expressed in terms of the concentration of CRiG monomer present in each sample), added AS to activate the AP, and quantified the per cent lysis spectrophotometrically following incubation at 37 °C for a fixed time interval (Fig. 2.5B). We found that unmodified CRiG-Mut-1 achieved mean values of 8.9%, 24.5%, and 58.6% inhibition of hemolysis at the concentrations used. By contrast, we found that CRiG-Mut-1-PEG₁₂ showed mean values of 17%, 32.2%, and 76.1% inhibition of hemolysis at the same concentrations. This represents a statistically significant increase in inhibition ($p < 0.0001$) relative to unmodified CRiG-Mut-1 at all three concentrations tested. Curiously, we found that CRiG-Mut-1-PEG₂₅ showed mean values of 8.9%, 19.9%, and 53% inhibition of hemolysis at these same concentrations. This represents a statistically significant loss in activity relative to the unmodified CRiG-Mut-1 control. Together, these observations reveal that crosslinked CRiG-Mut-1 can surpass the activity of the CRiG-Mut-1 protein alone, but also suggest that the length of the crosslinker is critical to enhancing this complement-inhibitory activity.

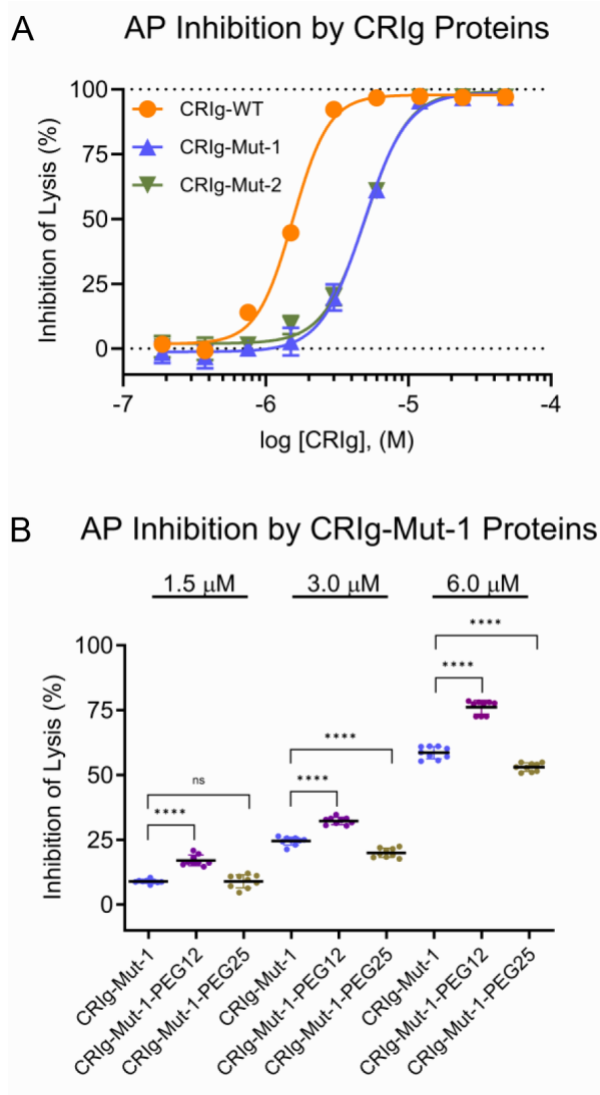


Figure 2.5 Inhibition of AP-Driven Hemolysis by Lysine-Substitution CRiG Mutants.

Rabbit erythrocytes were treated with 12% (v/v) AS either in the absence or presence of a dilution series of CRiG proteins. Hemolysis was assessed spectrophotometrically following a 30 min incubation at 37 °C (please see *Materials & Methods*). (A) Inhibition of hemolysis by CRiG-WT, CRiG-Mut-1, and CRiG-Mut-2, with a legend inset. Normalized values from three technical replicates are expressed as a function of CRiG protein concentration and were fit to a four-parameter dose-response curve. The IC₅₀ values for each protein are as follows: CRiG-WT= 1.6 μM, CRiG-Mut-1= 4.9 μM; and CRiG-Mut-2= 5.0 μM. (B) Inhibition of hemolysis in the presence of 12% (v/v) AS by crosslinked CRiG-Mut-1-PEG12 and CRiG-Mut-1-PEG25 was compared to that of unmodified CRiG-Mut-1 as a control. Three different CRiG concentrations were used and nine technical replicates were performed. Each experimental group was compared to the corresponding unmodified control by an unpaired t-test to assess statistical significance. ****, $p < 0.0001$; n.s., not statistically significant.

Discussion

CRiG belongs to a group of cell-surface exposed receptors that selectively bind to complement activation products and facilitate phagocytic uptake of complement-opsonized materials (58). In the absence of its transmembrane domain (Fig. 2.1A), however, the extracellular region of CRiG acts as complement inhibitor by virtue of its direct interactions with the C3 convertase component, C3b (44, 45, 58). CRiG shares some similarities to CR1 in this respect, which has been developed into a potent inhibitor of all three complement pathways since it binds to both C4b and C3b (77). Because CRiG binds only to C3b and not to C4b as well (44, 45), it

selectively inhibits the AP without any activity against the CP or LP. To capitalize on this useful property, Li and coworkers used structure-based affinity maturation to identify increasingly potent variants of the CRIg C3b-binding domain (i.e. its N-terminal Ig-V domain) on the premise that its intrinsic affinity for C3b was relatively weak ($K_D=1.1 \mu\text{M}$; (66)). However, our results show that the affinity of wild-type CRIg for C3b is significantly higher than previously reported, with values in 10-100 nM range depending upon the specific experimental conditions used (Fig. 2.3, Fig. S2.2-S3, & Table 2.2). CRIg-Mut-1 and CRIg-Mut-2 likewise display C3b-binding affinities in this low-nanomolar range (Fig. 2.3 & Table 2.2). Although the reasons for the differences between our results and those reported previously are not certain, the use of Fc-fusion proteins of CRIg and/or dimers of C3b in earlier binding studies could have been a factor (44). Since our experiments relied on soluble, monomeric forms of CRIg and C3b to obtain binding information, we propose that the affinity of CRIg for C3b is at least an order of magnitude tighter than initially believed.

We also found that all three forms of CRIg examined here displayed progressively weaker binding to downstream C3 activation products, particularly C3c (Table 2.2). Indeed, the observed affinity of CRIg-WT and both mutants for C3c is between ~10-100-fold weaker than what we measured for C3b and iC3b (Fig. 2.3, Fig. S2.2-S2.3 & Table 2.2). These observations stand in contrast to earlier reports, which suggested that CRIg binds with comparable affinity to C3b and C3c (45). The differences in affinity we observed correlate with minor structural changes in the CRIg-binding site that occur as C3b is processed to iC3b and then C3c, however; according to the EBI-PISA server (78), there is approximately 200 Å² more buried surface area at the CRIg/C3b interface when compared to that of CRIg/C3c. These changes are delocalized in nature, which might be expected given the discontinuous CRIg binding site on these C3 fragments (45). Since the proteolytic cleavages that mark the transition from C3b to iC3b to C3c happen at some distance

from the CRiG-binding site itself (58), we suspect that changes in protein dynamics might occur upon cleavage that affect the CRiG-binding site in C3b/iC3b/C3c. From a biological standpoint, though, the differences in affinity of CRiG for C3c relative to C3b and iC3b could also serve a functional purpose: since C3c does not remain surface-associated and has no known biological activity, having a lower affinity for this decoy ligand would make CRiG binding less likely while simultaneously favoring its interactions with the C3b and iC3b that remain covalently bound to complement opsonized cells and materials. Thus, we suspect that this differential affinity for C3 fragments is an important feature to proper functioning of CRiG as a complement receptor that has been overlooked until now.

Although the ability of CRiG to selectively inhibit the AP makes it a promising candidate for therapeutic development, information presented in earlier publications suggested that the C3b-binding affinity of CRiG was too weak to be used for this purpose (45, 66). In fact, this was the very reason Li and coworkers pursued a structure-based affinity maturation strategy to generate CRiG variants of increased affinity for C3b (66). Since multivalent molecules often exhibit enhanced functional properties through avidity effects, we sought to modify CRiG using an amine-reactive crosslinking approach. This required generating forms of CRiG that had minimal lysine content, which we thought was feasible since the structures of CRiG bound to C3b and C3c showed that its endogenous lysines play a minimal role in these protein-protein interactions (45). The two CRiG mutants we characterized here are essentially indistinguishable from CRiG-WT at the structural level (Figs. 2.1-2.2). Although they show a mild ~2-3-fold loss of activity in various functional assays (Fig. 2.3-2.5A), they still bind C3b and iC3b *in vitro* and on cells and inhibit the AP-driven lysis of erythrocytes. Most importantly, we found that a crosslinked CRiG-Mut-1 dimer prepared using a bifunctional PEG12-NHS ester displayed a significant increase in AP inhibition

across a range of concentrations when compared to the unmodified protein (Fig. 2.5B). It is worth noting that while CR Ig-Mut-1 contains only a single lysine residue (Fig. 1A), its N-terminus also contributes a primary amine functional group; because of this, it is possible that the crosslinked dimers we prepared are a mixture of three different species. In the future, it may be worthwhile to evaluate a CR Ig mutant that has no lysines such that any amine-based crosslinking reactions would be restricted to the N-terminus alone. Nevertheless, it is also interesting to consider preparation other types of crosslinked species, such as those arising through use of activated dendrimers (79). Given the polyvalency possible through such approaches, we think that CR Ig-modified dendrimers could exhibit quite potent AP inhibitory properties.

Another interesting outcome of our crosslinking study is that modification of CR Ig-Mut-1 with PEG25-NHS yields a product that is slightly, but significantly less active than CR Ig-Mut-1 alone (Fig. 2.5B). This result was unexpected, as we predicted that the longer length crosslinker would provide greater physical freedom for the modified CR Ig-Mut-1 to bind C3b and thereby inhibit the AP. However, this appears not to have been the case. Although characterization of the CR Ig-Mut-1-PEG25 adduct shows some evidence of chemical heterogeneity (Fig. 2.2A), we suspect that its diminished activity more likely results from physical incompatibility with C3b-containing species at the erythrocyte surface. Complement-mediated lysis of erythrocytes requires formation of the TCC, which requires cleavage of C5 by C3b-containing C5 convertases (3, 4). Since the AP C5 convertase (i.e. C3b3bBb) contains two copies of C3b yet becomes a progressively more efficient enzyme upon greater surface densities of C3b (55, 56), it is possible that CR Ig-Mut-1 modified by an overly long crosslinker might not bind the convertase in an ideal orientation to inhibit C5 activation. Whereas a shorter length crosslinker may favor binding both copies of C3b within the same C5 convertase, a longer length crosslinker could promote binding

between adjacent convertases or even other C3b molecules deposited on the surface, reducing the apparent inhibitory potency. Much remains to be learned insofar as the structure and function of C5 convertases are concerned. Consequently, we think that CR Ig mutants crosslinked by PEGs of various lengths could be useful experimental tools for uncovering new structure/function information on C5 convertases. Considering the importance of these enzymes as therapeutic targets, we believe a continuation of these studies may be warranted.

Acknowledgement

X-ray diffraction data were collected at Southeast Regional Collaborative Access Team (SER-CAT) 22-ID beamline at the Advanced Photon Source, Argonne National Laboratory. A list of supporting institutions may be found on the SER-CAT website. Use of the Advanced Photon Source was supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-Eng-38.

Footnotes

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^{**}The refined coordinates and structure factors have been deposited in the RCSB Protein Data Bank (<http://wwpdb.org/>) under the accession codes 8TE5 and 8TE6.

Abbreviation

aHUS; atypical hemolytic uremic syndrome
AMD; age-related macular degeneration
AP; alternative pathway of complement
AS; active human serum
BSA; bovine serum albumin
CP; classical pathway of complement
CR1; complement receptor 1
CRIg; complement receptor of the Ig superfamily
CRIg-Mut-1; CRIg Ig-V domain mutant K15R/K36R/K62R/K72R/K110R
CRIg-Mut-2; CRIg Ig-V domain mutant K15R/K36R/K62Q/K72R/K110R
C3; complement component C3
C3b; complement component C3b
iC3b; complement component iC3b
FB; complement factor B
FI; complement factor I
FH; complement factor H
HBS; HEPES buffered saline
HBS-T; HEPES buffered saline with tween-20
HIS; heat-inactivated human serum
HSA; human serum albumin
Ig-V; variable-class Immunoglobulin repeat
LP; lectin pathway of complement
MG; macroglobulin-like domain
NHS; N-hydroxy-succinimide
PBS; phosphate buffered saline
PEG; polyethylene glycol
SCR; short consensus repeat domain
SPR; surface plasmon resonance
TBS-T; tris buffered saline with tween-20
TCC; terminal complement complex
THB; Todd Hewitt broth

Supplementary information

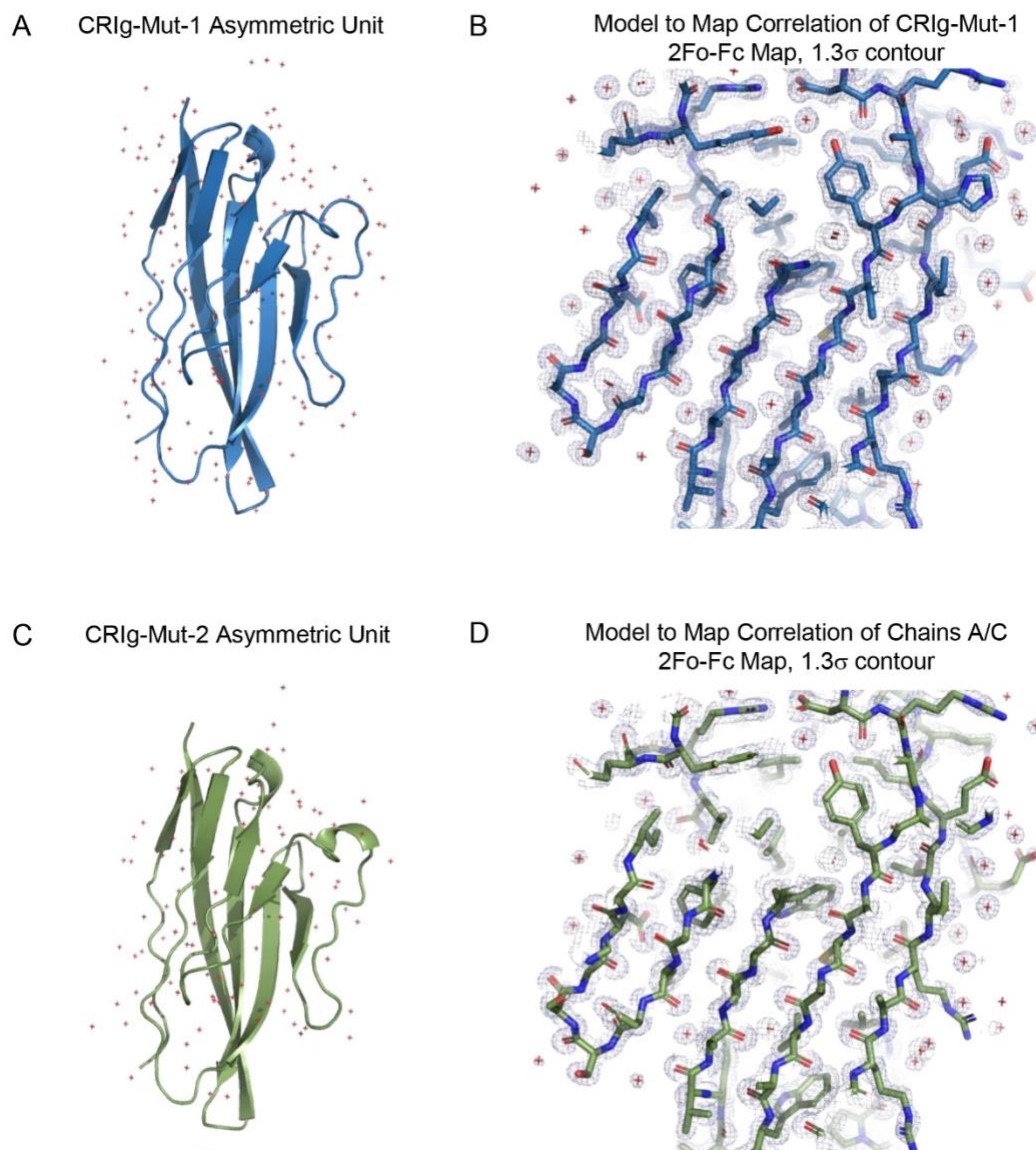


Figure S2.1. Supporting Information Pertaining to Crystal Structure Determinations of CRlg-Mut-1 and CRlg-Mut-2.

(A) Representation of the complete asymmetric unit for the CRlg-Mut-1 structure. The CRlg-Mut-1 protein is depicted as a ribbon diagram (blue), while ordered solvent molecules are shown as asterisks (red). (B) Correlation of the CRlg-Mut-1 model in ball-and-stick convention (carbon atoms in blue) with the final 2Fo-Fc electron density map (blue mesh) calculated at 1.25 Å limiting resolution and contoured at 1.3 σ . (C) Representation of the complete asymmetric unit for the CRlg-Mut-2 structure. The CRlg-Mut-2 protein is depicted as a ribbon diagram (green), while ordered solvent molecules are shown as asterisks (red). (D) Correlation of the CRlg-Mut-2 model in ball-and stick convention (carbon atoms in green) with the final 2Fo-Fc electron density map (blue mesh) calculated at 1.50 Å resolution and contoured at 1.3 σ .

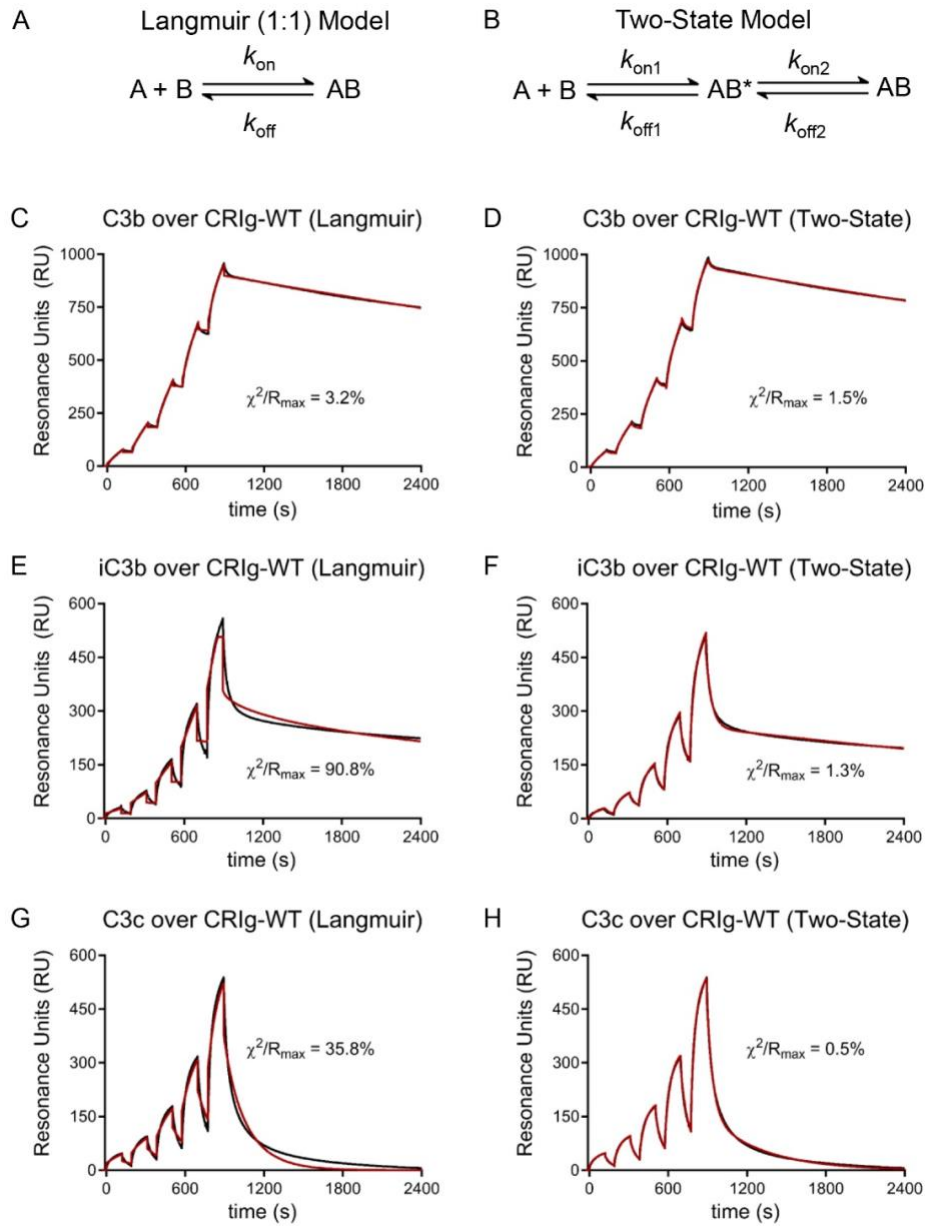


Figure S2.2. Supporting Information Pertaining to Direct Binding Studies of C3b, iC3b, and C3c to Surface-Captured His-Tagged CR1g Proteins: Part I. (A) Kinetic model used to evaluate binding of C3 fragments to his-tagged CR1g proteins. The Langmuir model incorporates a single association (k_{on}) and dissociation (k_{off}) rate constant. (B) Alternative kinetic model used to evaluate binding of C3 fragments to his-tagged CR1g proteins. The two-state model incorporates two sets of association and dissociation rate constants. (C) Single-cycle kinetic study of C3b injected over CR1g-WT captured on a sensor chip surface. Experimental data (black trace) were fit to a Langmuir model (red trace). (D) Identical experiment to panel C, except the experimental data (black trace) were fit to a Two-State model (red trace). (E) Single-cycle kinetic study of iC3b injected over CR1g-WT captured on a sensor chip surface. Experimental data (black trace) were fit to a Langmuir model (red trace). (F) Identical experiment to panel E, except the experimental data (black trace) were fit to a Two-State model (red trace). (G) Single-cycle kinetic study of C3c injected over CR1g-WT captured on a sensor chip surface. Experimental data (black trace) were fit to a Langmuir model (red trace). (H) Identical experiment to panel G, except the experimental data (black trace) were fit to a Two-State model (red trace). Note that values showing the quality of fit (χ^2) relative to the R_{max} for each experiment shown in panels C-H are inset.

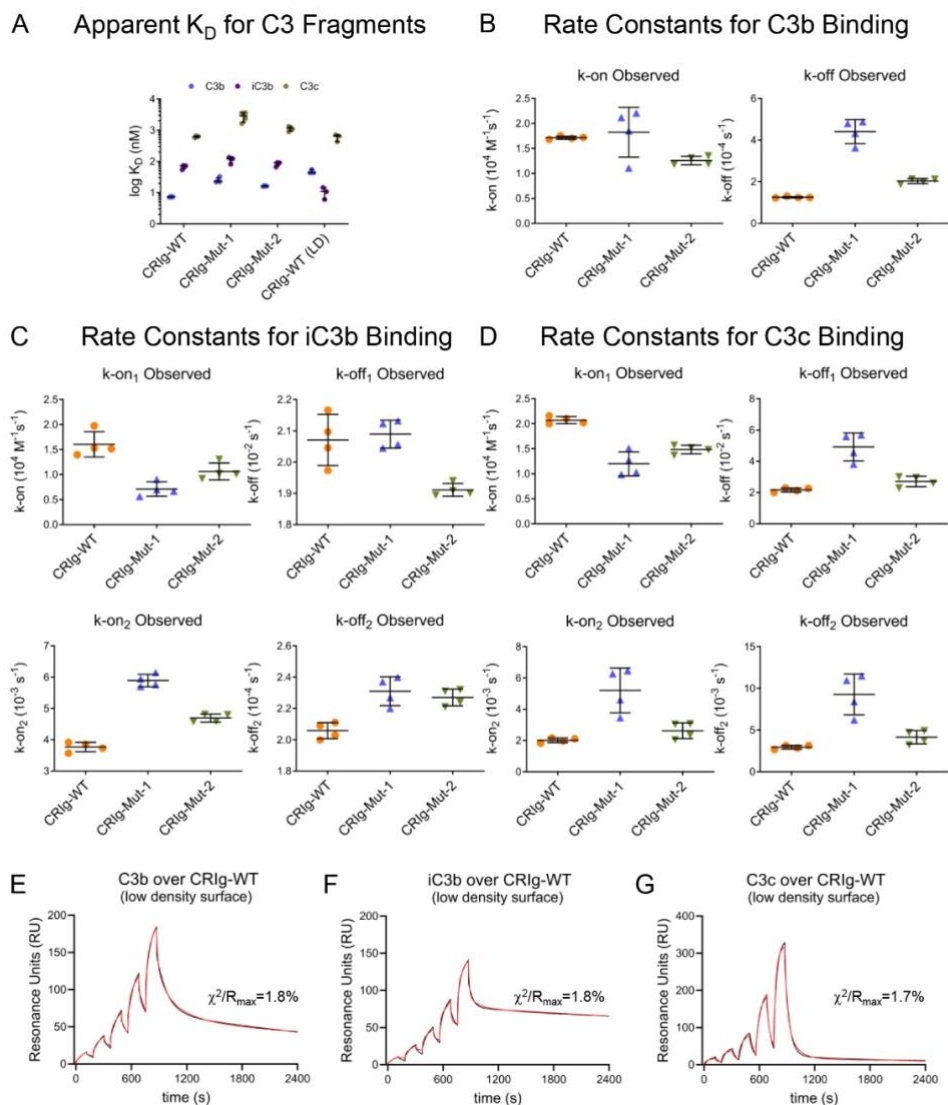
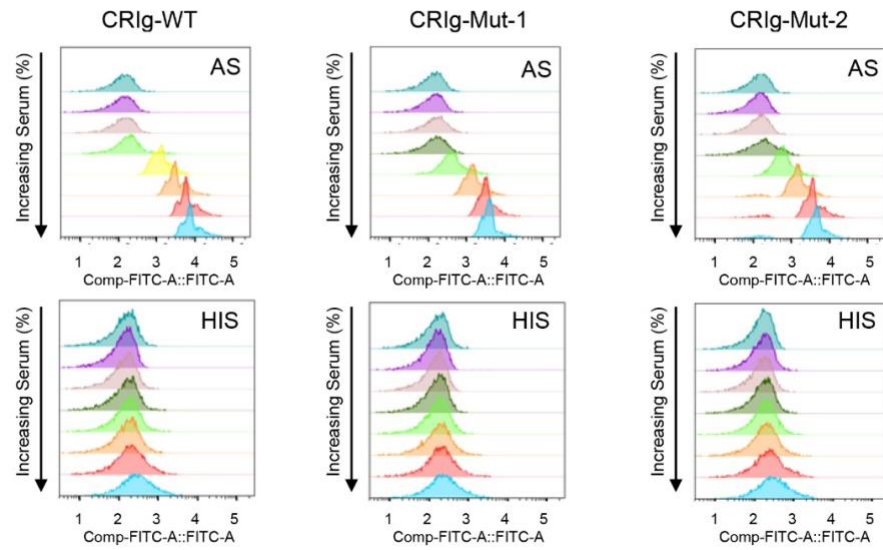


Figure S2.3. Supporting Information Pertaining to Direct Binding Studies of C3b, iC3b, and C3c to Surface-Captured His-Tagged CRiG Proteins: Part II. (A) Comparison of the apparent K_D values from replicate injections of C3b, iC3b, and C3c ($n = 4$) across all three forms of CRiG, as well as for CRiG-WT ($n = 3$) captured at a low surface density (CRiG-WT (LD)). Note the roughly 10-fold difference in affinity between C3b, iC3b, and C3c observed independently of the CRiG protein on the surface. While the apparent K_D for iC3b binding to CRiG-WT captured at a low density was tighter than for C3b alone, both C3b and iC3b still bound with at least 10-fold higher affinity than C3c. (B) Observed association and dissociation rate constants for C3b binding to his-tagged CRiG proteins from four technical replicates. Values were obtained following fitting to a Langmuir model. (C) Observed association and dissociation rate constants for iC3b binding to his-tagged CRiG proteins from four technical replicates. Values were obtained following fitting to a Two-State model. (D) Observed association and dissociation rate constants for C3c binding to his-tagged CRiG proteins from four technical replicates. Values were obtained following fitting to a Two-State model. Note that in panels B-D the black bars represent the mean plus or minus one standard deviation. (E) Single-cycle kinetic study of C3b injected over CRiG-WT captured at a low surface density on sensor chip surface. Experimental data (black trace) were fit to a Two-State model (red trace). (F) Single-cycle kinetic study of iC3b injected over CRiG-WT captured at a low surface density on sensor chip surface. Experimental data (black trace) were fit to a Two-State model (red trace). (G) Single-cycle kinetic study of C3c injected over CRiG-WT captured at a low surface density on sensor chip surface. Experimental data (black trace) were fit to a Two-State model (red trace).

A Binding of CRlg Proteins to *S. aureus* : Serum Dose-Dependence



B Binding of CRlg Proteins to *S. aureus* : CRlg Dose-Dependence

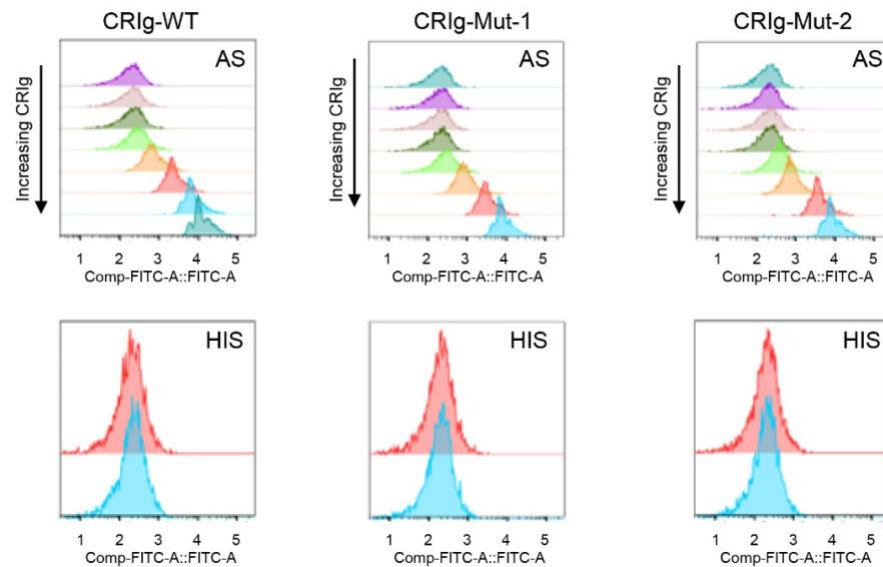


Figure S2.4. Supporting Information Pertaining to Binding of His-tagged CRlg Proteins to C3-Opsonized *S. aureus* Cells. (A) Cells of an *S. aureus* strain lacking Ig-binding activity were treated with a dilution series of human serum prior to assessment of his-tagged CRlg binding by flow cytometry (*please see Materials & Methods*). Representative flow cytometry analyses of his-tagged CRlg-WT, CRlg-Mut-1, and CRlg-Mut-2 binding to *S. aureus* cells treated with increasing concentrations of Normal Human Serum (AS) are shown in the three upper panels, while representative flow cytometry analyses of the same proteins binding to *S. aureus* cells treated with increasing concentrations of Heat Inactivated Normal Human Serum (HIS) are shown in the three lower panels. (B) Cells of an *S. aureus* strain lacking Ig-binding activity were treated with a fixed dilution of human serum (3% (v/v)), followed by incubation with increasing concentrations of his-tagged CRlg and assessment of binding by flow cytometry (*please see Materials & Methods*). Representative flow cytometry analyses of his-tagged CRlg-WT, CRlg-Mut-1, and CRlg-Mut-2 binding to *S. aureus* cells treated with AS are shown in the three upper panels, while the corresponding HIS-treated controls are shown in the three lower panels. Note that all panels in A and B have x-axis values given as $\log_{10}$ of observed fluorescence intensity.

Chapter 3 - The Inhibitory Effects of a Factor B-Binding DNA Aptamer Family Supersede the Gain-of-Function of Factor B Variants Associated with Atypical Hemolytic Uremic Syndrome[#]

This chapter has been published as a journal article in the Journal of Immunology

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Abstract

Aptamers are short, single-stranded oligonucleotides that selectively bind to target biomolecules. Although they generally exhibit good binding specificity, their affinities are often limited due to the relative lack of hydrophobic groups in nucleic acids. To address this obstacle, chemically modified nucleotides incorporating hydrophobic structures into uracil have been synthesized. Modified DNA aptamers containing such non-standard nucleotides have been developed for over twenty different complement proteins. These modified aptamers show increased affinity and enhanced serum stability and have potential value as therapeutic agents. We recently conducted a structure/function study on a family of modified DNA aptamers that bind specifically to complement factor B (FB). This work revealed that these aptamers selectively inhibit the complement alternative pathway (AP) by preventing the formation of the AP C3 proconvertase complex, C3bB. Certain patients with atypical hemolytic uremic syndrome (aHUS) express gain-of-function variants of FB that enhance the formation of the proconvertase complex and/or decrease the efficacy of endogenous regulators against the C3 convertases they form. To investigate whether these FB-binding aptamers could override the effects of disease-causing mutations in FB, we examined how they interacted with several FB variants, including D279G, F286L, K323E, and K350N, in various assays of complement function. We found that the inhibitory effect of the FB-binding aptamers superseded the gain-of-function mutations in FB, although the aptamers could not dissociate preformed C3 convertases. These findings suggest that FB-binding aptamers could be further developed as a potential treatment for certain aHUS patients or those with other diseases characterized by excessive complement activity.

Introduction

Complement is a critical component of the innate immune system that helps remove pathogens and damaged cells, clear circulating immune complexes, and promote downstream inflammatory reactions (1, 2). There are three canonical routes for complement activation known as the classical (CP), lectin (LP), and alternative (AP) pathways; although each pathway is triggered by distinct biochemical cues, they coalesce upon the activation of complement component C3 (C3) (58). When serving as an initiation route, the AP begins with spontaneous hydrolysis of the intramolecular thioester found in C3. Subsequent conformational change in hydrolyzed C3 (C3(H₂O)) allows it to bind the pro-protease Factor B (FB), generating a fluid-phase proconvertase complex (C3(H₂O)B); activation of this initial proconvertase by Factor D (FD) yields a fluid-phase C3 convertase (C3(H₂O)Bb) that can proteolytically cleave C3 into its bioactive fragments, C3a and C3b. Whereas C3a serves as a chemotactic molecule, C3b can covalently attach itself to nearby surfaces and biomaterial. This leads to the formation of the surface-bound AP C3 convertase (C3bBb) through an analogous process to that described above. Surface-bound C3 convertases are more efficient enzymes than their fluid-phase counterparts (80). This is due partly to the effects of the positive regulator, properdin (FP), which promotes formation of surface-bound convertases and increases their stability (81). Moreover, localized generation of high C3b levels on surfaces facilitates self-amplification of the AP (82, 83). Thus, the AP provides the primary means of complement amplification under most settings (84, 85). Indeed, one well-known study has suggested that the AP contributes over 80% of downstream C5 activation products when complement is initiated by the CP (84).

The self-amplifying nature of the AP requires that its activity be strictly controlled to avoid damaging otherwise healthy cells and tissues. This is achieved through conceptually distinct yet

ultimately overlapping mechanisms (1, 2, 58). On one hand, the AP C3 convertase itself is intrinsically labile and dissociates irreversibly with a half-life of approximately one minute under physiological conditions (80). On the other hand, a dedicated suite of soluble and cell surface-exposed proteins act as negative regulators of AP activity (86). These so-called Regulators of Complement Activation (RCA) accelerate the irreversible decay of preformed convertases into their individual components. They also act as cofactors for Factor I (FI), which cleaves C3b into iC3b and C3c, neither of which support the formation of new AP C3 convertases. Among the RCA proteins are Factor H (FH), which, though soluble, binds to host cells through sialylated carbohydrates, as well as Decay Accelerating Factor (DAF) and Membrane Cofactor Protein (MCP), which are membrane-spanning glycoproteins. Together, these intrinsic and extrinsic regulatory mechanisms help shield host cells from indiscriminate complement attack (26).

Atypical hemolytic uremic syndrome (aHUS) is a rare, chronic, and potentially life-threatening thrombotic microangiopathy characterized by thrombocytopenia, hemolytic anemia, and acute kidney injury (87). Whereas typical HUS is often triggered by Shiga toxin-producing bacterial infections, the central pathogenic mechanism in aHUS is dysregulation of the complement AP (88, 89). Mutations in several RCA proteins are most commonly associated with the development of aHUS, and have highlighted the role of excessive AP activity in pathogenesis of this disease (90-92). Several mutations in FH have been identified that diminish binding of this regulator to endothelial cells, thereby resulting in insufficient complement regulation at the cell surface (93-95). Mutations in MCP (CD46) that disrupt its C3b-binding and cofactor activities have also been described (96). Finally, mutations in FI have also been reported that either lead to loss of its proteolytic activity toward C3b or lack of FI protein altogether (97). While these examples describe loss-of-function in negative regulators of the AP, gain-of-function mutations in

other AP components have also been linked to aHUS. Several mutations in C3 have been identified that lead to reduced binding to RCA proteins, including FH and MCP (98). aHUS-associated mutations have also been described in FB (99). Although these are rare and account for less than 2% of all aHUS patients (92), molecular analyses of these disease-linked FB variants have revealed interesting features including an enhanced rate of formation of the C3 proconvertase (i.e. C3bB) and/or formation of a fully active C3 convertase (i.e. C3bBb) that is resistant to both intrinsic and regulatory decay (100-102).

The treatment of aHUS has evolved significantly over the years. The initial first-line therapy was plasma treatment, which is based upon plasma infusion and exchange (103). This method aimed to supply functional complement regulators and to remove abnormal complement components. However, the introduction of eculizumab - a monoclonal antibody that directly binds with C5, prevents C5 cleavage, and the formation of the terminal complement complex - revolutionized aHUS treatment (104). Eculizumab showed high efficacy and safety and quickly became the gold standard for managing aHUS (105, 106). Whereas terminal complement inhibition via eculizumab is effective for treating aHUS resulting from a variety of molecular lesions, including gain-of-function mutation in FB, one clinical study reported ongoing C5 cleavage and terminal pathway activation in a patient with an FB mutation after receiving eculizumab (107). An additional concern with anti-C5 treatment is its lack of complement pathway selectivity, that can reduce the overall protection against bacterial infection (103), although this can be managed through preventative vaccination against problematic species like *Neisseria*. Nevertheless, the recent regulatory approvals of molecules targeting upstream complement components have renewed interest in the use of proximal complement inhibitors for the treatment

of aHUS. Indeed, the small-molecule FB inhibitor iptacopan is currently undergoing a phase III trial for this indication (108).

We previously characterized a class of FB-binding modified DNA aptamers that are potent inhibitors of the AP (47). These aptamers bind directly to FB, prevent its interaction with C3b, and thereby block the formation of the C3 proconvertase complex, C3bB (47). In turn, this leads to decreased levels of the enzymatically active C3 convertase, C3bBb (47). Since certain FB variants linked to aHUS have been shown to increase the formation rate of the C3 convertase and/or generate C3 convertases resistant to decay, we considered whether the inhibitory effect of these FB-binding aptamers could counteract the gain-of-function caused by these mutations in FB. In this study, we tested this concept using a panel of FB mutants, all of which showed either an increased affinity for C3b, an increased rate of proconvertase formation, and/or an increased stability of the active C3 convertase (100-102). Our findings demonstrate that the FB-binding modified DNA aptamers effectively blocked these gain-of-function FB mutants from binding to C3b, thereby preventing the formation of the C3 convertase and counteracting the increased complement activity caused by these gain-of-function mutations. Our results suggest that it should be possible to use these FB-binding aptamers therapeutically in a subset of aHUS patients who express gain-of-function variants in FB. Considering their potency, use of these aptamers could also be extended to other disease settings where excessive complement amplification via the AP is a contributing factor (62).

Materials and Methods

Protein Samples

Samples of complement components FB, FD, FH, FP, and C3b that were purified from human serum were obtained commercially from Complement Technologies.

Plasmids for expression of recombinant wild-type and mutant forms of FB were obtained from GeneScript. DNA fragments encoding proteins of interest were synthesized with a C-terminal octahistidine-tag and then subcloned into pcDNA3.1+. Sufficient plasmid for large-scale transient transfection was prepared using a QIAGEN Plasmid Maxi kit. Individual expression plasmids were transiently transfected into Human Embryonic Kidney cells (HEK293T) using polyethyleneimine (PEI) MAX according to the general methods described previously (109). Following transfection, approximately 1 L of conditioned cultured medium was collected and concentrated by tangential flow filtration to a volume of 150 mL. The buffer was then exchanged into native binding buffer (20 mM Tris [pH 8.0], 20 mM imidazole, 500 mM NaCl) in preparation for immobilized metal ion affinity chromatography. The sample was applied to a 5-mL HisTrap HP column using an AKTA FPLC system (Cytiva Life Sciences). Following extensive washing using native binding buffer, the bound protein was eluted using a linear gradient to full-strength native elution buffer (20 mM Tris [pH 8.0], 500 mM imidazole, 500 mM NaCl) while collecting 1 mL fractions. Fractions containing recombinant FB protein were collected, concentrated, and further purified by gel filtration chromatography on a Superdex 75 26/60 column using HEPES-buffer saline (HBS; 20 mM HEPES [pH 7.40], 140 mM NaCl) as a mobile phase. The column eluent was collected in 2 mL fractions, which were subsequently analyzed by SDS-PAGE. Fractions containing purified, recombinant FB were pooled, concentrated, quantitated, and stored at either 4°C or -80°C until further use.

Aptamer Synthesis

The modified, 31-base DNA aptamer SL1100 was used for all studies described here (47). Its sequence is 5'-dC-dG-dC-2NEdU-dG-dA-dG-dA-dA-2NEdU-dA-dG-dA-dA-dG-2NEdU-dA-dG-dA-dG-2NEdU-dA-2NEdU-dG-dC-2NEdu-2NEdU-dG-dC-dG-3'. SL1100 was synthesized by standard DNA phosphoramidite methods as described previously (110). The phosphoramidite for incorporating 5-(N-2-naphthylethylcarboxamide)-29-deoxyuridine (2NEdU) was likewise prepared according earlier publications (111, 112). Following synthesis, SL1100 was purified using ion-pairing reversed-phase liquid chromatography, desalted using a Hi-Trap Sephadex G-25 column, and evaporated to dryness. The purified SL1100 product was analyzed using ultra-performance liquid chromatography and its identity was confirmed using MALDI-TOF mass spectrometry. A form of SL1100 that was used for FB-binding studies, which incorporated a 5'-modified base containing both a polyethylene glycol linker and a biotin moiety, was also prepared using the methods outlined above.

Surface Plasmon Resonance Binding Studies

Surface plasmon resonance (SPR) experiments were performed on a BiaCore T-200 instrument at 25°C using HBS-T (20 mM HEPES [pH 7.4], 140 mM NaCl, 0.005% [v/v] Tween 20, and 10 mM MgCl₂) as the running buffer and a flow rate of 30 µL/min. A streptavidin-derivatized SAD200L chip (Xantec Bioanalytics; Dusseldorf, Germany) was used to capture human C3b that had been site-specifically biotinylated at Cys-1010 via EZ-link Maleimide-PEG2-Biotin (Thermo Fisher Scientific) according to a previously published method (113). A reference surface was prepared by 3 injections of biotin alone (10 nM) on one flow cell (fc1). Replicate experimental surfaces containing 2158 (fc2), 2150 (fc3), and 2027 (fc4) RU of C3b-biotin were prepared thereafter by injecting dilute solutions of C3b-biotin (8 ng/ml) across the remaining flow cells. Binding of various forms of FB to C3b was assessed by injecting a buffer blank, followed

by a 2-fold dilution series of FB across seven different concentrations spanning 15.625 nM to 1 μ M. Each injection cycle consisted of a 1-minute association phase, a 2-minute dissociation phase, and three consecutive pulses of a mildly acidic, high salt regeneration buffer (10 mM sodium acetate [pH 4.0], 2M NaCl).

The binding of various forms of FB to 5'-biotinylated SL1100 aptamer was also studied using a separate SAD200L chip. A reference surface was prepared using the method described above (fc1), while biotinylated SL1100 was immobilized on experimental flow cells with an initial capture level of 59 (fc2) 59 (fc3), and 34 (fc4) RU. Interactions were assessed using single-cycle kinetics with 5 concentrations of FB (i.e. 62.5 nM, 125 nM, 250 nM, 500 nM, and 1000 nM). Surfaces were regenerated between analyte series using three consecutive pulses of 4 M MgCl_2 , and the experimental flow cells were reloaded with biotinylated SL1100 as described above. The performance of the surface across multiple analytes was assessed by injecting the same analyte (i.e. FB-K350N) at the beginning and end of the entire experiment. These internal standard data showed excellent agreement with one another, yielding apparent K_D values of 5.9 ± 2.8 and 7.0 ± 4.4 for the initial and final injections, respectively.

Reference-subtracted sensorgrams from all experiments were analyzed using Biacore T-200 Evaluation software. Data were fitted to a two-state reaction model to derive an apparent K_D using a mechanism that incorporates a single second-order rate constant (i.e. K_{a-1}) and three first-order rate constants (i.e. K_{a-2} , K_{d-1} , and K_{d-2}). Values from fits are presented as the mean plus or minus the standard deviation from 3 technical replicates (Table 3.1).

SPR Competition Binding Studies

Competition binding assays were conducted on the same C3b-immobilized experimental surface prepared above using a method described previously (47). A two-fold dilution series of

various forms of FB (spanning seven different concentrations from 15.625 nM to 1 μ M), either in the absence or in the presence of an increasing concentration of SL1100, was injected over the surface. Analysis of the reference-corrected sensorgram series was carried out as described above. Data were normalized by assigning a value of 100% to the signal resulting from the injection of the competitor-free, 1 μ M FB sample immediately before the end of the association phase. Each experimental flow cell was normalized independently to account for the slight differences in C3b density. All plots were generated using GraphPad Prism v10.0.0.

SPR Measurements of Alternative Pathway C3 Convertase Formation and Decay

The AP C3 convertase formation assay was performed using the same surface described above using a previously described SPR-based method (114). Each experiment consisted of two separate injection events: the first injection of a 100 nM mixture of FD and FB occurred for 1 min, followed by a 15-second dissociation phase and a brief stabilization period, whereas the second injection occurred for 1 min, followed by a 2-min dissociation phase. For the second injection, HBS-T was used as a negative control, whereas FH served as a positive control. 3 concentrations (100 nM, 500 nM, and 1000 nM) of SL1100 aptamer were injected to test for any potential effects on convertase stability and/or decay. Regeneration of the surface between experimental cycles was achieved by three consecutive pulses of a mildly acidic, high salt buffer (10 mM sodium acetate [pH 4.0], 2M NaCl).

Additional SPR experiments were conducted to investigate the effects of FP on AP C3 convertase formation and decay. These studies were performed using the same general methodology described above, with the exception that streptavidin-modified SPR surfaces were generated by coupling NeutrAvidin (ThermoFisher Scientific) to a CMD200 sensor chip (Xantec Bioanalytics) as previously described (113). The reference surface, fc1, was prepared by 3

injections of biotin (10 nM), while experimental surfaces were prepared by injecting C3b-biotin (5 ng/ml) to achieve 978 RU on fc2 and 970 RU on fc3. FP (100 nM) was injected for 90 sec over fc2 to saturate the surface prior to any experimental injections over other flow cells. Each experiment was comprised of two sequential injection events. The first injection consisted of a 100 nM mixture of FB and FD lasting for 1 min, and was followed by a 3 min dissociation phase. The second injection consisted of 100 nM FH lasting for 1 min, and was followed by a 3 min dissociation phase. Regeneration of the surface between experimental cycles was achieved by three consecutive pulses of a mildly-acidic, high salt buffer (10 mM sodium acetate [pH 4.0], 2M NaCl). This same general procedure was modified to assess binding differences of various FB and FB/SL1100 complexes in the presence or absence of FP. In those experiments, the only experimental injection consisted of either 250 nM FB or a solution of FB/SL1100 containing 250 nM of each component. Surface regeneration between experimental cycles was achieved using three consecutive pulses of 2M NaCl, 10 mM EDTA.

Hemolytic Assays for Alternative Pathway Activity

AP hemolysis assays were conducted in a 100 μ L reaction mixture consisting of 12 μ L FB-depleted serum (Complement Technologies), 53 μ L of 0.1% (w/v) gelatin veronal buffer (GVB; Complement Technologies), 10 μ L of 0.1 M MgEGTA (Complement Technologies), 5 μ L of FB solution in GVB to achieve desired concentration, and 20 μ L of rabbit erythrocytes (3×10^8 /mL in GVB; Complement Technologies). Following a 30-min incubation at 37°C, samples were centrifuged for 10 min at 200 $\times g$, the supernatant was diluted 4-fold in ddH₂O, and the absorbance was determined at 412 nm. The percentage of lysis was determined by the following equation: $[100 \times (\text{OD}_{412, \text{ sample}} - \text{OD}_{412, \text{ buffer}}) / (\text{OD}_{412, \text{ water}} - \text{OD}_{412, \text{ buffer}})]$, and the EC₅₀ was determined by a four-parameter fit using GraphPad Prism v10.0.0.

A similar procedure was employed for studying inhibition by SL1100, except that the concentrations of the 5 μ L FB stock solutions added were adjusted to give the desired final concentration (i.e. 20 nM and/or 60 nM), and the GVB buffer volumes were reduced to allow for 5 μ L SL1100 stock solution to achieve the final inhibitor concentration. The percentage of lysis inhibition was determined by the following equation: $100 - [100 * (OD_{412, \text{sample}} - OD_{412, \text{buffer}} / (OD_{412, \text{water}} - OD_{412, \text{buffer}}))]$. The IC_{50} was determined by a four-parameter fit using GraphPad Prism v10.0.0.

Finally, an analogous protocol was also employed for comparing inhibition of hemolysis by SL1100 in either normal human serum (NHS; Innovative Research) or FP-depleted serum (FP-Dep; Complement Technologies). In these experiments, the final concentration of both sera was increased to 20% (v/v). The reaction using FP-Dep was also incubated at 37°C for 3 h, as opposed to 30 min for NHS. The inhibition percentage of lysis was determined as described above.

Results

Generation and Characterization of aHUS-associated FB mutants

FB is a 93 kDa, single-chain glycoprotein comprised of two distinct functional regions (Fig. 3.1A): the N-terminal Ba region consists of three consecutive complement control protein (CCP) domains followed by a 40-amino acid linker region, while the C-terminal Bb region consists of a metal-binding von Willebrand factor (vWF) domain followed by a serine protease (SP) domain (115). By contrast, the FB-binding aptamers described previously are approximately 11 kDa and are between 29 to 31 bases in length (47). Crystallographic analysis of two members of this aptamer family, SL1102 and SL1103, bound to human FB revealed that these aptamers bind FB at a groove-like surface lying at the juncture of the CCP1 and CCP3 domains from Ba and the vWF domain from Bb (Fig. 3.1B). Approximately half of the FB-associated surface area in these

complexes is derived from the CCP1 domain with the remainder divided equally between the CCP3 and vWF domains; however, these aptamers do not bind well to either the isolated Ba or Bb regions themselves. Nevertheless, because the Ba region is responsible for the initial interaction of FB with C3b, binding of these aptamers at this site inhibits the formation of the C3 proconvertase through a steric hindrance-type mechanism (Fig. 3.1C; (47)).

Several gain-of-function forms of FB associated with aHUS have been described. These variants result in excessive AP activity by increasing the rate of formation and/or overall stability of the C3 convertases they form (100-102). Since many of these aHUS-linked mutations map to FB regions outside the aptamer binding site (Fig. 3.1B), we hypothesized that these specific mutations would not diminish aptamer binding to these FB variants. To test this possibility, we first designed expression vectors to produce a panel of FB mutants for further studies. We focused on four single mutations, FB-D279G, FB-F286L, FB-K323E, and FB-K350N, identified in aHUS patients (100-102, 116). We also designed a combinatoric double mutant, FB-D279G/K350N (FB-2Mut); although this variant has not been described in any aHUS patient, we sought to test it for any synergistic effects that might arise from incorporating two gain-of-function mutations simultaneously. Finally, we created a triple mutant, FB-D279G/K350N/S699A (FB-3Mut), to serve as a negative control since this variant lacked the catalytic residue within the serine protease domain (i.e. S699). We expressed wild-type FB (FB-WT) and all six FB mutants in transfected HEK293T cells and purified the recombinant proteins from conditioned culture medium. Each form of recombinant FB maintained its integrity and displayed a molecular weight comparable to FB purified from human serum (FB-Native), as judged by SDS-PAGE (Fig. 3.1D).

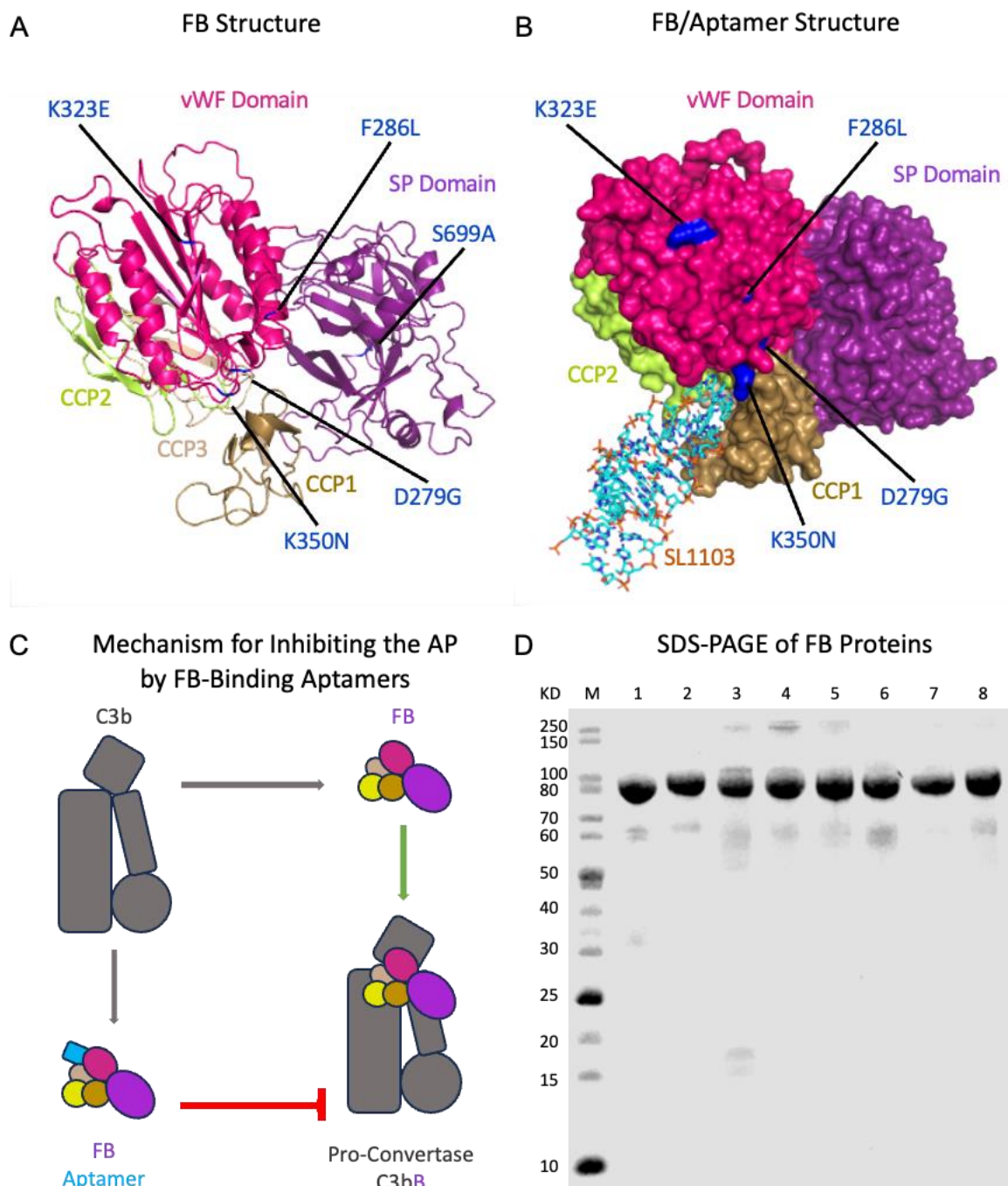


Figure 3.1 Description and Characterization of Gain-of-Function Variants in FB.

(A) The structure of human FB as drawn from the PDB entry 7JTN is shown as a ribbon diagram. The individual domains of FB are colored as follows: CCP1, sand; CCP2, yellow; CCP3, tan; vWF, magenta; SP, purple. (B) Structure of the modified DNA aptamer, SL1103, bound to human FB as drawn from the PDB entry 7JTN. SL1103 is shown in ball-and-stick convention with carbon atoms colored teal. FB is shown as a molecular surface with domains colored identically to panel A. The locations of residues D279, F286, K323, and K350 are highlighted in blue. Missing

residues were added via Modeller, using the FB structure from PDB entry 7JTN as a starting point. Note that the CCP3 domain and S699 are not visible in this orientation. (C) Illustrated mechanism for AP inhibition by members of a FB-binding aptamer family. These aptamers block C3 proconvertase formation by occupying a site normally required for FB binding to C3b. (D) Samples of serum-purified and recombinant FB proteins were processed under reducing conditions, separated by SDS-PAGE, and visualized by Coomassie Blue staining. Lane M, molecular mass marker; lane 2, FB-Native; lane 3, FB-WT; lane 4, FB-D279G; lane 5, FB-F286L; lane 6, FB-K323E; lane 7, FB-K350N; lane 8, FB-2Mut; lane 9, FB-3Mut.

To test whether these FB variants exhibited increased affinity to C3b, we characterized the interaction of each FB protein with site-specifically biotinylated C3b that had been captured on an SPR surface (Fig. 3.2, Fig. S3.1A, & Table 3.1). As has been reported previously (58, 117, 118), we employed a two-state binding model to evaluate FB/C3b binding and determined the apparent affinity for each form of FB. Both serum-isolated FB-Native and recombinant FB-WT exhibited similar affinities of 109 nM and 180 nM, respectively (Fig. 3.2A-B). The apparent affinities of FB-D279G and FB-K323E were likewise within a similar range as FB-Native and FB-WT at 71 nM and 159 nM, respectively (Fig. 3.2C & 3.2E). FB-F286L displayed a higher apparent affinity for C3b of 9 nM (Fig. 3.2D), which was driven primarily by an enhanced association rate with C3b (Fig. S3.1A & Table 3.1). In contrast, FB-K350N displayed a weaker apparent affinity for C3b of 322 nM (Fig. 3.2F). Both the FB-2Mut and FB-3Mut variants exhibited a similar affinity to FB-D279G at 88 nM and 104 nM, respectively (Fig. 3.2G-H). This observation suggested that the effect of the D279G mutation was dominant to that of K350N. Furthermore, it also revealed that there was also no synergistic effect between these two mutations insofar as C3b binding was concerned.

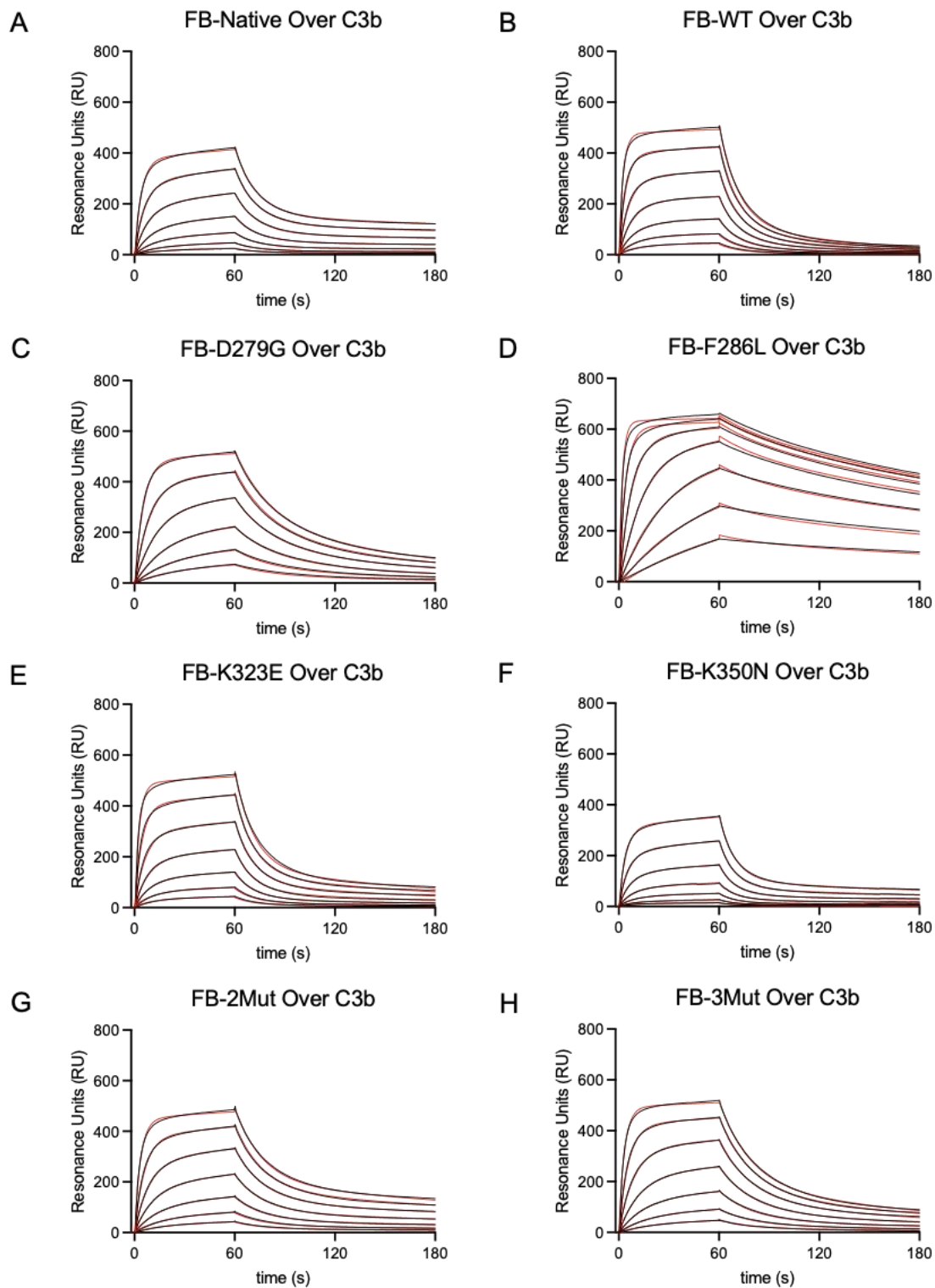


Figure 3.2 Direct Binding Studies of Various FB Proteins to C3b.

Site-specifically biotinylated C3b was captured on three experimental flow cells of streptavidin-coated biosensor, leaving one flow cell unmodified for reference subtraction. Protein-protein

interactions were monitored by injecting increasing concentrations of FB in multi-cycle mode, where 1 μ M was used as the highest concentration. Reference-subtracted sensorgrams (black lines) were analyzed using a two-state kinetic model (red lines). (A) Binding of FB-Native to C3b. (B) Binding of FB-WT to C3b. (C) Binding of FB-D279G to C3b. (D) Binding of FB-F286L to C3b. (E) Binding of FB-K323E to C3b. (F) Binding of FB-K350N to C3b. (G) Binding of FB-2Mut to C3b. (H) Binding of FB-3Mut to C3b. For panels A-H, the data shown represents one of three independent replicates.

The FB-binding Aptamer SL1100 Binds Gain-of-Function Forms of FB with Nanomolar Affinity

With a panel of aHUS-linked forms of FB now available, we examined whether members of the previously described family of FB-binding DNA aptamers retained the ability to bind these forms of FB (47). To test this possibility, we first synthesized a 5'-biotinylated version of the modified DNA aptamer, SL1100 (47). SL1100 is a 31-base, single-stranded DNA oligonucleotide that contains seven 5-(N-2-naphthylethylcarboxamide)-2'-deoxyuridine (2NEdU) residues in lieu of deoxythymidine (47); it is closely related to aptamers SL1102 and SL1103, which have been crystallized bound to FB, although it lacks the DNA backbone 2'-O-methylation at certain positions designed to increase serum-stability of SL1102 and SL1103 (47). We then characterized the interaction of each FB protein with biotinylated SL1100 that had been captured on an SPR surface using single-cycle kinetics (Fig. 3.3, Fig. S3.1B, & Table 3.1). We again used a two-state binding model to analyze the experimental sensorgrams and obtained two sets of association and dissociation rate constants, along with an apparent affinity. Significantly, we found that each form of FB displayed an apparent affinity for SL1100 in the range from 4.1 nM to 14.6 nM (Table 3.1). Although serum-isolated FB-Native had a higher maximal response value than expected (i.e. R_{max}), all recombinant forms of FB studied here, including FB-WT, had a similar R_{max} value to the others (Table 3.1). Together, these data supported our hypothesis by showing that SL1100 retained high-affinity binding to these aHUS-linked variants in FB.

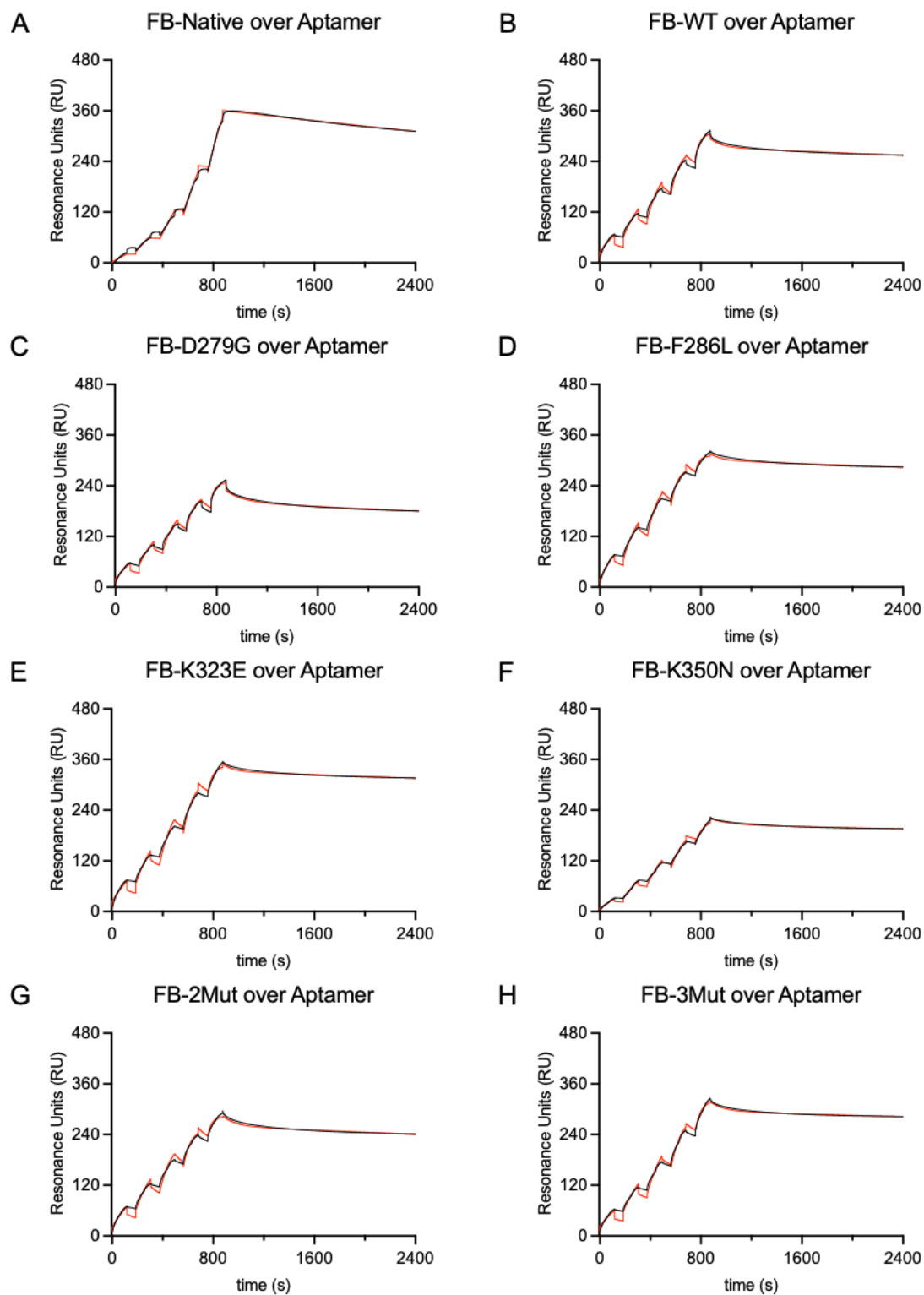


Figure 3.3 Direct Binding Studies of Various FB Proteins with Modified DNA Aptamer SL1100.

5'-biotinylated SL1100 was captured on three experimental flow cells of a streptavidin-coated biosensor, leaving one flow cell unmodified for reference subtraction. Protein-aptamer interactions were monitored by injecting increasing concentrations of FB in single-cycle mode, where 1 μ M was used as the highest concentration. Reference-subtracted sensorgrams (black lines) were analyzed using a two-state kinetic model (red lines). (A) Binding of FB-Native to SL1100. (B) Binding of FB-WT to SL1100. (C) Binding of FB-D279G to SL1100. (D) Binding of FB-F286L to SL1100. (E) Binding of FB-K323E to SL1100. (F) Binding of FB-K350N to SL1100. (G) Binding of FB-2Mut to SL1100. (H) Binding of FB-3Mut to SL1100. For panels A-H, the data shown represents one of three independent replicates.

Table 3.1 Interaction parameters of various FB proteins with immobilized C3b or SL1100a,b

Analyte	Biotinylated-Ligand	K_{a-1} ($10^4 \text{ M}^{-1} \text{ s}^{-1}$)	K_{d-1} (10^{-3} s^{-1})	K_{a-2} (10^{-3} s^{-1})	K_{d-2} (10^{-4} s^{-1})	K_D (nM)	Rmax (RU)
FB-Native	C3b	16.8 $\pm$ 1.1	70.2 $\pm$ 0.7	7.1 $\pm$ 0.2	24.9 $\pm$ 2.4	109 $\pm$ 15	579 $\pm$ 38
	SL1100	5.2 $\pm$ 1.2	0.1 $\pm$ 0.0	2.1 $\pm$ 2.7	89.2 $\pm$ 82.5	14.6 $\pm$ 7.5	651 $\pm$ 126
FB-WT	C3b	32.5 $\pm$ 1.2	79.6 $\pm$ 5.0	5.0 $\pm$ 0.6	138.3 $\pm$ 10.2	180 $\pm$ 7	641 $\pm$ 23
	SL1100	24.2 $\pm$ 7.4	3.9 $\pm$ 1.4	5.2 $\pm$ 1.1	2.4 $\pm$ 1.2	7.3 $\pm$ 4.0	270 $\pm$ 35
FB-D279G	C3b	30.4 $\pm$ 1.7	37.6 $\pm$ 1.5	7.0 $\pm$ 0.7	94.1 $\pm$ 4.4	71 $\pm$ 1	659 $\pm$ 33
	SL1100	26.2 $\pm$ 3.4	2.7 $\pm$ 0.7	2.7 $\pm$ 1.0	2.3 $\pm$ 0.6	8.3 $\pm$ 3.2	228 $\pm$ 9
FB-F286L	C3b	55.1 $\pm$ 7.3	22.9 $\pm$ 9.6	28.9 $\pm$ 8.5	83.0 $\pm$ 6.5	9 $\pm$ 0	710 $\pm$ 42
	SL1100	25.1 $\pm$ 9.8	2.4 $\pm$ 2.5	3.5 $\pm$ 2.8	1.7 $\pm$ 0.7	4.1 $\pm$ 1.8	240 $\pm$ 90
FB-K323E	C3b	28.0 $\pm$ 1.6	80.7 $\pm$ 4.2	5.9 $\pm$ 0.5	72.3 $\pm$ 9.2	159 $\pm$ 4	690 $\pm$ 28
	SL1100	18.3 $\pm$ 6.4	2.4 $\pm$ 2.2	4.5 $\pm$ 2.6	2.5 $\pm$ 1.5	6.0 $\pm$ 3.3	270 $\pm$ 99
FB-K350N	C3b	13.1 $\pm$ 0.5	99.5 $\pm$ 3.7	5.7 $\pm$ 0.1	41.9 $\pm$ 2.6	322 $\pm$ 11	594 $\pm$ 27
	SL1100	14.7 $\pm$ 1.9	1.2 $\pm$ 0.4	3.2 $\pm$ 1.4	2.4 $\pm$ 1.1	5.9 $\pm$ 2.8	229 $\pm$ 60
FB-2Mut	C3b	22.5 $\pm$ 0.9	52.6 $\pm$ 1.6	7.4 $\pm$ 0.2	45.0 $\pm$ 3.8	88 $\pm$ 4	607 $\pm$ 30
	SL1100	26.0 $\pm$ 6.4	2.8 $\pm$ 1.6	2.9 $\pm$ 1.9	2.4 $\pm$ 1.2	9.1 $\pm$ 5.7	207 $\pm$ 78
FB-3Mut	C3b	25.6 $\pm$ 0.8	46.9 $\pm$ 1.9	6.3 $\pm$ 0.5	83.6 $\pm$ 6.3	104 $\pm$ 2	620 $\pm$ 31
	SL1100	15.8 $\pm$ 4.1	2.2 $\pm$ 1.0	3.7 $\pm$ 1.9	1.7 $\pm$ 0.8	6.9 $\pm$ 4.1	273 $\pm$ 65

^aAll values are presented as the mean $\pm$ standard deviation obtained for a minimum of three replicate experiments.

^bValues result from fitting to a two-state binding model.

SL1100 Blocks C3 Proconvertase Formation by Gain-of-Function Variants of FB but Does Not Affect C3 Convertase Stability

Our earlier work established that the FB-binding aptamer family typified by SL1100 inhibits the AP by blocking the formation of the C3 proconvertase complex, C3bB (Fig. 3.1C; (47)). However, most of the aHUS-associated FB variants described in previous publications exhibit increased affinity for C3b and/or increase the stability of the AP C3 convertases they form

(100-102); Fig 3.2 & Table 3.1). Therefore, we considered whether the high-affinity binding by SL1100 could supersede the functional consequences of these FB mutations. To investigate this possibility, we employed two different SPR-based methods that monitor the formation of the C3 proconvertase and the stability of the already formed C3 convertase, respectively (47, 114).

We initially examined whether SL1100 could effectively block C3b-binding by the gain-of-function forms of FB (Fig. 3.4). We monitored FB binding to site-specifically immobilized C3b across a dilution series consisting of seven different FB concentrations, and as a function of increasing molar ratios of SL1100 competitor relative to FB (i.e. no competitor, 0.25:1, 0.5:1, 0.75:1, and 1:1 stoichiometry; (47)). Following normalization of the signal for each data point relative to the signal generated by the highest concentration of FB in the absence of any competitor, we plotted the residual SPR signal as a function of FB concentration and the level of SL1100 present. We found that increasing concentrations of SL1100 resulted in progressively greater inhibition of FB binding to C3b. Regardless of the specific mutation under investigation, we observed that SL1100 could completely block FB binding to C3b when present at equimolar concentrations. Together, these results showed that SL1100 reduced the effective concentration of FB, thereby inhibiting C3 proconvertase formation by each of the FB variants available.

We then explored whether SL1100 could affect the stability of pre-formed C3 convertases generated with each FB variant (Fig. 3.5). We used an SPR-based assay to monitor C3 convertase formation and decay in real time (114). In this first phase of such experiments, a mixture of FB and FD are injected over captured C3b-biotin to generate the C3 convertase; following a brief period wherein the rate of intrinsic C3 convertase decay is measured, the second phase is begun by injecting proteins (e.g., FH) or other molecules to determine if they accelerate the rate of C3 convertase decay. We validated this approach by showing that both serum-purified (i.e., FB-

Native) and recombinant FB (i.e., FB-WT) formed transiently stable C3 convertases whose decay was significantly accelerated by injecting FH over each surface (Fig. 3.5A-B). However, we did not detect appreciable decay-accelerating activity when we injected SL1100 at 100 nM, 500 nM, or 1000 nM instead of FH over C3 convertases formed by FB-Native or FB-WT.

We conducted analogous experiments on the remaining FB variants in our panel. Although FB-D279G displayed only a ~2.5-fold increase in affinity for C3b (Table 3.1), we found that its fully formed C3 convertase was significantly more stable than that of FB-WT (Fig. 3.5C). The FB-D279G convertase was only mildly decayed by FH, and not at all when SL1100 was injected instead. By contrast, the increased rate of proconvertase formation seen for FB-F286L did not translate into an increased level of convertase stability, as we found that its fully formed C3 convertase decayed faster than all other FB variants studied here (Fig. 3.5D). In fact, the FB-F286L convertase seemed insensitive to decay acceleration by both FH and SL1100, but this was most likely due to an already significant decay rate in the absence of any exogenous factors. Whereas both FB-K323E (Fig. 3.5E) and FB-K350N (Fig. 3.5F) behaved similarly to each other and to FB-WT insofar as C3 convertase formation was concerned, the FB-K323E convertase was more resistant to decay acceleration by FH; however, neither of these convertases could be dissociated by injections of SL1100. Finally, the C3 convertases formed by FB-2Mut (Fig. 3.5G) and FB-2Mut (Fig. 3.5H) exhibited the increased stability found in the FB-D279G convertase but appeared to be fully resistant to decay acceleration by FH and SL1100. Although the FB-K350N convertase was not as resistant to decay acceleration as the FB-D279G convertase, including both mutations concurrently in the FB-2Mut and FB-3Mut variants seemed to have an additive effect in promoting convertase stability. While our observations here largely confirmed previous work showing that these FB variants generated intrinsically stabilized C3 convertases and/or convertases resistant to

decay (100-102), we further found that SL1100 could not diminish the stability of any C3 convertases once they had formed.

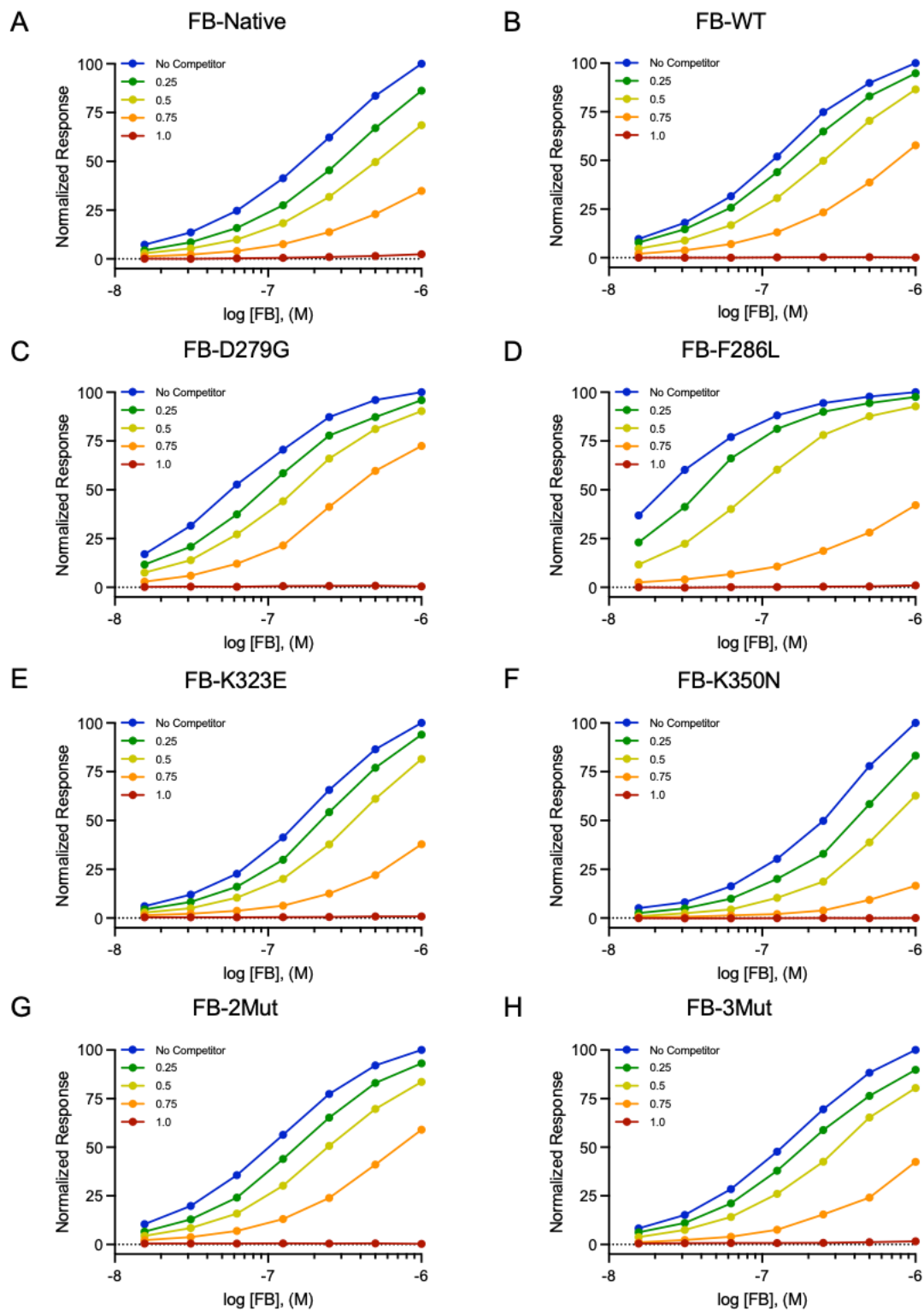


Figure 3.4 Modified DNA Aptamer SL1100 Inhibits C3 Proconvertase Formation by Gain-of-Function FB Variants.

Site-specifically biotinylated C3b was captured on three experimental flow cells of streptavidin-coated biosensor, leaving one flow cell unmodified for reference subtraction. A two-fold dilution series of FB spanning seven different concentrations from 15.625 nM to 1 μ M was injected over the surface, either in the absence or presence of increasing molar ratios of SL1100 to FB. SPR signals were normalized for each flow cell by treating the response immediately prior to injection stop for the highest concentration of FB without any inhibitor as 100%. The residual signals were then plotted as a function of FB for each ratio of SL1100 to FB. (A) Normalized SPR response of FB-Native binding to C3b. (B) Normalized SPR response of FB-WT binding to C3b. (C) Normalized SPR response of FB-D279G binding to C3b. (D) Normalized SPR response of FB-F286L binding to C3b. (E) Normalized SPR response of FB-K323E binding to C3b. (F) Normalized SPR response of FB-K350N binding to C3b. (G) Normalized SPR response of FB-2Mut binding to C3b. (H) Normalized SPR response of FB-3Mut binding to C3b. Increasing aptamer levels diminished FB binding to the surface at each FB concentration examined. For panels A-H, the data shown represents one of three independent replicates.

Inhibition by SL1100 Supersedes the Increased Alternative Pathway Activity Resulting from FB Gain-of-Function

Although the results presented above showed that SL1100 blocked proconvertase formation by gain-of-function variants of FB in purified settings (Fig. 3.4), we sought further insight into whether this could also be seen in more complex assays of complement activity. To investigate this issue, we monitored hemolysis of rabbit erythrocytes resulting from AP activity in the absence or presence of SL1100 using FB-depleted human serum reconstituted with various forms of FB (47). We first evaluated hemolytic activity as a function of FB concentration using 12% (v/v) FB-depleted serum (Fig. 3.6A). We found that FB-Native and FB-WT had similar activities to one another as judged by EC_{50} values of 19.8 nM and 15.8 nM, respectively. Both FB-F286L and FB-K323E exhibited a ~4-fold increase in potency, as judged by their EC_{50} values of 3.6 nM and 3.8 nM, respectively, while FB-K350N was ~8-fold more potent with an EC_{50} value of 2.0 nM. The most potent hemolytic activities were observed for FB-D279G and FB-2Mut, with

EC₅₀ values of 0.9 nM and 1.0 nM, respectively. Curiously, neither of these FB variants achieved full hemolysis at the highest concentrations of FB examined; they even showed some evidence of inhibition at concentrations above 100 nM in this assay. FB-3Mut did not support any hemolytic activity, as expected, since this variant contains a serine protease catalytic site mutation.

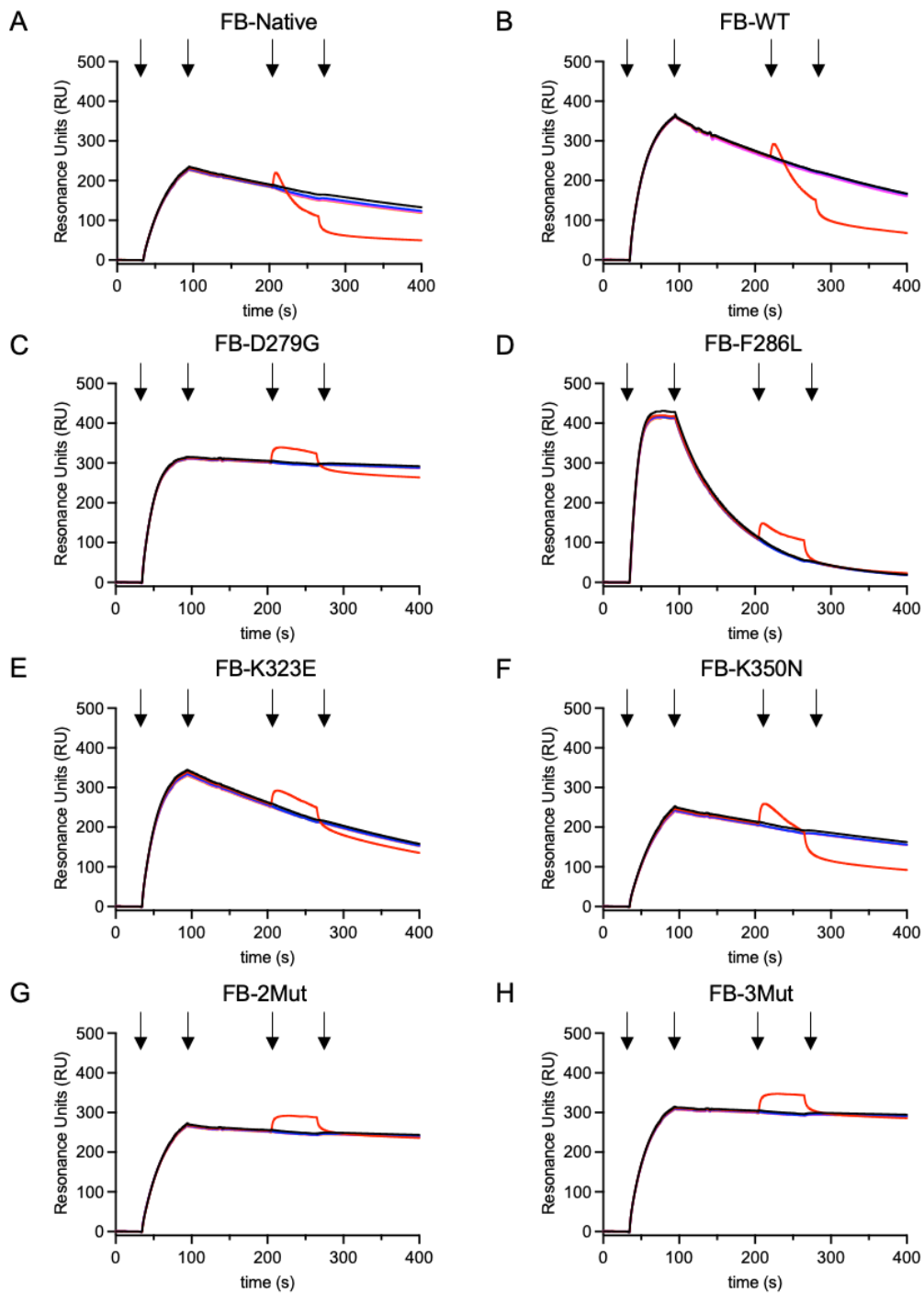


Figure 3.5 Modified DNA Aptamer SL1100 Does Not Alter C3 Convertase Stability.

Site-specifically biotinylated C3b was captured on three experimental flow cells of a streptavidin-coated biosensor, leaving one flow cell unmodified for reference subtraction. Reference-corrected sensorgrams were used to follow the formation and decay of the AP C3 convertase in real-time. The first experimental cycle involved injection of a solution of 100 nM FB/FD followed by HBS-T buffer alone to monitor formation and decay of the C3 convertase, respectively (black trace). The second cycle involved FB/FD injection followed by FH to illustrate decay acceleration of the C3 convertase (red trace). The third, fourth, and fifth cycles involved FB/FD injection followed by 100 nM SL1100 (blue trace), 500 nM SL1100 (magenta trace), or 1000 nM SL1100 (orange trace) to determine the effect of SL1100 on C3 convertase decay. Arrows indicate the start and stop points of the FB/FD injection and the decay injection sequences. (A) C3 convertase formed by FB-Native. (B) C3 convertase formed by FB-WT. (C) C3 convertase formed by FB-D279G. (D) C3 convertase formed by FB-F286L. (E) C3 convertase formed by FB-K323E. (F) C3 convertase formed by FB-K350N. (G) C3 convertase formed by FB-2Mut. (H) C3 convertase formed by FB-3Mut. For panels A-H, the data shown represents one of three independent replicates.

To examine whether SL1100 could supersede the enhanced AP activity induced by these gain-of-function FB variants, we evaluated its inhibitory potency by adding increasing concentrations of SL1100 into hemolysis experiments that used 12% (v/v) FB-depleted human serum fortified with FB. We used 60 nM FB-Native and FB-WT to ensure sufficient hemolytic activity in the absence of any inhibitor and found that SL1100 blocked the AP with IC_{50} values of 36.7 nM and 31.7 nM, respectively (Fig. 3.6B). We repeated these experiments for the gain-of-function variants, with the exception that we used 20 nM FB for these forms of FB since they showed increased AP activity relative to wild-type (Fig. 3.6A). We found that SL1100 inhibited the AP activity arising from each FB variant within a relatively narrow IC_{50} range from 23.8 nM to 33.9 nM. We also carried out these experiments using the same concentration of FB (i.e. 60 nM) for both the wild-type FB and various mutants (Fig. S3.2A). These results were in good agreement with the previous experiment, and showed that increased levels of SL1100 were required to inhibit the gain-of-function arising from mutations in FB. It should be noted that these experiments collectively reflected several functionally critical parameters, including the affinity of each FB

variant for C3b (Fig. 3.2), the affinity of SL1100 for each form of FB (Fig. 3.3), and the intrinsic features of the C3 convertases these FB variants form (Fig. 3.5). Nevertheless, these observations were consistent with the prediction that SL1100 counteracts the enhanced hemolytic activity induced by gain-of-function F B mutants.

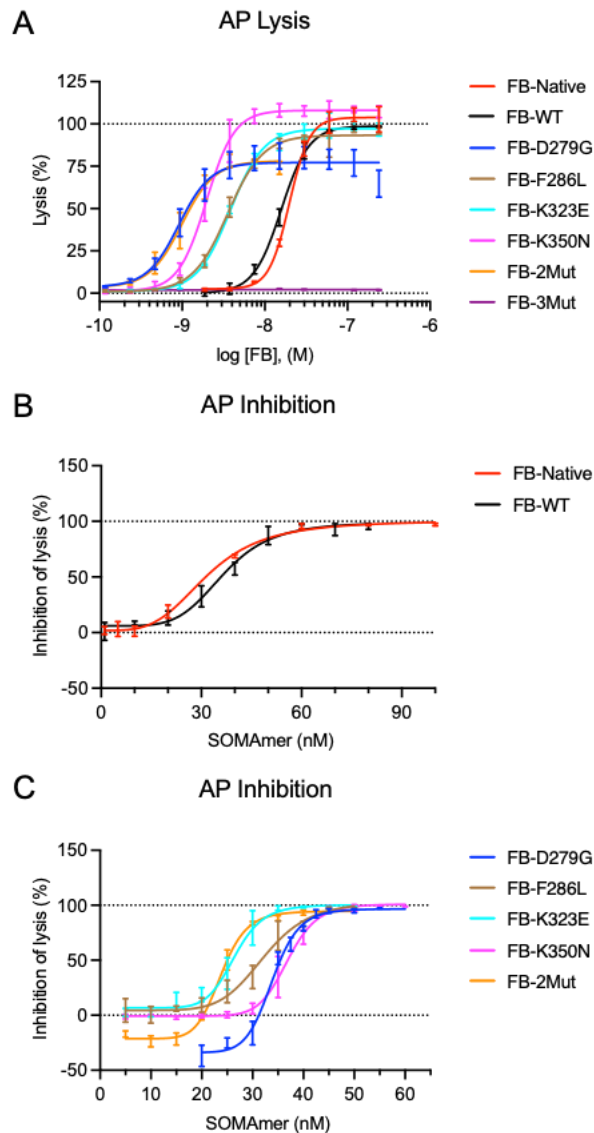


Figure 3.6 Inhibition by the Modified DNA Aptamer SL1100 Supersedes the Increased Alternative Pathway Activity Resulting from FB Gain-of-Function.

Rabbit erythrocytes were incubated for 30 min at 37 °C with 12% (v/v) FB-depleted serum that had been fortified with various forms of FB, either in the absence or presence of SL1100. Hemolysis was assessed spectrophotometrically, and the data were fit to dose-response curve (*please see Materials and Methods*). (A) AP-driven hemolysis using 12% (v/v) FB-depleted serum fortified with increasing concentrations of various FB proteins. Observed EC_{50} values were as follows: FB-Native, 19.8 nM; FB-WT, 15.8 nM; FB-D279G, 0.9 nM; FB-F286L, 3.6 nM; FB-K323E, 3.8 nM; FB-K350N, 2.0 nM; FB-2Mut, 1.0 nM; FB-3Mut, no activity. (B) Inhibition of hemolysis using 12% (v/v) FB-depleted serum fortified with 60 nM FB-Native or FB-WT in the presence of increasing concentrations of SL1100. Observed IC_{50} values for SL1100 were as follows: FB-Native, 36.7 nM; FB-WT, 31.7 nM. (C) Identical to panel B, except that 12% (v/v) FB-depleted serum was fortified with 20 nM of each gain-of-function FB variant. Observed IC_{50} values for SL1100 were as follows: FB-D279G, 33.9 nM; FB-F286L, 32.4 nM; FB-K323E, 26.5 nM; FB-K350N, 36.9

nM; FB-2Mut, 23.8 nM). For panels A-C, note that normalized values from six technical replicates were expressed as a function of FB protein or SL1100 concentration and were fit to a four-parameter dose-response curve.

Discussion

aHUS is characterized by hemolytic anemia, thrombocytopenia, and renal failure, but a dysregulated complement AP is widely acknowledged as the molecular basis of this disease (88, 102). Genetic studies have established that aHUS is most often associated with loss of function in AP regulatory proteins, including FH, MCP, and DAF (89-91, 102). However, a smaller subset of aHUS patients expresses gain-of-function mutations in AP components themselves, including FB (100-102). Several of these FB mutations increase the rate at which the C3 convertase forms or convertase stability thereafter (100-102). This suggests that inhibiting the initial interaction of FB with C3b could be an effective strategy for preventing excessive AP activity resulting from these gain-of-function mutations in FB. In this report, we provided proof-of-concept for this approach. We showed that a prototypic member of a previously described family of FB-binding modified DNA aptamers effectively bound to disease-causing gain-of-function FB variants (Fig. 3.3, Fig. S3.1 & Table 3.1), thereby blocking C3 proconvertase formation (Fig. 3.4 & Fig. S3.4) and inhibiting activity of the AP (Fig. 3.6 & Fig. S3.2).

Molecular studies on FB variants identified from aHUS patients have been a rich source of information on the formation, decay, and regulation of the AP C3 convertase (100-102). Because most of these FB variants were described by different investigator teams at separate times, there has not been a concurrent, systematic investigation of these FB variants until now. Here we compared four aHUS-linked FB variants using well-established assays to interrogate relevant properties of the AP C3 convertase, including the rate of proconvertase formation (Fig. 3.2) and how efficiently it is activated (Fig. 3.5), its intrinsic stability and sensitivity to decay acceleration (Fig. 3.5), and its ability to cleave C3 and support downstream complement activity (Fig. 3.6). Our results largely agree with the observations from other investigators, with a few potentially

interesting exceptions. First, we found that the FB-D279G and FB-K350N variants have weaker apparent affinities for C3b than earlier work might have predicted (100). Roumenina et al. reported that FB-D279G and FB-K350N bound to hydrolyzed C3 (i.e. C3(H₂O)) with apparent affinities of 1.4 nM and 1.8 nM, while wild-type FB bound with an apparent affinity of 105 nM (100); here, we found that FB-D279G and FB-K350N bound to C3b with apparent affinities of 71 nM and 322 nM, while wild-type FB had an apparent affinity of 180 nM (Table 3.1). Although C3(H₂O) and C3b are not structurally identical to one another (119), the long-standing observation that C3(H₂O) and C3b share many functional properties suggests that the values should have been more similar (120, 121). Second, the apparent affinity for C3b of FB-K350N we observed here was surprisingly weaker than that of wild-type FB (Table 3.1). Earlier work reported the apparent affinity of FB-K350N for C3(H₂O) was comparable to that of FB-D279G and nearly ~60-fold tighter than wild-type FB. The reasons for this disparity are unclear since the FB-K350N convertase was partly resistant to decay by FH (Fig. 3.5) and exhibited gain-of-function in hemolytic assays as expected (Fig. 3.6).

A final noteworthy difference we observed while characterizing FB variants pertains to the unusual functional properties of FB-D279G. As mentioned in the paragraph above, we found that FB-D279G was not as dramatically enhanced in the rate of proconvertase formation as previously reported ((100, 101); Table 3.1). Once the FB-D279G convertase was formed, however, we found that it was remarkably stable and nearly completely resistant to decay acceleration by FH (Fig. 3.5); these observations extend those of Rodríguez de Córdoba et al., who showed that the FB-D279G convertase was significantly stabilized against decay acceleration by DAF (102). We further found that simultaneous D279G and K350N mutations in FB variants resulted in convertases that appeared completely resistant to FH decay, as judged by studies on FB-2Mut and

FB-3Mut (Fig. 3.5). This suggested that the convertase-stabilizing attributes of these individual mutations are additive. Perhaps most unusually, though, we noticed that FB-D279G did not support full activity in hemolytic assays of complement function (Fig. 3.6); in fact, we even found evidence of dose-dependent inhibition in these experiments at FB-D279G concentrations above 30 nM. Both features were shared by FB-2Mut, suggesting these effects were likely not the result of an artifact. While we cannot provide a definitive explanation for these observations at this time, it seems the effective levels of FB available for productive convertase formation were reduced under these conditions. This could have been caused by the binding of FB-D279G and FB-2Mut to alternative ligands like iC3b (100), which may not generate catalytically efficient C3 convertases. Future studies could explore this possibility in greater detail.

The hemolytic assays we performed utilized FB-depleted serum that was fortified with various forms of FB under investigation (Fig. 3.6). This serum contained otherwise normal levels of complement components, including FP, which is the only positive regulator of the AP (81). Since the activities of FP oppose those of the FB-binding aptamers studied here, we sought additional information on how SL1100 might impact FP function. We found that the concentration of SL1100 needed to inhibit the hemolytic activity of normal human serum (NHS) was only slightly greater than that needed to inhibit FP-depleted serum (Fig. S3.2B), even though the rate at which FP-depleted serum lysed rabbit erythrocytes was considerably slower than NHS. Furthermore, while FP promoted C3 convertase formation for each FB variant examined and stabilized their respective convertases after activation (Fig. S3.3), we observed that inclusion of SL1100 in the initial stage of this experiment completely blocked C3 proconvertase formation regardless of whether FP was present or not (Fig. 3.4). This indicates that the inhibitory effect of SL1100 supersedes the stimulatory activities of FP. It is also in agreement with our earlier

observations that the IC₅₀ of SL1100 in hemolytic assays reflects the levels of FB present in the serum (Fig. 3.6 & Fig. S3.2).

Whereas earlier work suggested that the affinity of these aptamers for FB lay in the 10-100 pM range (47), here we found that the interaction was weaker in the realm of 1-10 nM (Fig. 3.3 & Table 3.1). This discrepancy could be due to differences in the solution-based bead-binding assays used previously instead of the solid-phase SPR method used here. Differences could also be attributed to the specific 5'-biotinylated linker we used, which may have somehow influenced FB binding to the immobilized aptamer. Nevertheless, the SL1100 aptamer used in our current studies showed comparable binding to all forms of FB independent of any mutations they contained (Fig. 3.3 & Table 3.1). We attribute the generally similar affinities we observed to the fact that the aptamer binding site on FB is removed from the region of its vWF domain where these aHUS-linked mutations cluster. In hindsight, assuming this would have been the case seems reasonable. However, we thought it was essential to rule out any differences in affinity due to unexpected changes in protein dynamics or other minor structural alterations caused by these mutations. Regardless of the genuine affinity of these aptamers for FB, the concentration of FB in human plasma is relatively high (~200 µg/mL or ~2.1 µM); thus, the concentrations of aptamer needed to approach stoichiometric equivalence would almost certainly yield FB/aptamer complexes, resulting in potent inhibition of C3 proconvertase formation and blocking the AP. Because of this, affinity seems less of a limiting factor to potentially using these aptamers for therapeutic purposes than their pharmacologic properties, which are currently unknown.

Considering that aptamers offer several significant advantages over traditional protein therapeutics, including synthetic accessibility, high solubility, and an apparent lack of immunogenicity (122, 123), the developmental landscape of aptamer drugs has been relatively

weak for the past two decades. The first aptamer-based drug, Macugen (pegaptanib), was approved by the FDA in 2004 (123). Macugen is a pegylated, 27-mer RNA aptamer that targets and inhibits vascular endothelial growth factor for treatment of wet age-related macular degeneration (AMD), a major cause of vision loss. Although Macugen foreshadowed the potential of aptamers as therapeutics, it took till 2023 for the next aptamer-based drug, Izervay (avacinaptad), to receive FDA approval (39). Coincidentally, Izervay is also used to treat AMD. This pegylated, 39-mer RNA aptamer is a complement C5 inhibitor that prevents C5 activation, leading to the blockade of the terminal complement pathway (124). The limited portfolio of aptamer-based drugs likely reflects technical challenges they face during development, including metabolic instability, susceptibility to rapid renal filtration due to their small size, and unpredictable pharmacokinetic properties, all of which can diminish their efficacy *in vivo* (122, 123, 125, 126). Indeed, both approved aptamer drugs are modified RNAs that include polyethylene glycol chains to extend their half-lives *in vivo*, and inverted thymidine nucleotides, fluorinated and O-methylated ribose bases to increase their resistance to degradation. Although the FB-binding DNA aptamer we investigated here (i.e. SL1100) lacks such modifications, other members of this aptamer family (i.e. SL1102 and SL1103) contain 2'-O-methylations that improve their serum stability without significantly affecting their potency (47). Moving forward, the extensive structure/function information available for this aptamer family should facilitate the design of pegylation strategies compatible with preserving their bioactivity (47).

While effective therapies for aHUS are already in the clinic (106), further development of these FB-binding aptamers could provide valuable new tools for treating other complement-driven diseases aside from aHUS (62). In this regard, there are many other diseases where excessive or uncontrolled complement activity is believed to play a contributing, if not causal role (62). Since

the AP is the main driver of complement amplification (84), molecules that block any components of its convertase (i.e. C3b, FB, and FD) or its substrate (i.e. C3) could be useful therapeutic modalities for these diseases. Indeed, inhibitors of C3, FD, and FB have received FDA approvals in recent years for various conditions. Although the FB-binding aptamers we describe here would not be unique in their ability to block complement amplification via the AP, their synthetic nature, high solubility, and potent activities may impart certain advantages that justify continued research and development of these unusual molecules throughout the coming years.

Acknowledgements

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Footnotes

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Abbreviations

aHUS; atypical hemolytic uremic syndrome
AMD; age-related macular degeneration
AP; alternative pathway of complement
C3; complement component C3
C3b; complement component C3b
C4b; complement component C4b
C5; complement component C5
CCP; complement control protein domain
CP; classical pathway of complement
DAF; decay accelerating factor
FB-2Mut; recombinant FB with D279G and K350N
FB-3Mut; recombinant FB with D279G , K350N, and S699A
FB-D279G; recombinant FB with D279G mutation
FB-F286L; recombinant FB with F286L mutation
FB-K323E; recombinant FB with K323E mutation
FB-K350N; recombinant FB with K350N mutation
FB-Native; Native FB purified from human serum
FB-WT; recombinant wild-type FB
FB; complement factor B
FD; complement factor D
FH; complement factor H
FI; complement factor I
FP; properdin
HBS-T; HEPES buffered saline with tween-20
HBS; HEPES buffered saline
LP; lectin pathway of complement
MAC; membrane attack complex
MCP; membrane cofactor protein
MIDAS; metal ion-dependent adhesion site
PEG; polyethylene glycol
RCA; regulator of complement activity
SP; serine protease domain
SPR; surface plasmon resonance
vWF; von Willebrand factor domain

Supplementary information

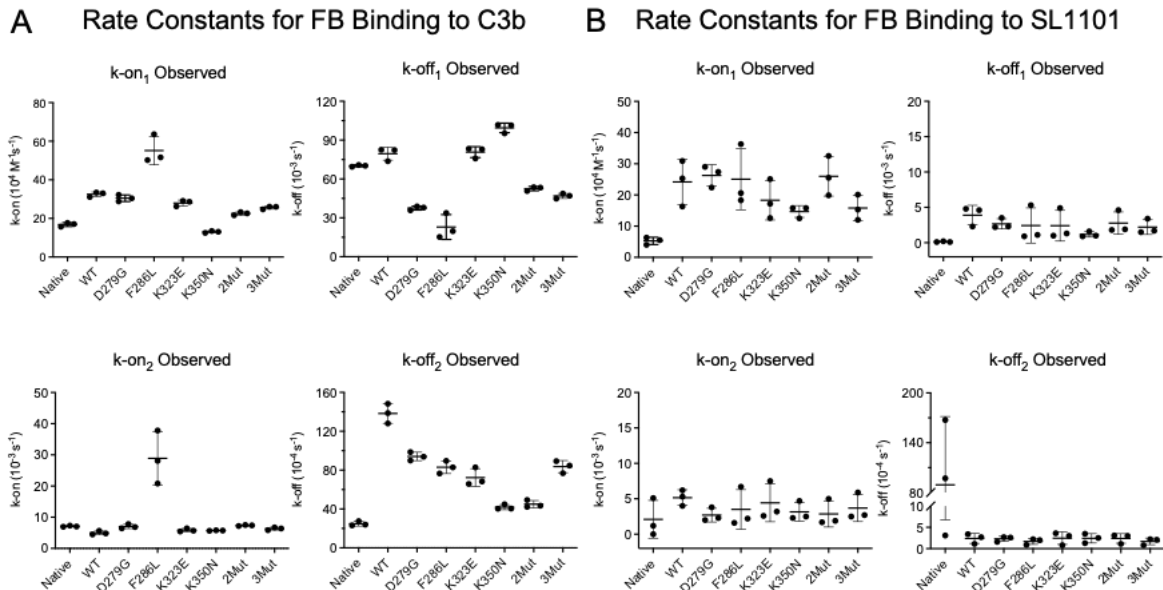


Figure S3.1. Supporting Information Pertaining to Binding Studies of FB to Biotinylated Forms of C3b and SL1100. (A) Observed association and dissociation rate constants for FB binding to site-specifically biotinylated C3b from three technical replicates. (B) Observed association and dissociation rate constants for FB binding to 5'-biotinylated SL1100 from three technical replicates. Note that the black bars in each panel represent the mean plus or minus one standard deviation.

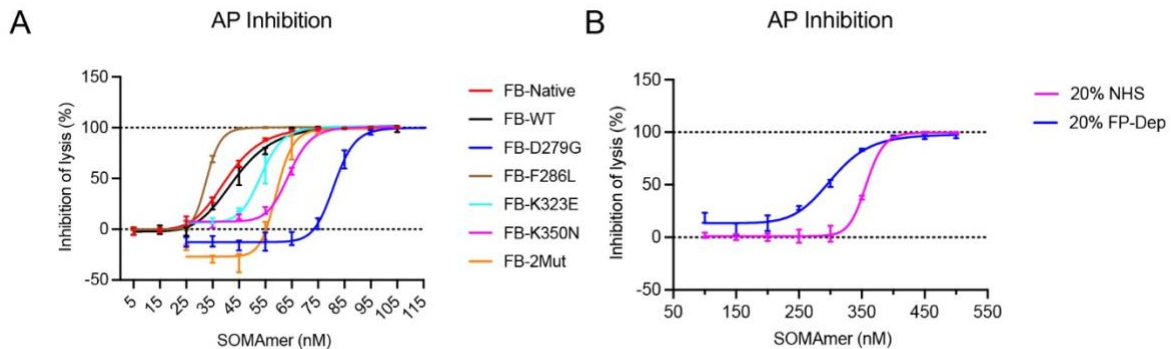


Figure S3.2. Supporting Information Pertaining to Hemolytic Assays for Alternative Pathway Activity. (A) Inhibition of hemolysis using 12% (v/v) FB-depleted serum fortified with 60 nM FB variants in the presence of increasing concentrations of SL1100. Observed IC_{50} values for SL1100 were as follows: FB-Native, 40.6 nM; FB-WT, 43.6 nM; FB-D279G, 81.1 nM; FB-F286L, 32.8 nM; FB-K323E, 53.6 nM; FB-K350N, 64.0 nM; FB-2Mut, 59.2 nM. (B) Inhibition of hemolysis using 20% (v/v) Normal Human Serum (NHS) or 20% (v/v) FP-depleted serum (FP-Dep) in the presence of increasing concentrations of SL1100. The reactions using NHS were incubated for 30 min at 37 °C, while those using FP-Dep were incubated for 3 h at 37 °C. Observed IC_{50} values for SL1100 were as follows: NHS, 357 nM; FP-Dep, 303 nM. For panels A and B, note that normalized values from at least six technical replicates were expressed as a function of SL1100 concentration and were fit to a four-parameter dose-response curve.

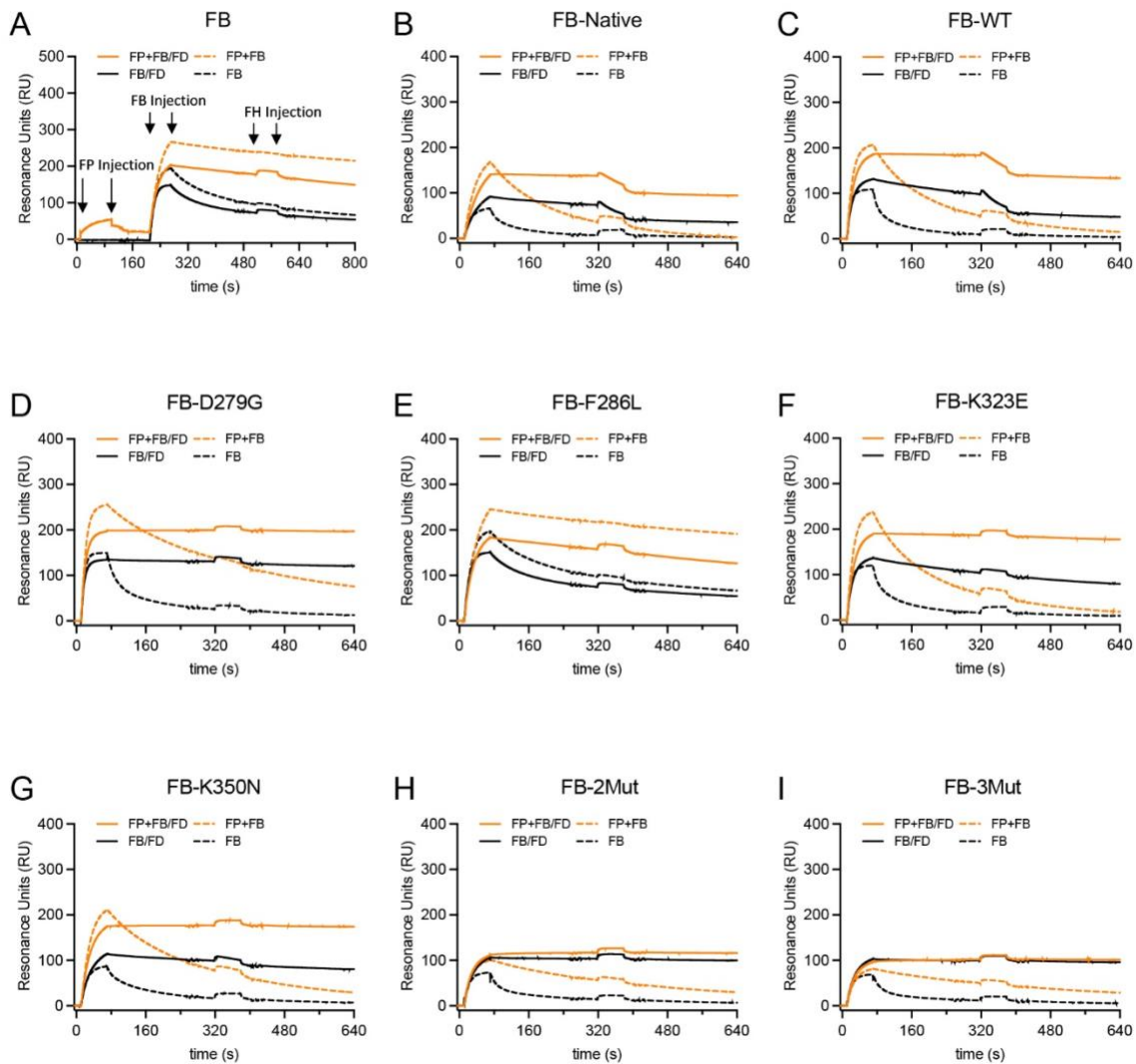


Figure S3.3. The Effects of Properdin on AP C3 Convertase Formation and Decay using FB Variants. Site-specifically biotinylated C3b was captured on fc2 and fc3 of a streptavidin-modified SPR chip, leaving fc1 to serve as a basis for reference subtraction. Properdin (FP) was injected on fc2 at the beginning of each experimental cycle to achieve FP saturation on that flow cell only; thereafter, an FB/FD mixture was injected on fc2 (orange, solid line) and fc3 (black, solid line) to form the AP C3 convertase. Following an observation period, decay acceleration was assessed by injecting FH on fc2 and fc3. This sequence was repeated where FB alone was injected on fc2 (orange, dashed line) and fc3 (black, dashed line) to serve as a control. Reference-subtracted sensorgrams are shown for all experiments. (A) Illustration of the overall experimental design. Note that the FP presaturation phase is omitted from the remaining panels for clarity. (B) AP C3 convertase formation and decay by FB-Native. (C) AP C3 convertase formation and decay by FB-WT. (D) AP C3 convertase formation and decay by FB-D279G. (E) AP C3 convertase formation and decay by FB-F286L. (F) AP C3 convertase formation and decay by FB-K323E. (G) AP C3 convertase formation and decay by FB-K350N. (H) AP C3 convertase formation and decay by FB-2Mut. (I) AP C3 convertase formation and decay by FB-3Mut. For panels B-I, the data shown represents one of three independent replicates.

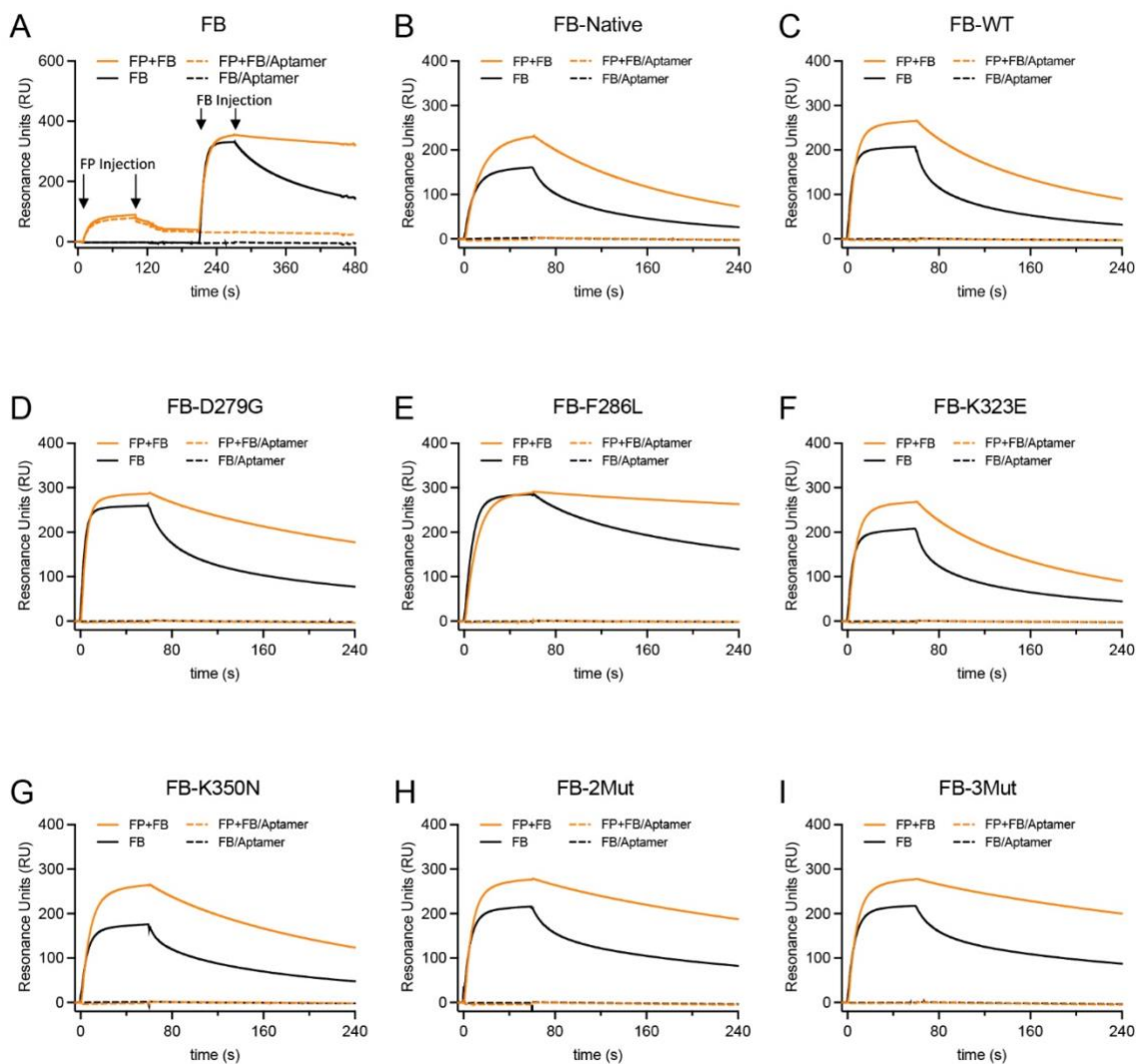


Figure S3.4. The Effects of Properdin and SL1100 on C3 Proconvertase Formation by FB Variants. Site-specifically biotinylated C3b was captured on fc2 and fc3 of a streptavidin-modified SPR chip, leaving fc1 to serve as a basis for reference subtraction. Properdin (FP) was injected on fc2 at the beginning of each experimental cycle to achieve FP saturation on that flow cell only. Reference-subtracted sensorgrams corresponding to injections of FB over fc2 (orange, solid line) or fc3 (black, solid line) are shown, as are those corresponding to injections of FB/SL1100 at a 1:1 molar ratio over fc2 (orange, dashed line) or fc3 (black, dashed line). (A) Illustration of the overall experimental design. Note that the FP presaturation phase is omitted from the remaining panels for clarity. (B) C3b-binding of FB-Native. (C) C3b-binding of FB-WT. (D) C3b-binding of FB-D279G. (E) C3b-binding of FB-F286L. (F) C3b-binding of FB-K323E. (G) C3b-binding of FB-K350N. (H) C3b-binding of FB-2Mut. (I) C3b-binding of FB-3Mut. For panels B-I, the data shown represents one of three independent replicates.

Chapter 4 - Blocking Activation of the C1r Zymogen Defines a Novel

Mode of Complement Inhibition[#]

This chapter has been published as a journal article in the Journal of Biological Chemistry

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Abstract

Many hematophagous organisms secrete inhibitors of the coagulation and complement systems as constituents of their salivary fluid. Whereas previous studies on salivary gland extracts from the sandfly *Lutzomyia longipalpis* identified SALO as a potent inhibitor of the classical complement pathway (CP), its precise mechanism of action has remained elusive. Here we show that SALO inhibits the CP by binding selectively to the C1r zymogen. Using surface plasmon resonance, we found that SALO expressed by HEK293(T) cells (eSALO-WT) bound with nanomolar affinity to the zymogen of complement protease C1r (pro-C1r), but that it did not bind the enzymatically-active form of C1r. To gain insight into the structural basis for CP inhibition by SALO, we solved a 3.3 Å resolution crystal structure of eSALO-WT bound to a recombinant form of C1r that was engineered to remain in a zymogen-like state (zC1r-12SP). eSALO-WT formed extensive interactions with the zymogen activation loop of zC1r-12SP, including groups derived from residues R463 and I464, which comprise its scissile peptide bond. Although the interactions with R463 and I464 were mediated by sidechain sulfation of eSALO-WT at position Y51, we found that this modification enhanced the potency of SALO but was not required for its activity. Consistent with our structural observations, subsequent studies showed that eSALO-WT binding to pro-C1r blocked its activation and thereby inhibited the CP in hemolytic assays of complement function. Together, our results define a new mode of inhibiting complement by blocking the farthest upstream enzymatic reaction of the CP.

Introduction

The complement system is an ancient arm of innate immunity (1, 2). Although its best understood roles lie in opsonizing and eliminating microorganisms, facilitating clearance of circulating immune complexes, and promoting recruitment of inflammatory and phagocytic cells, contributions of complement to new and unexpected aspects of human physiology continue to emerge (3). Complement activity can be initiated through three conventional routes, known as the classical (CP), lectin (LP), and alternative (AP) pathways, each of which responds to specific immunological stimuli. While several immunological triggers of the CP have been described (1, 127), its canonical activator is surface antigen-bound IgG hexamers. In this scenario, hexamerized IgG molecules serve as an efficient platform for binding the first component of complement (C1) (9).

The C1 complex is a large, ~766 kDa Ca^{2+} -dependent assembly that exhibits specific proteolytic activity upon activation. It is comprised of a hexavalent pattern recognition component, known as C1q, bound to a heterotetramer of two serine proteases, known as C1r and C1s (1, 127). Both C1r and C1s are present within resting C1 as zymogens. The earliest events in the CP lead to C1 activation, and can be understood as a series of discrete steps (Fig. 4.1A): (i) the C1q component binds specifically to antigen-bound IgGs clustered on surfaces (e.g. microbes), (ii) the C1r zymogen undergoes autoactivation into C1r enzyme, and (iii) the C1r enzyme cleaves the C1s zymogen into its enzymatically-active state. With its C1s constituent now active, C1 can cleave its initial substrate, which is the downstream complement component C4. This leads to deposition of the C4 fragment, C4b, on complement-reactive surfaces.

Complement component C2 binds to surface-associated C4b and is also cleaved by active C1. This generates the multi-subunit protease known as the CP C3 convertase (i.e. C4b2a) that

activates native C3 into C3a and C3b (1, 127, 128). Surface-bound C3b serves as a platform for assembly of the AP C3 convertase (i.e. C3bBb) that facilitates self-amplification of the complement response. This same convertase also initiates the terminal complement pathway by cleaving complement component C5 into C5a and C5b. The terminal pathway activates powerful complement effector mechanisms, including the recruitment of phagocytic cells via C5a and formation of the so-called terminal complement complex (TCC) via C5b (1, 2). Thus, a well-orchestrated series of protein-protein interactions and proteolytic transformations underlies initiation of the CP and indeed all of complement activity.

Lutzomyia longipalpis is a species of sandfly common to Central and South America. *L. longipalpis* serves as a vector for the transmission of *Leshmania* parasites, which pose a significant public health burden in many tropical regions of the world (129). *Leshmania* infection is enabled by the contents of sandfly saliva (130, 131), which contains various anti-coagulant and immunomodulatory proteins similar to other hematophagous arthropods including ticks and mosquitos (128, 132). Some of these inhibitors present within insect salivary fluid may also help alleviate gut tissue damage caused by complement components found within the bloodmeal. In 2016, Ferreira et al. reported the identification of one such protein, which they named SALO (short for Salivary Anti-complement from *Lutzomyia longipalpis*) (50). These investigators showed that SALO was a potent, dose-dependent inhibitor of the CP that acted upstream of the level of C4 (50). Although they further showed that SALO did not block other complement pathways and that it did not act as a protease inhibitor (50), the mechanistic basis for its activity has remained unclear.

SALO is ~11 kDa and is comprised of four α -helices, but bares no sequence relationship to proteins of known function (51). This has largely precluded the use of structural information to formulate hypotheses regarding the mechanism through which SALO inhibits complement. To

circumvent this limitation, we employed a biochemical screening strategy in an attempt to decipher the SALO mechanism. We found that SALO inhibits the CP by binding specifically to the zymogen form of C1r. We provide two crystal structure determinations and supporting functional studies which show that SALO binds tightly to the zymogen activation loop of C1r, preventing cleavage of its scissile bond. By blocking formation of active C1r, SALO prevents formation of proteolytically-active C1. Our results define a novel mode of inhibiting complement by blocking the farthest upstream enzymatic reaction within the CP. They also provide a first ever description of regulating protease zymogen activation by shielding its scissile bond from proteolytic cleavage.

Materials and Methods

Protein Samples

Native C1 complex, C1r proenzyme, C1r enzyme, C1s proenzyme, C1s enzyme, and C4 purified from human serum were obtained from Complement Technologies.

For prokaryotic expression, DNA encoding wild-type SALO was subcloned into the pT7HMT vector and transformed into *E. coli* BL21(DE3) cells (67, 68). Cultures were grown to an OD600 of 0.6–0.8, induced with 1 mM IPTG, and incubated for 16 h at 37 °C. Cells were harvested, lysed in 100 mM Tris-HCl (pH 8.0), 10 mM imidazole, and 6 M guanidine HCl, and clarified by centrifugation. Recombinant SALO proteins were purified using Ni-NTA affinity chromatography under denaturing conditions. Eluted proteins were reduced with 1 mM TCEP at 37 °C for 30 min and then dialyzed overnight against 2 L of 100 mM Tris-HCl (pH 8.6), 20 mM glycine, 2 mM L-cysteine, 0.5 mM EDTA, and 2.5 M urea. Each sample was then dialyzed overnight against 4 L of PBS (pH 7.4). The affinity tag was removed by incubating with recombinant Tobacco Etch Virus protease at a 100:1 mass ratio overnight at room temperature. The sample was then applied to a 5-mL HisTrap HP column using AKTA FPLC (Cytiva), and the

unbound fraction was collected thereafter. The protein was further purified on a Superdex 75 26/60 column equilibrated in HBS (pH 7.4). Purified proteins were analyzed by SDS-PAGE, pooled, and stored at 4 °C or –80 °C.

For eukaryotic expression, DNAs encoding recombinant SALO proteins and zC1r-12SP were synthesized with a C-terminal octahistidine tag and subcloned into the pcDNA3.1+ vector (GeneScript). Large-scale plasmid preparations were performed using a QIAGEN Plasmid Maxi kit. Transient transfections into HEK293(T) cells were achieved with polyethyleneimine (PEI) MAX (109, 133). After collecting 1 L of medium, the conditioned medium was concentrated to 150 mL via tangential flow filtration and buffer-exchanged into native binding buffer (20 mM Tris [pH 8.0], 20 mM imidazole, 500 mM NaCl). The samples were then loaded onto a 5-mL HisTrap HP column using AKTA FPLC (Cytiva) and eluted with native elution buffer (20 mM Tris [pH 8.0], 500 mM imidazole, 500 mM NaCl). Fractions containing the target protein, as determined by SDS-PAGE, were further purified on a Superdex 75 26/60 column equilibrated in HBS (pH 7.4). Purified proteins were pooled, quantified, and stored at 4 °C or –80 °C for later use.

Circular Dichroism Spectropolarimetry

The structural integrity of various SALO proteins was assessed using circular dichroism (CD) spectropolarimetry. Experiments were performed on a Jasco J-815 CD spectrophotometer with a 1-mm pathlength jacketed cylindrical quartz cuvette. Proteins were dissolved in 5 mM HEPES (pH7.4), 35 mM NaCl, and concentrations were determined by absorbance at 280 nm using a DeNovix DS-11 spectrophotometer. CD measurements were conducted at a final protein concentration of 37.5 μ M. Spectra were recorded over a wavelength range of 260–190 nm at a scan rate of 50 nm/min with a step interval of 1 nm. For each sample, an average of five scans was used to generate a composite spectrum, followed by subtraction of a blank measurement. The

resulting spectra were smoothed using the Savitzky-Golay algorithm provided in the Spectra Analysis software (Jasco Inc., Easton, MD).

Mass Spectrometry

The intact mass of purified SALO proteins was determined using liquid chromatography-mass spectrometry (LC-MS). The protein sample, at an approximate concentration of 1.0 mg/mL, was filtered through a 0.2 μ m PTFE centrifugal filter (Millipore) prior to analysis. The filtered sample was introduced into a Waters LC-MS system, where it underwent initial desalting on a trapping column (ACQUITY UPLC M-Class Symmetry C₁₈ Trap Column, 100 Å, 5 μ m, 300 μ m $\times$ 50 mm; Waters). Separation was on an analytical column (ACQUITY UPLC M-Class BEH C₄ Column, 1.7 μ m, 300 μ m $\times$ 150 mm; Waters) under a gradient elution profile. Mobile phase A consisted of 0.1% (v/v) formic acid in water and mobile phase B consisted of 0.1% (v/v) formic acid in acetonitrile. The gradient ranged from 15% (v/v) to 60% (v/v) mobile phase B over a duration of 20 min. Mass spectrometry was performed in positive and MSe mode with the capillary voltage set to 3.0 kV and the cone voltage set to 100 V. All data processing was conducted using Waters MassLynx software.

Surface Plasmon Resonance Binding Studies

Surface plasmon resonance (SPR) experiments were performed on a BiaCore T-200 instrument at 25 °C using HBS-T (20 mM HEPES [pH 7.4], 140 mM NaCl, 0.005% [v/v] Tween 20) as the running buffer with a flow rate of 30 μ L/min.

Initial SPR screening experiments were performed by immobilizing eSALO-WT on a CMD200M SensorChip (Xantec Bioanalytics, Düsseldorf, Germany). Standard NHS/EDC activation was used to facilitate amine coupling to the surface (113). The experimental flow cell (fc2) had a final immobilization level of 720 RU eSALO-WT, while an ethanolamine-quenched

flow cell (fc1) was used as a reference. Serial two-fold dilutions (15.6–1000 nM) of C1 complex, C4, C1r proenzyme, C1r enzyme, C1s proenzyme, and C1s enzyme were injected over the sensorchip in reference subtraction mode; additional experiments were performed for C1 complex and C1r proenzyme using a lower concentration dilution series (1.56–100 nM). Each injection included a 90 s association phase followed by a 180 s dissociation phase. Surface regeneration was achieved by a 60 s pulse of 20 mM glycine (pH 2.2), 1.5 M NaCl. Reference-subtracted sensorgrams were processed using Biacore T-200 Evaluation Software v3.2.1 (Cytiva Life Sciences). In this initial series of experiments, sensorgrams were analyzed using a 1:1 binding model to derive an apparent K_D and estimates of the association and dissociation rate constants.

To compare binding of eSALO-WT to C1r proenzyme and zC1r-12SP, a low immobilization level surface was prepared using capture of biotinylated eSALO-WT. Biotinylation was achieved by mixing EZ-Link™ NHS-PEG4-Biotin (Thermo Fisher Scientific) with eSALO-WT in a 2:1 molar ratio according to the manufacturer's suggestions. After a 30 min incubation at room temperature, the reaction was terminated by adding 50 mM Tris (pH 8.0). Streptavidin was immobilized on two flow cells of a CMD200M chip (Xantec Bioanalytics, Düsseldorf, Germany) via the amine-coupling method described above (113). The reference surface (fc1) was prepared by injecting biotin (10 nM) three times, while the experimental surface (fc2) was prepared by injecting biotinylated eSALO-WT (50 nM) to achieve an immobilization level of 97 RU. Single-cycle kinetic analysis was performed using five concentrations of C1r proenzyme (25, 50, 100, 200, and 400 nM) and zC1r-12SP (6.25, 12.5, 25, 50, and 100 nM). Surface regeneration between experimental cycles was achieved using a single pulse of 20 mM glycine (pH 2.2), 1.5 M NaCl. Reference-subtracted sensorgrams were analyzed using Biacore T200 evaluation software v3.2.1 (Cytiva Life Sciences).

An analogous ligand-capture protocol was used to compare eSALO-WT with its mutants. Two separate CMD200M sensor chips (Xantec Bioanalytics) were prepared with streptavidin immobilized on all four flow cells. For both chips, fc1 served as the reference surface after three injections of biotin (10 nM). On the first chip, biotinylated eSALO-WT was captured on fc2 (66 RU), biotinylated eSALO-Y51F was captured on fc3 (56 RU), and biotinylated eSALO-E58A was captured on fc4 (50 RU). On the second chip, biotinylated eSALO-WT was captured on fc2 (69 RU), biotinylated eSALO-DN was captured on fc3 (56 RU), and biotinylated eSALO-DNYE was captured on fc4 (73 RU). Binding of C1r proenzyme and zC1r-12SP to SALO proteins was assessed using single-cycle kinetics as described above. Due to improved quality of fitting to the experimental data, sensorgrams were analyzed using a two-state binding model to derive an apparent K_D with a mechanism that incorporates a single second-order rate constant (i.e., k_{a-1}) and three first-order rate constants (i.e., k_{a-2} , k_{d-1} , and k_{d-2}). Values from the fits are presented as the mean $\pm$ SD from a minimum of three technical replicates and are included in the accompanying table (Table 4.2).

Crystallization, X-ray Diffraction Data Collection, Structure Solution, and Refinement

The complex of eSALO-WT and zC1r-12SP was prepared by mixing the individual monomers at molar ratios of 2:1, followed by purification via size-exclusion chromatography using a Superdex S75 26/60 column (Cytiva Life Sciences). Fractions containing the eSALO-WT/zC1r-12SP complex were identified by SDS-PAGE, buffer exchanged into 10 mM HEPES (pH 7.5), 50 mM NaCl, and concentrated to 1.5 mg/mL. Initial crystallization conditions were determined through vapor diffusion of sitting drops using a Crystal Gryphon liquid handling system (Art Robbins Instruments, LLC) along with the Index HT and PEGRx HT screening kits (Hampton Research Corp). Individual crystals for X-ray diffraction studies were grown through vapor

diffusion of hanging drops at 20 °C by mixing 1 μ L of sample with 1 μ L of precipitant solution (0.2 M ammonium sulfate, 0.1 M BIS-TRIS (pH 5.5), and 20% (w/v) polyethylene glycol 3350) and equilibrating over 500 μ L of precipitant solution. Rod-like crystals appeared within 2 days and grew to their final size over the course of 2 weeks. Crystals were cryopreserved after a short incubation in precipitation buffer containing an additional 15% (w/v) polyethylene glycol 3350, followed by flash cooling in liquid N₂.

The complex of eSALO-Y51F and zC1r-12SP was prepared using same method described above, with the exception that the sample was concentrated to 3 mg/mL. Individual crystals for X-ray diffraction studies were grown using the same general procedure as above, but used a different precipitant solution (0.2 M Ammonium citrate tribasic (pH 7.0), 20% (w/v) polyethylene glycol 3350). Crystals were cryopreserved after a short incubation in precipitation buffer containing an additional 15% (v/v) glycerol, followed by flash cooling in liquid N₂.

X-ray diffraction data for the eSALO-WT/zC1r-12SP complex were collected at NSLS-II beamline 19-ID of Brookhaven National Laboratory. Reflections were indexed, integrated, and scaled using HKL-2000 (69). The structure was solved by molecular replacement using PHASER (70) as implemented in the PHENIX suite (72) with the structures of SALO (PDB entry 5KX4 (51)) and C1r-12SP (PDB entry 2QY0 (134)) as search models. Iterative cycles of model building and reciprocal space positional, B-factor, and TLS refinement were carried out using COOT (135) and PHENIX.REFINE (72), respectively. The final model contains two copies of the eSALO-WT/zC1r-12SP complex in the asymmetric unit. Positive Fo-Fc electron density near the side chain of Y51 in both copies of eSALO-WT was observed during model building; upon confirmation of an increased m/z ratio consistent with tyrosine sulfation in the eSALO-WT sample by mass spectrometry, this position was modeled as sulfo-tyrosine X51 in both copies of eSALO-

WT. Both copies of eSALO-WT in the final model consist of residues 24–112 (UniProtKB Q5WPZ4). The first molecule of zC1r-12SP (Chain C) consists of residues 308–416, 421–600, and 604–703, while the second molecule (Chain D) consists of residues 308–415, 421–598, and 604–703 (human C1r, UniProtKB P00736). Although these chain breaks were included due to weak electron density, the zymogen activation loop spanning residues 458–468, including the scissile bond, remained intact in both zC1r-12SP molecules.

X-ray diffraction data for the eSALO-Y51F/zC1r-12SP complex were collected at ALS beamline 5.0.1 at Berkeley National Laboratory. Reflections were processed as described above and the structure was solved by molecular replacement using the eSALO-WT/zC1r-12SP structure as a search model. Model building and refinement followed the same general procedures described above. The final model consists of two molecules of eSALO-Y51F/zC1r-12SP in the asymmetric unit. Both copies of eSALO-Y51F in the final model consist of residues 24–112 (UniProtKB Q5WPZ4), with the exception of the Y51F mutation. The first molecule of zC1r-12SP (Chain C) consists of residues 308–417, 420–508, 515–597, and 605–703, while the second molecule (Chain D) consists of residues 308–417, 423–507, 513–597, and 604–703 (human C1r, UniProtKB P00736). As with the eSALO-WT/zC1r-12SP structure, chain breaks were included due to weak electron density, but the zymogen activation loop was intact in both zC1r-12SP copies.

Refined models for eSALO-WT/zC1r-12SP and eSALO-Y51F/zC1r-12SP have been deposited in the PDB under accession codes 9EKD and 9EKE, respectively. Additional descriptive statistics for X-ray diffraction data collection and model refinement are included in the accompanying table (Table 4.1).

C1r-proenzyme Autoactivation Assay

Inhibition of C1r-proenzyme autoactivation was assessed by detecting the cleavage of C1r-proenzyme using SDS-PAGE. All assays were established on ice in 10 μ L total volume and contained 3.3 μ M C1r-proenzyme and 8.3 μ M SALO protein. Each trial contained 6 μ L of C1r-proenzyme at 0.5 μ g/ μ L (supplied in a buffer of 10 mM imidazole (pH 6.0), 400 mM NaCl, 1 mM CaCl₂) and 4 μ L of SALO at 0.25 μ g/ μ L in HBS; an identical sample containing C1r-proenzyme alone incubated at 4 °C served as a negative control for cleavage, while a sample containing 1 μ g of C1r-enzyme alone was included as a positive control for cleavage. Reaction mixtures were then incubated as appropriate for 10 h at 37 °C. Reactions were terminated by adding SDS-PAGE sample buffer containing a reducing agent, followed by heating at 95 °C for 5 min. The cleavage products were separated by SDS-PAGE and visualized by Coomassie staining.

Hemolysis Assays

CP hemolysis assays were conducted in a 100 μ L reaction mixture containing 0.5 μ L of normal human serum (Innovative Research), 59.5 μ L of 0.1% (w/v) gelatin veronal buffer (GVB++; Complement Technologies), 20 μ L of SALO protein in GVB++ at the desired concentration, and 20 μ L of antibody-sensitized sheep erythrocytes (3×10^8 /mL in GVB++). The mixture was incubated at 37 °C for 30 min, followed by centrifugation at 200 $\times$ g for 10 min. The supernatant was then diluted four-fold in water, and the absorbance was measured at 412 nm. The percentage of lysis inhibition was calculated using the formula: $100 - [100 * (OD_{412, \text{sample}} - OD_{412, \text{buffer}}) / (OD_{412, \text{water}} - OD_{412, \text{buffer}})]$, and the IC₅₀ was determined by a four-parameter fit using GraphPad Prism v10.1.1.

Data availability

The atomic coordinates and structure factors for crystal structure determinations have been deposited in the Protein Data Bank (<http://wwpdb.org/>) and are publicly available through the

accession codes provided in Table 4.1. All other experimental data are available upon request by contacting the corresponding author B.V.G. (geisbrechtb@ksu.edu).

Results

SALO Binds Selectively to the Zymogen Form of Complement Component C1r

Ferreria et al. showed that SALO inhibited the CP upstream of the activation of complement component C4 (C4) (50). Based upon their observations, we hypothesized that SALO must somehow affect the early part of the CP involving C1 activation (Fig. 4.1A). If this were true, we reasoned that SALO might bind to either C1q, a protease zymogen, or an enzymatically-active protease to enable its complement inhibitory activity. To test this hypothesis, we prepared two different versions of recombinant SALO protein (Fig. S4.1A-D). The first version of SALO was expressed as an N-terminal polyhistidine-tagged fusion protein in *E. coli* in an insoluble form, purified under denaturing conditions using metal-ion affinity chromatography, refolded by slow dilution of the denaturant, and the monomeric, monodisperse fraction was isolated by size-exclusion chromatography (rSALO-WT). The second form of SALO was expressed as a C-terminal polyhistidine-tagged fusion protein by transiently-transfected HEK293(T) cells, secreted into the culture medium, and purified therefrom using a combination of metal ion-affinity and size-exclusion chromatographies (eSALO-WT). We then compared the activity of rSALO-WT to eSALO-WT using a hemolytic assay performed under conditions that stimulate the CP. Curiously, we found that eSALO-WT ($IC_{50}=84.9$ nM) was over 100-fold more potent than rSALO-WT ($IC_{50}\approx 16$ μ M) (Fig. 4.1B). As rSALO-WT was properly folded and maintained some potency in hemolytic assays, these observations suggested that eSALO-WT might undergo post-translational modification during expression in HEK293(T) cells that contributed to its activity.

We then used SPR to screen for interactions between covalently immobilized eSALO-WT with various components of the CP. We found that eSALO-WT displayed dose-dependent binding to intact C1 (Fig. 4.1C). However, eSALO-WT did not bind to C4 (Fig. 4.1D), which serves as the initial substrate for activated C1. As C1 consists of several discrete subcomponents (1, 127), we further screened for interactions between eSALO-WT and both the zymogen and enzymatically-active forms of C1r and C1s. Interestingly, we found that eSALO-WT displayed dose-dependent binding to the zymogen form of C1r (Fig. 4.1E), but that eSALO-WT did not bind to enzymatically-active C1r (Fig. 4.1F). eSALO-WT also failed to bind either to the zymogen (Fig. 4.1G) or the activated form of C1s (Fig. 4.1H). Thus, these screening data strongly suggested that SALO inhibits the CP by binding to the zymogen form of C1r.

SALO Binds to the Activation Loop of the C1r Zymogen

We sought structural information on the complex formed by SALO and the C1r zymogen. However, since the C1r zymogen undergoes autoactivation over time, we expressed an engineered fragment of human C1r consisting of its two CCP repeats and its serine protease domain, wherein the catalytic serine (S654) had been mutated to alanine (zC1r-12SP). We then used SPR to compare the affinity of surface captured eSALO-WT for the zymogen form of C1r that had been purified from human serum (Fig. 4.2A) with our zC1r-12SP fragment (Fig. 4.2B). Using a single-cycle kinetics approach, we found that eSALO-WT formed a slowly dissociating complex with both the native and recombinant forms of the C1r zymogen. This was consistent with high affinity interactions of eSALO-WT for both targets.

Thereafter, we crystallized eSALO-WT bound to zC1r-12SP, collected X-ray diffraction data extending to 3.3 Å limiting resolution, and solved the structure by molecular replacement (Table 4.1). Following iterative cycles of model building and refinement, our final model had R_{work}

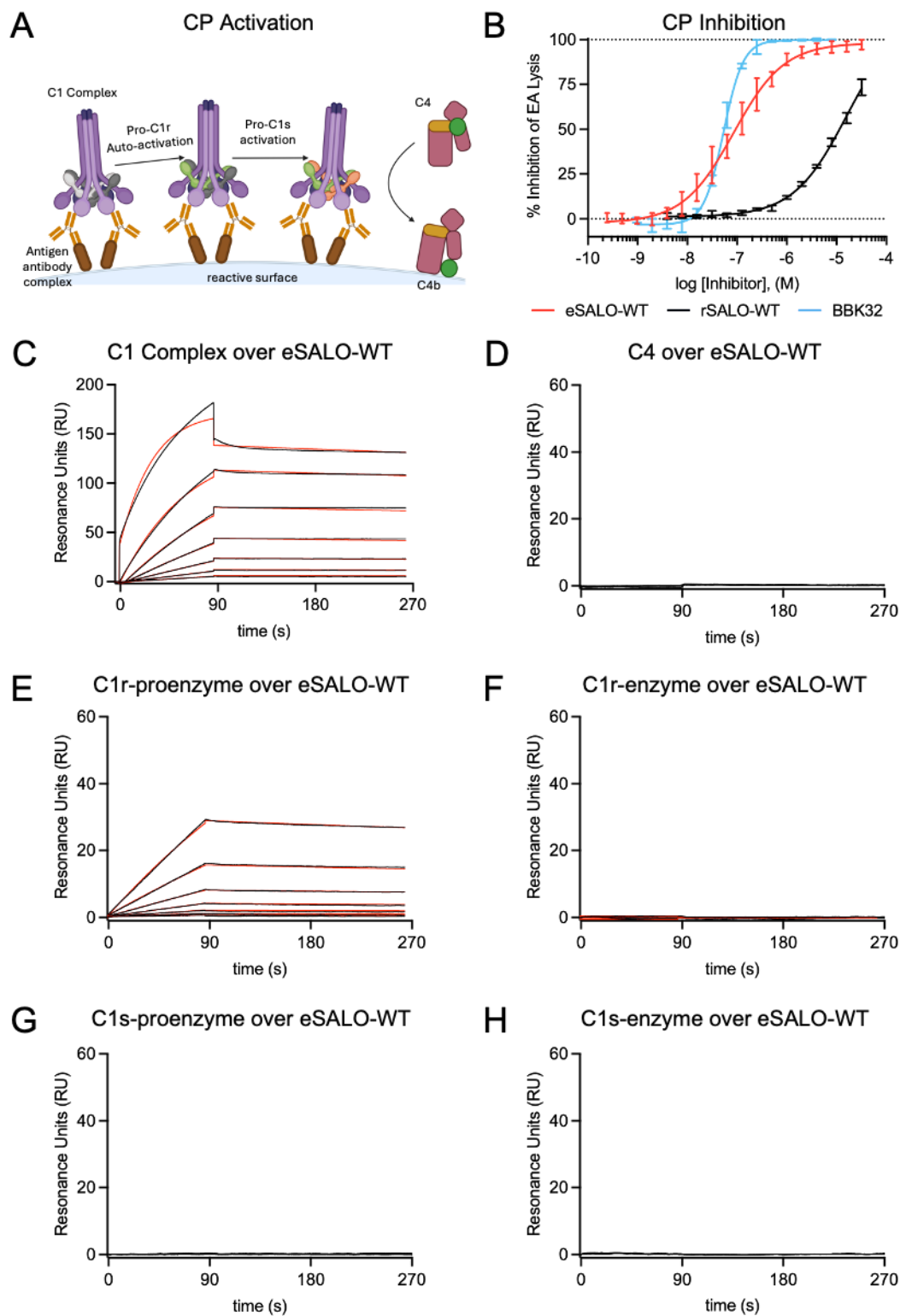


Figure 4.1 SALO Binds to the C1r Zymogen.

(A) Schematic representation of the earliest steps in the CP. The C1 complex recognizes antigen-bound antibodies at surfaces, leading to activation of the C1r zymogen. Active C1r converts the

C1s zymogen into its enzymatically-active form, resulting in cleavage of C4 and deposition of C4b on the surface. (B) Dose-dependent inhibition of the CP by eSALO-WT and rSALO-WT. Antibody-sensitized sheep erythrocytes were incubated with 0.5% (v/v) NHS, resulting in activation of the CP. The extent of complement-dependent lysis was monitored spectrophotometrically and normalized to a control that yielded 100% lysis. The effects eSALO-WT, rSALO-WT, and the *B. burgdorferi* C1r-inhibitor, BBK32 (136), were plotted as a function of concentration prior to curve fitting. Error bars represent the standard deviation from six technical replicates (n=6). (C) Binding of a two-fold concentration series of C1 to immobilized eSALO-WT. The apparent K_D was 0.8 nM. (D) Binding of a two-fold concentration series of C4 to immobilized eSALO-WT. (E) Binding of a two-fold concentration series of C1r proenzyme to immobilized eSALO-WT. The apparent K_D was 8.5 nM. (F) Binding of a two-fold concentration series of C1r enzyme to immobilized eSALO-WT. (G) Binding of a two-fold concentration series of C1s proenzyme to immobilized eSALO-WT. (H) Binding of a two-fold concentration series of C1s enzyme to immobilized eSALO-WT. Note that in the experiments shown in panels D, F, G, and H, the highest concentration used for each analyte was 1 μ M, while in panels C and E, the highest concentration used for each analyte was 100 nM. The black lines represent the reference-subtracted sensorgrams, while the red lines represent the outcome of fitting to a kinetic model. Representative sensorgrams are shown from a minimum of three technical replicates (n=3).

and R_{free} values of 24.2% and 28.7%, respectively (Fig. S4.2A-B & Table 4.1). The asymmetric unit of the crystal contained two copies of the eSALO-WT/zCr-12SP complex, which superimposed well upon each other (RMSD = 0.344 Å across 444 equivalent C α positions); thus, our subsequent analyses focused on the eSALO-WT/zC1r-12SP complex represented by chains A and C. Whereas the eSALO-WT/zC1r-12SP interaction buried 1098.5 Å² of surface area at the interface, the most extensive interactions between the two proteins were centered upon the zymogen activation loop of zC1r-12SP (Fig. 4.2C). By contrast, there were no contacts between eSALO-WT and either of the CCP domains of the protease, which may be less accessible within the context of the larger C1 structure (137). Thus, the eSALO-WT/zC1r-12SP complex structure was in good agreement with the pronounced binding selectivity of eSALO-WT for the C1r zymogen when compared to enzymatically-active C1r (c.f. Fig. 4.1E vs Fig. 4.1F).

We noted several other interesting features upon closer inspection of the eSALO-WT/zC1r-12SP structure. Foremost, the α -helical bundle fold of eSALO-WT appeared well-suited to binding an extended conformation polypeptide (Fig. 4.2D & Fig. S4.3A). eSALO-WT displayed numerous

potential hydrogen bond donors and acceptors along the concave channel that is a defining feature of its tertiary structure. Consistent with this, EBI-PISA (78) identified 13 likely hydrogen bonds and 4 candidate salt bridges between the residues lining this channel, including N33 and Y/X51 (explained further in the following subsection), and residues from the zymogen activation loop of zC1r-12SP, which is comprised of residues R461-K471 inclusive. At the periphery of the eSALO-WT channel, the sidechain of E58 formed a salt bridge with the sidechain of R461 from zC1r-12SP. It seems that this interaction might help constrain the zymogen activation loop, which constitutes the primary eSALO-WT binding site. Finally, outside the concave channel, residues on the first α helix of eSALO-WT, including D32, appeared to contact a secondary site on zC1r-12SP, which is comprised of residues H642-Q647 inclusive. It is possible this secondary site could help contribute to the selectivity of SALO for C1r over other protease zymogens.

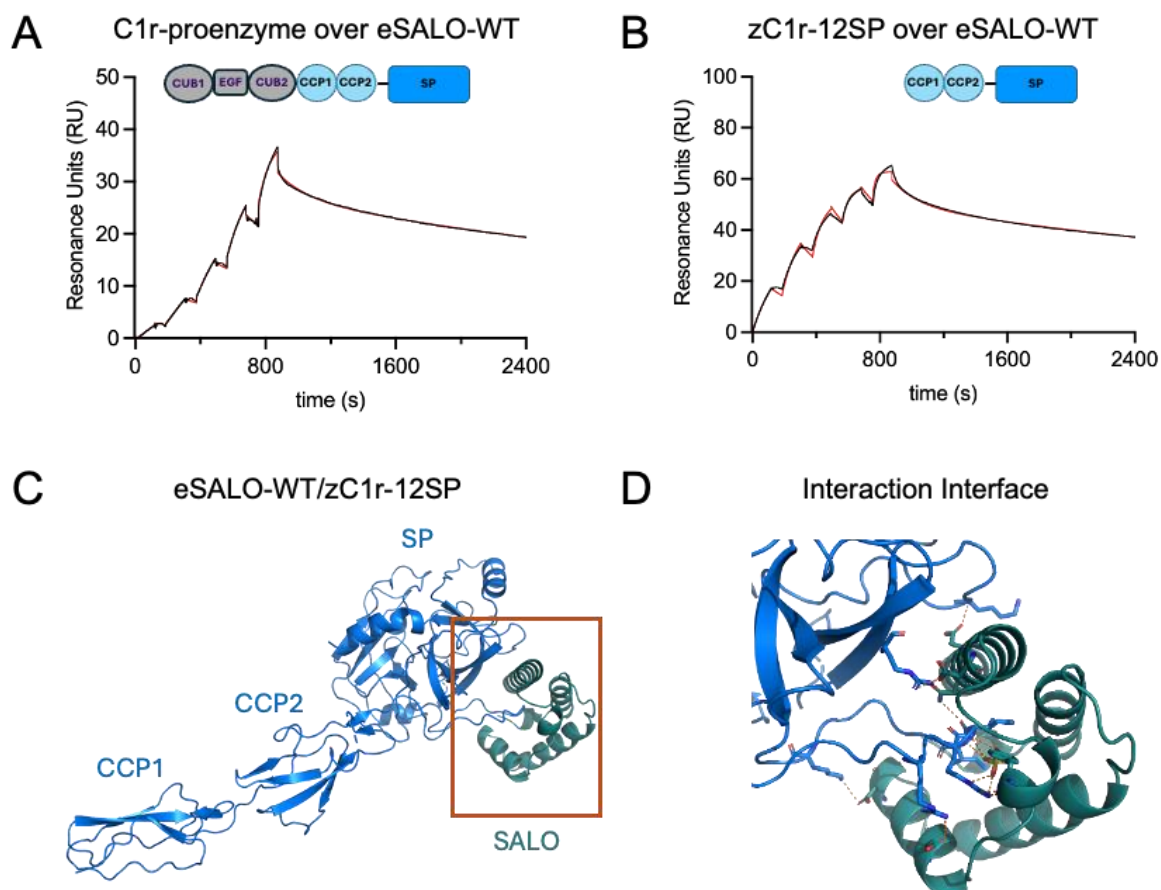


Figure 4.2 SALO Binds the Activation Loop of the C1r Zymogen.

(A) Binding of C1r proenzyme to surface-captured, biotinylated eSALO-WT. Interactions were monitored by injecting C1r proenzyme in single-cycle mode, using 6.25, 12.5, 25, 50, and 100 nM concentrations of analyte. A representative reference-subtracted sensorgram (black line) was analyzed using a two-state kinetic model (red line); the apparent K_D was 11.1 nM. A cartoon diagram of the domain structure of C1r proenzyme is inset. (B) Binding of zC1r-12SP to surface-captured, biotinylated eSALO-WT. Interactions were monitored by injecting zC1r-12SP in single-cycle mode, using 25, 50, 100, 200, and 400 nM concentrations of analyte. A representative reference-subtracted sensorgram (black line) was analyzed using a two-state kinetic model (red line); the apparent K_D was 0.7 nM. A cartoon diagram of the domain structure of zC1r-12SP is inset. (C) Representation of the eSALO-WT/zC1r-12SP structure, determined at 3.3 Å limiting resolution. zC1r-12SP is shown as a blue ribbon diagram, while eSALO-WT is shown as a teal ribbon diagram. Note the only contacts of eSALO-WT with zC1r-12SP involved the SP domain. (D) Close-up view of the interaction interface between eSALO-WT and zC1r-12SP. The region shown in this image corresponds to the area within orange rectangle in panel C.

Evidence for Sulfation of SALO Tyrosine-51

During the course of model building and refinement, we noticed a strong, positive feature in the Fo-Fc electron density map distal to the hydroxyl group of eSALO-WT residue Y51 (Fig. 4.3A). Based on the geometry of this electron density, we suspected that Y51 had undergone post-translational sulfation during eSALO-WT expression and secretion from HEK293(T) cells. We obtained several pieces of analytical information that supported this hypothesis. First, mass spectrometry analyses on samples of eSALO-WT detected a species whose m/z ratio was 80 units greater than expected for unmodified eSALO-WT, which was consistent with the addition of a sulfate group to Y51 (Fig. S4.1C-D). Second, corresponding analyses on samples of a site-directed mutant wherein Y51 was exchanged for F (eSALO-Y51F) did not show a similar species at this increased m/z value (Fig. S4.1C-D). Similarly, this increased m/z species was not observed in additional site-directed mutants that were prepared for subsequent structure/function studies so long as the Y51F substitution was present (Fig. S4.1C-D). Finally, the presence of tyrosine sulfation could explain the difference in potency between eSALO-WT and rSALO-WT (Fig. 4.1B), since *E. coli* is not known to incorporate this modification into its proteins (138). Therefore,

we subsequently modeled this residue as sulfo-tyrosine, X51 (Fig. 4.3B). In the final model this sulfate group formed three different hydrogen bonds ranging from 2.6-3.2 Å in length with groups derived from R463 and I464, which are the two residues linked by the scissile bond in the C1r zymogen (Fig. 4.3C). It also formed an additional hydrogen with the backbone nitrogen from I465, which is immediately C-terminal to the C1r zymogen residues that share the scissile bond.

Table 4.1 X-ray Diffraction Data, Structure Solution, and Refinement Statistics

X-ray Diffraction Data, Structure Solution, and Refinement Statistics^a		
PDB Accession Code	eSALO-WT/zC1r-12SP 9EKD	eSALO-Y51F/zC1r-12SP 9EKE
<i>Data Collection</i>		
Beamline	NSLS-II 19-ID	ALS 5.0.1
Space Group	<i>P</i> ₃ ₂ ₂₁	<i>P</i> ₃ ₂ ₂₁
Wavelength (Å)	1.000	0.9744
Cell Dimensions		
a, b, c (Å)	117.83, 117.83, 190.58	117.81, 117.81, 195.59
Resolution (Å)	50.00-3.30	50.00-3.10
Wilson B-Factor (Å ²)	128.1	75.7
Completeness (%)	100.0 (100.0)	100.0 (100.0)
I / σ I	11.5 (1.5)	13.2 (1.0)
R _{pim}	0.055 (0.585)	0.062 (0.749)
CC _{1/2}	0.930 (0.514)	0.995 (0.441)
Redundancy	17.7 (15.2)	18.1 (16.9)
<i>Refinement</i>		
Resolution (Å)	49.29-3.28	49.36-3.09
Number of Reflections	23,486	25,373
R _{work} / R _{free} (%)	24.2 / 28.7	23.1 / 27.8
Atoms Modeled	7,644	7,515
Ramachandran Plot		
Favored/Allowed (%)	89.04 / 9.46	90.94 / 7.64
Average B-Factors (Å ²)	149.0	91.0
R.M.S. Deviations		
Bond Lengths (Å)	0.002	0.002
Bond Angles (°)	0.43	0.46

^aValues in parentheses are for the highest-resolution shell.

To further investigate this modification, we crystallized the eSALO-Y51F mutant with zC1r-12SP, collected X-ray diffraction data extending to 3.1 Å limiting resolution, and solved the structure by molecular replacement (Table 4.1). After completing model building and refinement, our final model had R_{work} and R_{free} values of 23.1% and 27.8%, respectively (Fig. S4.2C-D & Table 4.1). We found that the eSALO-Y51F/zC1r-12SP structure largely mirrored that of eSALO-WT/zC1r-12SP (Fig. 4.3D & Fig S4.2E). Although the contributions from the sulfate modification were expectedly absent (Fig. 4.3E), many of the interactions present in the wild-type structure were maintained. This included the hydrogen bonds formed by D32 and the salt bridges formed by E58, but not the hydrogen bonds formed by N33 (Fig. S4.3B). Overall, these structures showed that sulfation of SALO Y51 promoted interactions with residues sharing the scissile bond of the C1r zymogen, but that sulfation was not necessary for SALO binding to the C1r zymogen.

Mutagenesis Studies Identify SALO Residues Required for Binding the C1r Zymogen

We used the crystal structures described above as basis for investigating the function of various SALO residues. In addition to eSALO-Y51F, we overexpressed and purified a mutant where E58 was exchanged for alanine (eSALO-E58A) and a double mutant where both D32 and N33 were exchanged for alanine (eSALO-DN); we also prepared a combinatoric mutant where D32, N33, and E58 were exchanged for alanine along with the Y51F substitution (eSALO-DNYE). Each of these eSALO variants was characterized by SDS-PAGE, CD spectropolarimetry, and mass spectrometry (Fig. S4.1C-D). We then used SPR to examine their affinity for both C1r proenzyme that had been purified from human serum as well as zC1r-12SP (Table 4.2). Similar to the experiment described above (Fig. 4.2A-B), we found that eSALO-WT bound with low nanomolar affinity to both analytes as judged by their apparent K_D values of 5.6 ± 1.2 nM and 1.1 ± 0.0 nM, respectively (Fig. 4.4A-B). However, we found that the affinity of eSALO-Y51F for C1r

proenzyme and zC1r-12SP was diminished 7.5-fold and 8.2-fold, respectively (Fig. 4.4C-D). Whereas eSALO-E58A displayed only a mild 1.8-fold and 2.3-fold loss of affinity for both analytes (Fig. 4.4E-F), we found that neither eSALO-DN (Fig. 4.4G-H) nor eSALO-DNYE (Fig. 4.4I-J) bound to C1r proenzyme or zC1r-12SP in these experiments.

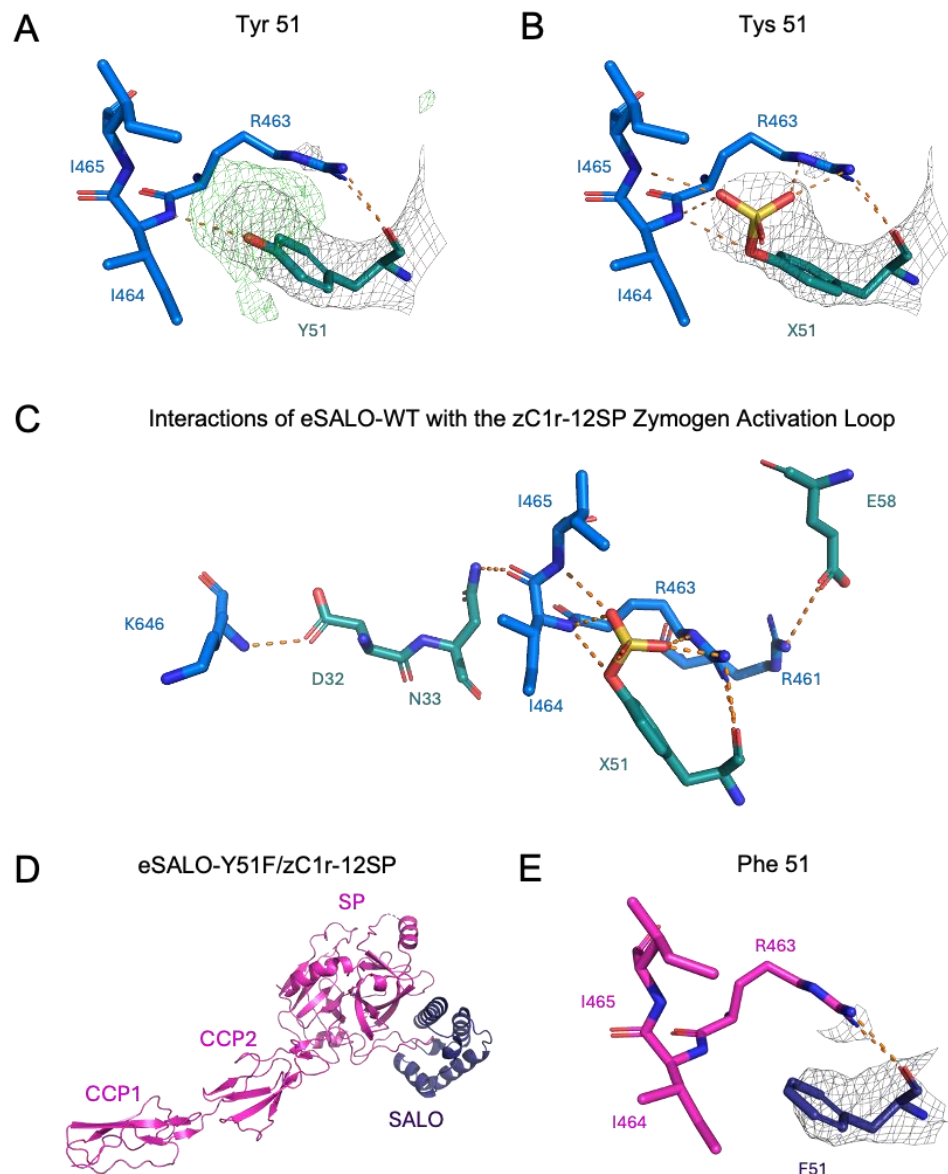


Figure 4.3 Sulfation of SALO Tyrosine 51 Contributes to But Is Not Necessary for Binding the C1r Zymogen.

(A) Close-up view of eSALO-WT Y51 (carbon atoms in teal) and its interactions with residues sharing the scissile bond in zC1r-12SP (carbon atoms in blue). Likely hydrogen bonds are drawn with dashed orange lines. The 2Fo-Fc map ($1.2\ \sigma$) in the vicinity of Y51 is shown as a grey mesh, while the Fo-Fc map ($3.0\ \sigma$) is shown as a green mesh. (B) Identical diagram to panel A, except that a tyrosine sulfate (X51) was included in the eSALO-WT model. Note the presence of additional hydrogen bonds formed between X51 and groups from the zC1r-12SP. (C) Schematic representation of the interactions found at the interface of eSALO-WT (carbon atoms in teal) and zC1r-12SP (carbon atoms in blue). The identities of relevant residues are labeled in teal for eSALO-WT and blue for zC1r-12SP. (D) Representation of the eSALO-Y51F/zC1r-12SP structure, determined at 3.1 Å limiting resolution. zC1r-12SP is shown as a magenta ribbon diagram, while eSALO-Y51F is shown as a purple ribbon diagram. (E) Close-up view of eSALO-Y51F F51 (carbon atoms in purple) and its interactions with residues sharing the scissile bond in zC1r-12SP (carbon atoms in magenta). Likely hydrogen bonds are drawn with dashed orange lines.

SALO Inhibits the Classical Pathway by Blocking Activation of the C1r Zymogen

Purified C1r proenzyme is unstable and will undergo spontaneous autoactivation at physiological temperatures. Although this feature reflects the functional role of C1r in complement activation, whether C1r autoactivation occurs in cis (139) or in trans (140, 141) within the C1 complex remains a topic of investigation. Nevertheless, this process can be monitored by SDS-PAGE as autoactivation of the C1r zymogen results in cleavage of its 92 kDa polypeptide into 57 kDa and 35 kDa fragments. Since our structural observations showed that eSALO-WT binds the zymogen activation loop of zC1r-12SP (Figs 4.2-4.3), we hypothesized that eSALO-WT binding would prevent C1r zymogen autoactivation. To test this idea, we incubated C1r proenzyme with either eSALO-WT, the mutant forms of eSALO described above, or buffer alone at 37 °C for 10 hr. We then separated the reaction products by SDS-PAGE and visualized the contents by Coomassie Brilliant Blue staining (Fig. 4.5A). While C1r zymogen underwent efficient autoactivation in this experiment, we found that eSALO-WT blocked the autoactivation reaction. Moreover, we noticed that the ability of the eSALO variants to block autoactivation correlated with their affinity for C1r proenzyme as measured by SPR (Table 4.2). eSALO-E58A was less effective than eSALO-WT, but was better than eSALO-Y51F in blocking C1r autoactivation. Both

eSALO-DN and eSALO-DNYE failed to block C1r autoactivation altogether. Thus, SALO binding was required to block autoactivation of the C1r zymogen.

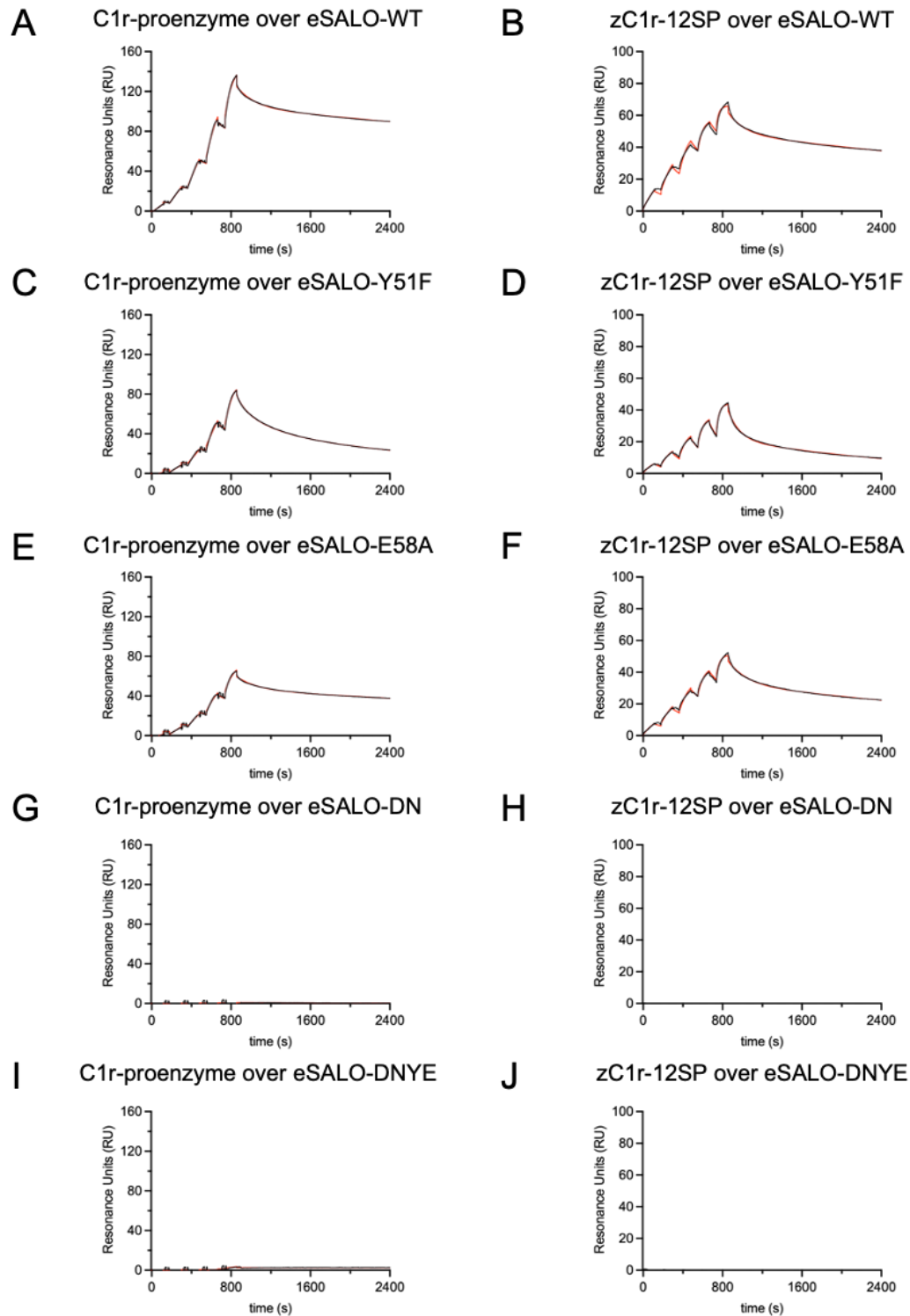


Figure 4.4 Characterization of Site-Directed SALO Mutants by SPR.

Binding of C1r proenzyme and zC1r-12SP to surface-captured, biotinylated eSALO proteins was analyzed by SPR. Interactions were monitored by injecting analytes in single-cycle mode, using 25, 50, 100, 200, and 400 nM concentrations for all experiments. Reference-subtracted sensorgrams (black line) were analyzed using a two-state kinetic model (red line). Representative experiments from three technical replicates (n=3) are shown, while the final fitting parameters are presented in Table 4.2. (A) Binding of C1r proenzyme to eSALO-WT. (B) Binding of zC1r-12SP to eSALO-WT. (C) Binding of C1r proenzyme to eSALO-Y51F. (D) Binding of zC1r-12SP to eSALO-Y51F. (E) Binding of C1r proenzyme to eSALO-E58A. (F) Binding of zC1r-12SP to eSALO-E58A. (G) Binding of C1r proenzyme to eSALO-DN. (H) Binding of zC1r-12SP to eSALO-DN. (I) Binding of C1r proenzyme to eSALO-DNYE. (J) Binding of zC1r-12SP to eSALO-DNYE.

We previously found that eSALO-WT blocked CP-driven hemolysis with an IC_{50} value of 84.9 nM (Fig. 4.1B). Therefore, we used identical hemolytic assays to test if the apparent IC_{50} values of these eSALO variants correlated with their ability to bind C1r proenzyme and block its autoactivation (Fig. 4.5B & Table 4.2). We found that eSALO-E58A displayed an IC_{50} value diminished by 14.1-fold, while eSALO-Y51F was even less potent with an estimated 171-fold loss compared to eSALO-WT. We noted that rSALO-WT was of similar potency to eSALO-Y51F, which suggested that the differences between these two variants and eSALO-WT was largely due to sulfation of Y51. Finally, both eSALO-DN and eSALO-DNYE had little inhibitory activity, which was consistent with their behavior in the autoactivation assay, as well as in direct binding studies. Therefore, SALO binding to the C1r zymogen blocks formation of active C1r enzyme, which results in potent inhibition of the CP. Since activation of the C1r zymogen is the furthest upstream proteolytic event in the CP, our observations here define a novel mode of complement inhibition.

Table 4.2 Inhibitory Potencies^a and Protein-Protein Interaction Parameters^{b,c} for Various SALO Proteins

Analyte	ligand	IC ₅₀ (nM)	IC ₅₀ relative	K _D (nM)	K _D relative	K _{a-1} (10 ⁵ M ⁻¹ s ⁻¹)	K _{d-1} (10 ⁻³ s ⁻¹)	K _{a-2} (10 ⁻³ s ⁻¹)	K _{d-2} (10 ⁻⁴ s ⁻¹)
C1r-proenzyme	eSALO-WT	84.9 (75.0-96.1)	1	5.6±1.2	1	0.3±0.1	2.2±0.4	3.0±0.1	2.7±0.1
	eSALO-Y51F	14,520 (10,390-25,410)	171	41.9±22.3	7.5	3.7±4.3	18.3±18.6	0.8±0.7	6.9±3.0
	eSALO-E58A	1,197 (1,138-1,261)	14.1	10.3±2.8	1.8	0.5±0.5	2.7±1.1	2.3±0.3	3.7±0.9
	eSALO-DN	-	-	-	-	-	-	-	-
	eSALO-DNYE	-	-	-	-	-	-	-	-
zC1r-12SP	eSALO-WT	-	-	1.1±0.0	1	3.7±0.1	3.8±0.1	2.4±0.0	2.8±0.0
	eSALO-Y51F	-	-	9.0±1.8	8.2	2.8±0.1	8.0±1.0	1.7±0.0	7.7±0.7
	eSALO-E58A	-	-	2.5±0.1	2.3	2.6±0.1	3.4±0.0	1.8±0.1	4.1±0.1
	eSALO-DN	-	-	-	-	-	-	-	-
	eSALO-DNYE	-	-	-	-	-	-	-	-

^aValues in parentheses represent the lower and upper limits for the 95 % confidence interval following non-linear curve fitting.

^bAll values are presented as the mean ± SD obtained from three replicate injections over the biosensor surface, and are taken from the experiments presented in Fig. 4.4.

^cValues result from fitting to a two-state binding model.

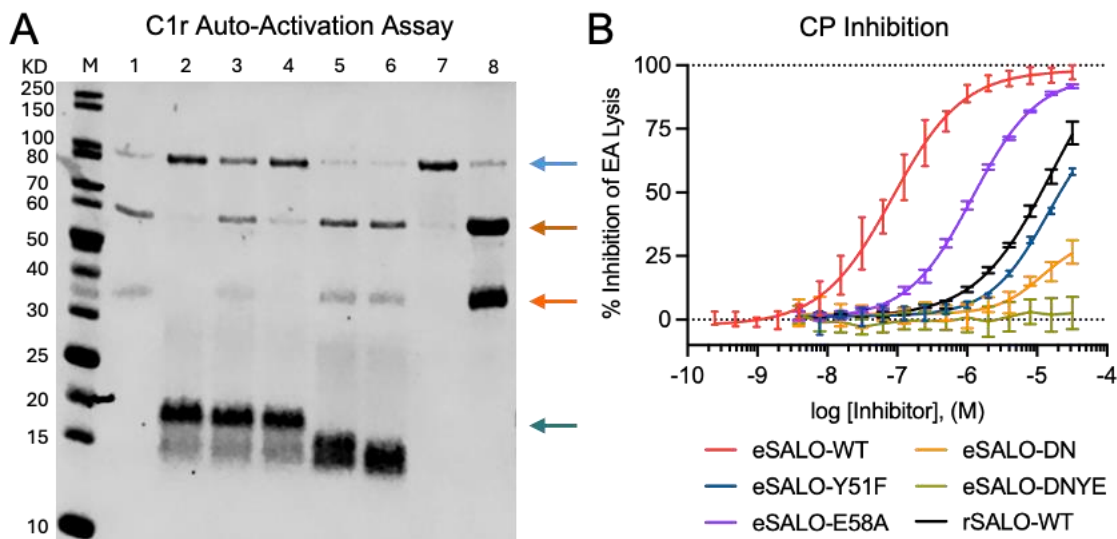


Figure 4.5 SALO Inhibits the CP by Blocking Activation of the C1r Zymogen.

(A) Autoactivation of C1r proenzyme was monitored by SDS-PAGE. Bands corresponding to polypeptides of interest are indicated by colored arrows: intact C1r proenzyme (92 kDa; light blue); activated C1r, heavy chain (57 kDa; light orange); activated C1r, light chain (35 kDa; dark orange); eSALO proteins (15-20 kDa; dark blue). The identities of individual lanes are as follows: M, molecular weight markers; 1, C1r proenzyme incubated with buffer alone; 2, C1r proenzyme incubated with eSALO-WT; 3, C1r proenzyme incubated with eSALO-Y51F; 4, C1r proenzyme incubated with eSALO-E58A; 5, C1r proenzyme incubated with eSALO-DN; 6, C1r proenzyme incubated with eSALO-DNYE; 7, C1r proenzyme incubated at 4 °C to prevent autoactivation (control); 8, C1r enzyme (control). The experiment shown is representative of three technical

replicates (n=3). (B) Dose-dependent inhibition of the CP by various SALO proteins. Antibody-sensitized sheep erythrocytes were incubated with 0.5% (v/v) NHS, resulting in activation of the CP. The extent of complement-dependent lysis was monitored spectrophotometrically and normalized to a control that yielded 100% lysis. The effects of various inhibitors were plotted as a function of concentration prior to curve fitting. Error bars represent the standard deviation from six technical replicates (n=6).

Discussion

Although SALO was identified as a potent, specific CP inhibitor nearly a decade ago (50), the molecular basis of its mechanism has remained unclear since that time. Here, we used a biochemical screening strategy to show that SALO binds selectively and with nanomolar affinity to the zymogen of C1r (Fig. 4.1). Our structural studies revealed that SALO binds to the activation loop of the C1r zymogen (Figs. 4.2-4.3 & Figs. S2-S3), shielding its scissile bond from proteolytic cleavage (Fig. 4.5). While we determined that the interaction between SALO and C1r proenzyme is enhanced by sulfation of SALO Y51 (Fig. 4.3, Fig. S4.1 & Fig. S4.3), we also found that this modification is not required for SALO binding or activity (Figs. 4.4-4.5). By blocking the first enzymatic step in C1 activation, SALO affects robust inhibition of the CP (Fig. 4.5). To our knowledge, this sort of mechanism defined by SALO has not been described previously. Therefore, it constitutes a novel mode of complement inhibition not seen in other naturally occurring regulatory molecules, immune evasion proteins, or therapeutic modalities.

During the course of this study, we determined that SALO expressed from HEK293(T) cells was modified by tyrosine sulfation at position Y51 (Fig. 4.3, Fig. S4.1 & Fig. S4.3). Although this finding was unanticipated, the presence of positive Fo-Fc electron density overlapping the phenolic oxygen of Y51 in the eSALO-WT/zC1r-12SP structure strongly suggested this possibility. We attempted to confirm the presence of tyrosine sulfation in eSALO-WT samples using immunoblotting, but were unsuccessful with that approach. However, using high accuracy mass spectrometry, we detected species of 80 m/z greater than expected in samples of eSALO-

WT, eSALO-E58A, and eSALO-DN, but not in samples of eSALO-Y51F or eSALO-DNYE, where the modification site (i.e. Y51) had been mutated (Fig. S4.1); this m/z change corresponded very well to the increased molecular weight expected from addition of a sulfate group. Since the mass spectrometry data we obtained are not quantitative, it is not possible to know the extent of tyrosine sulfation in these samples. Nevertheless, incorporation of tyrosine sulfate (i.e. X51) instead of tyrosine alone (i.e. Y51) in the eSALO-WT/zC1r-12SP structure improved the model to electron density map correlation and appeared to result in the formation of four additional hydrogen bonds, three of which involved R463 and I464, which are the residues that share the scissile bond in the C1r zymogen (Fig. 4.3 & Fig. S4.3). At the functional level, this modification appeared to result in an ~8-fold gain in affinity for the C1r zymogen and an ~170-fold gain in potency in CP-dependent hemolytic assays, as judged by comparison of eSALO-WT and eSALO-Y51F (Figs. 4.4-4.5 & Table 4.2). The existence of two likely tyrosine protein sulfotransferase isoforms in *L. longipalpis* that share 78% and 83% identity to the *Drosophila melanogaster* TPST, Tango-13, strongly suggests that natively expressed SALO will undergo tyrosine sulfation to some extent. Conceivably, this could explain the potent CP inhibitory activity ascribed to *L. longipalpis* salivary gland homogenates some years ago (142).

Given the complex structure/function relationships of C1 and its essential role in triggering CP activity (Fig. 4.1A), it is not surprising that several different immune evasion proteins acting at the level of C1 have been described (128). Perhaps the best understood among these come from the Lyme Disease spirochete, *B. burgdorferi*, which expresses at least two different classes of C1 inhibitors (143). The cell-surface lipoprotein BBK32 binds to the active site of C1r enzyme, thereby blocking not only C1r autoactivation but activation of the C1s zymogen as well (136, 144, 145). It also binds tightly to the zymogen of C1r, albeit through a distinct binding site from the

activation loop recognized by SALO (Fig. 4.2 & Fig. S4.2; (145)). Separately, the *B. burgdorferi* outer membrane lipoproteins ElpB and ElpQ both bind to the activated forms of C1r and C1s (143). Although no crystal structures for their representative complexes with either protease are available, biochemical studies have shown that both ElpB and ElpQ interfere with recognition of the substrate, C4, without occluding the C1s active site (146, 147). Despite this elaborate and seemingly redundant complement evasion program, no protein with a mechanism comparable to SALO has been identified from *B. burgdorferi* or related spirochetes (148). While this does not rule out the existence of such a molecule, the divergent mechanisms displayed by SALO versus BBK32, ElpB, and ElpQ could reflect evolutionary adaptations to different selective pressures faced by hematophagous arthropods like *L. longipalpis* versus bloodborne bacteria like *B. burgdorferi* (128). They may also underscore unique requirements for effectively inhibiting C1 in solution as opposed to on surfaces. Exploring this latter possibility further will be a topic for future investigations.

Several C1 inhibitors have either been approved for therapeutic use in humans or are undergoing investigation for treating CP-linked conditions, including hereditary angioedema (HAE), cold agglutinin disease (CAD), and immune thrombocytopenia, among others (Reviewed in (33, 62, 63)). The most established of these is the aptly named C1 inhibitor (C1-INH), which is marketed under various tradenames depending on the manufacturer for treatment of HAE. Forms of C1-INH are also being evaluated for treating ischemia/reperfusion injuries and organ transplantation in phase-II and phase-III clinical trials. C1-INH is a SERPIN, and inhibits the active forms of both C1r and C1s. However, it also inhibits activated mannose-binding lectin-associated serine proteases (MASPs) that function within the LP, as well as several activated coagulation factors, and is therefore not specific in its action against the CP. By contrast, the anti-

C1s monoclonal antibody, sutimlimab, specifically blocks cleavage of the substrates, C4 and C2, in response to CP activation. Sutimlimab was recently approved for treatment of CAD (149), providing transformative evidence that an individual subcomponent of C1 could be selectively targeted for therapeutic purposes. In that regard, we have shown that SALO inhibits generation of active C1r (Fig. 4.5), which is an essential event that precedes substrate cleavage by C1s (Fig. 4.1A). As SALO blocks an earlier event within the CP than sutimlimab, it is possible that SALO could hold certain therapeutic advantages insofar as C1 inhibition is concerned (Fig. 4.1A). Unfortunately, not much is known about the antigenicity of SALO in human populations. Nevertheless, its potential utility would seem to place a premium on identifying any molecules that mimic the SALO mechanism for inhibiting C1r activation.

Zymogen activation is a seemingly ubiquitous process used to regulate the catalytic properties of enzymes in bacteria, fungi, plants, and animals. Whereas some of the best understood examples have come from classical structure/function studies on digestive proteases like trypsin(ogen) and chymotrypsin(ogen) (150), cascades of protease zymogen activation also form the basis of biochemical networks that serve broad roles in defense and repair. This includes not only the complement (1-3) and coagulation systems (151) in humans and other mammals, but conceptually related pathways that fulfill analogous roles in various insect species such as *Anopheles gambiae*, *Drosophila melanogaster*, and *Manduca sexta*, among others (152). Despite myriad studies on such systems across the last several decades, we are unaware of any endogenous regulators or pathogen/parasite-derived inhibitory molecules that interfere with zymogen activation by shielding its scissile bond from cleavage, as does SALO. Considering the numerous zymogens present in blood, as well as the remarkable diversity among hematophagous organisms

and blood-borne pathogens, we suspect that additional inhibitors like SALO exist in nature, and remain to be discovered.

Acknowledgement

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Footnotes

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Abbreviations

C1; complement component C1
C1-INH; complement component C1 inhibitor
C2; complement component C2
C3; complement component C3
C3b; complement component C3b
C4; complement component C4
C4b; complement component C4b
C5; complement component C5
CCP; complement control protein domain
CP; classical pathway of complement
GVB; gelatin veronal buffer
GVB++; gelatin veronal buffer with magnesium and calcium
HBS-T; HEPES buffered saline with tween-20
HBS; HEPES buffered saline
MASP; mannose-binding lectin-associated serine protease
NHS; Normal Human Serum
PEG; polyethylene glycol
SP; serine protease domain
SPR; surface plasmon resonance
TCC; terminal complement complex
zC1r-12SP; C1r fragment consisting of its CCP repeats and serine protease domain with S654A mutation

Supplementary information

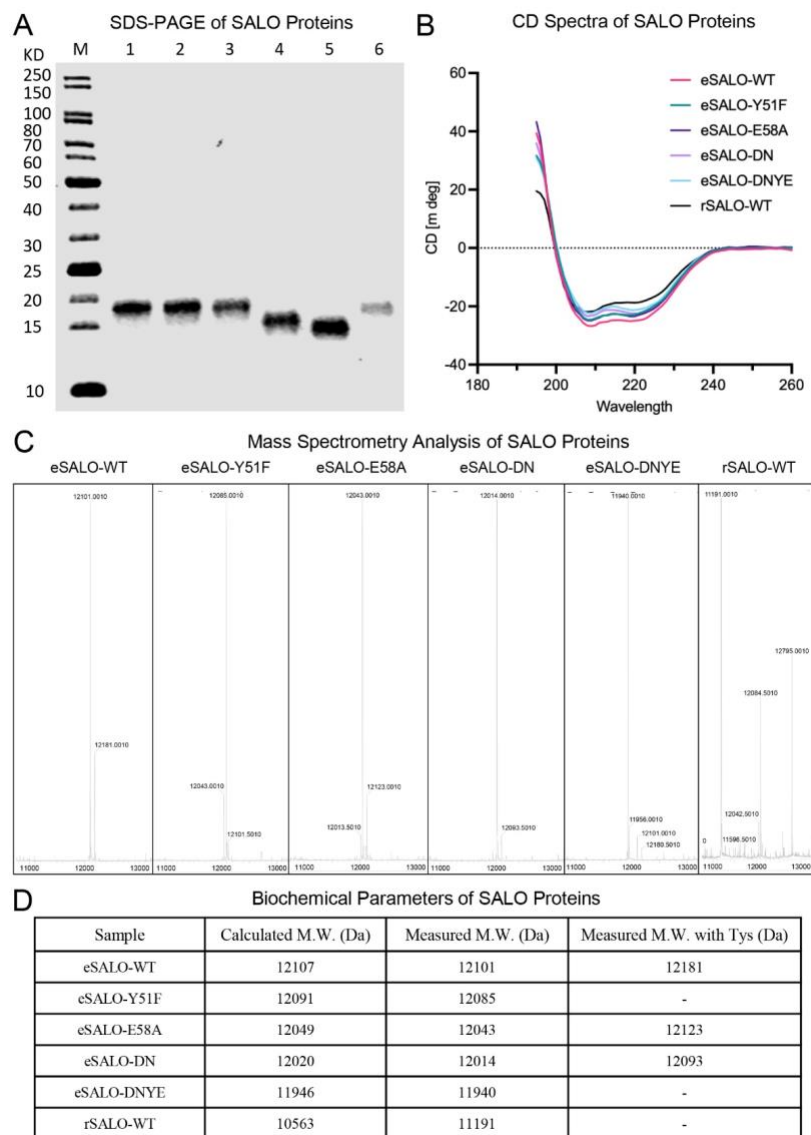


Figure S4.1. Supporting information pertaining the characterization of SALO proteins. (A) SDS-PAGE analysis of recombinant SALO proteins used in this study. 3 μ g of each protein were processed for SDS-PAGE under reducing conditions. The identities of individual lanes are as follows: M, molecular weight markers; 1, eSALO-WT; 2, eSALO-Y51F; 3, eSALO-E58A; 4, eSALO-DN; 5, eSALO-DNYE; 6, rSALO-WT. (B) CD Spectra of recombinant SALO proteins used in this study. Spectra were collected on 37.5 μ M protein dissolved in a buffer of 5 mM HEPES (pH7.4), 35 mM NaCl. A legend identifying the individual spectral traces is inset. (C) Mass spectrometry analysis of recombinant SALO proteins used in this study. Protein samples were separated by a C₄ UPLC column prior to collecting ESI-TOF data in positive and MSe mode. Regions corresponding to m/z values from 11,000 to 13,000 are presented for each SALO protein. Note the presence of an additional species at m/z ~80 units greater than the major species in all eSALO samples containing Y51, consistent with the presence of tyrosine sulfation. The major species identified also corresponds well to the expected molecular weight of each SALO protein, despite the fact that several SALO variants display anomalous migration in SDS-PAGE experiments. (D) Table of biochemical parameters for recombinant SALO proteins used in this study. Calculated molecular weights for HEK293(T)-cell expressed forms were determined from the predicted secretion signal peptide cleavage site using the SignalP 6.0 server. The difference of 6 units in m/z corresponds to loss of hydrogen atoms following formation of 3 pairs of disulfide bonds.

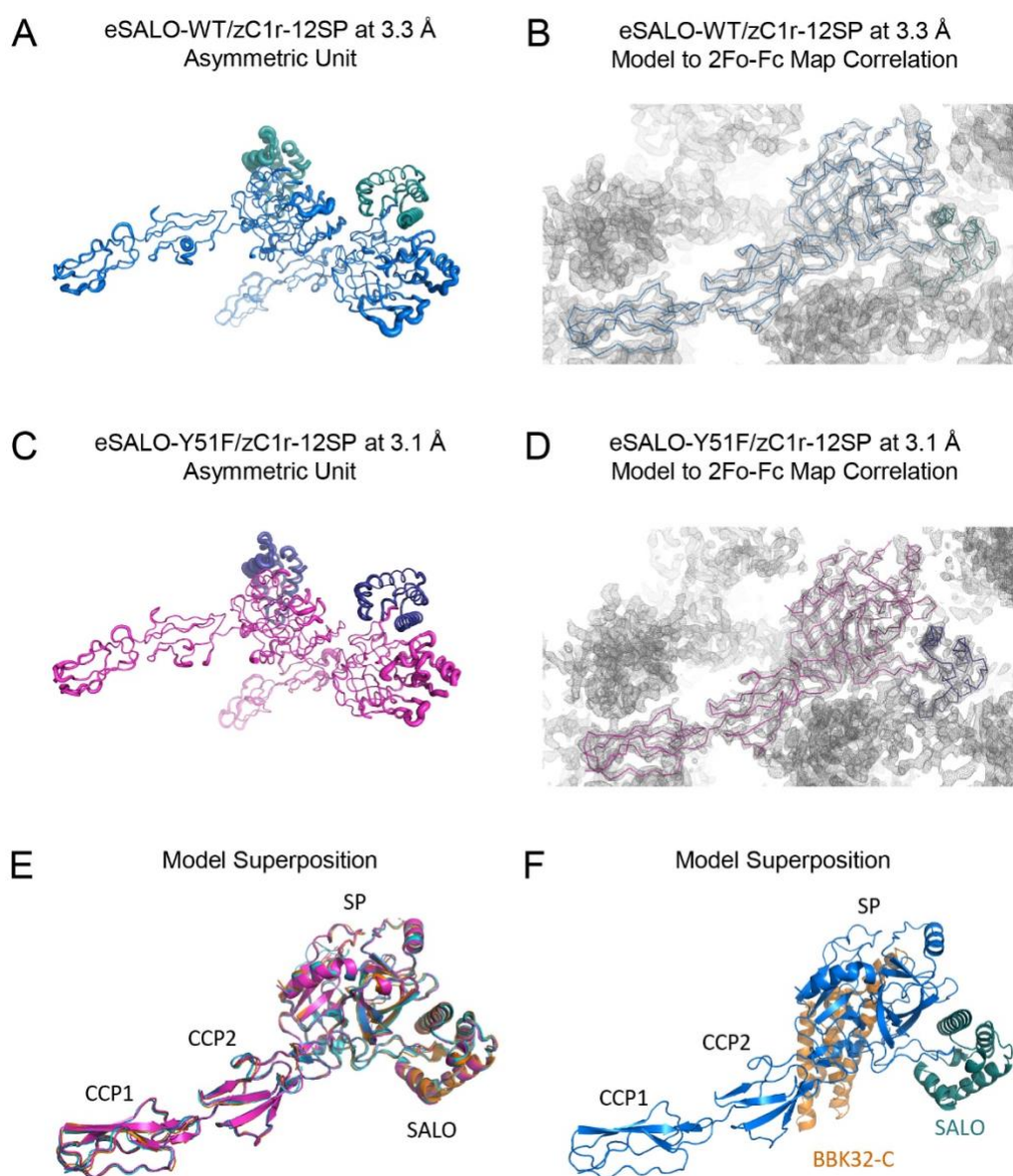


Figure S4.2. Supporting information pertaining to the structures of eSALO-WT and eSALO-Y51F bound to zC1r-12SP. (A) Model of the entire asymmetric unit of the eSALO-WT/zC1r-12SP crystal. eSALO-WT molecules are colored teal, while zC1r-12SP molecules are colored blue. The thickness of the tracing represents the final B-factor of that residue, with thicker tracing indicating higher B-factors. (B) Representative model to map correlation around chains A and C of the eSALO-WT/zC1r-12SP complex. The 2Fo-Fc map calculated at 3.3 Å limiting resolution and contoured at 1.2 σ is drawn as a grey mesh. Proteins are colored as in Panel A. (C) Model of the entire asymmetric unit of the eSALO-Y51F/zC1r-12SP crystal. eSALO-Y51F molecules are colored purple, while zC1r-12SP molecules are colored magenta. The thickness of the tracing represents the final B-factor of that residue, with thicker tracing indicating higher B-factors. (D) Representative model to map correlation around chains A and C of the eSALO-Y51F/zC1r-12SP complex. The 2Fo-Fc map calculated at 3.1 Å limiting resolution and contoured at 1.2 σ is drawn as a grey mesh. Proteins are colored as in Panel C. (E) Superposition of complexes from the eSALO-WT/zC1r-12SP and eSALO-Y51F/zC1r-12SP structures. The two copies of the eSALO-WT/zC1r-12SP structure are colored in blue and cyan, respectively, while the two copies of the eSALO-Y51F/zC1r-12SP structure are colored in magenta and orange, respectively. (F) Comparison of the binding sites for SALO and *B. burgdorferi* BBK32-C. SALO is colored teal, while BBK32-C is colored light orange. The BBK32-C structure is taken from PDB entry 7MZT.

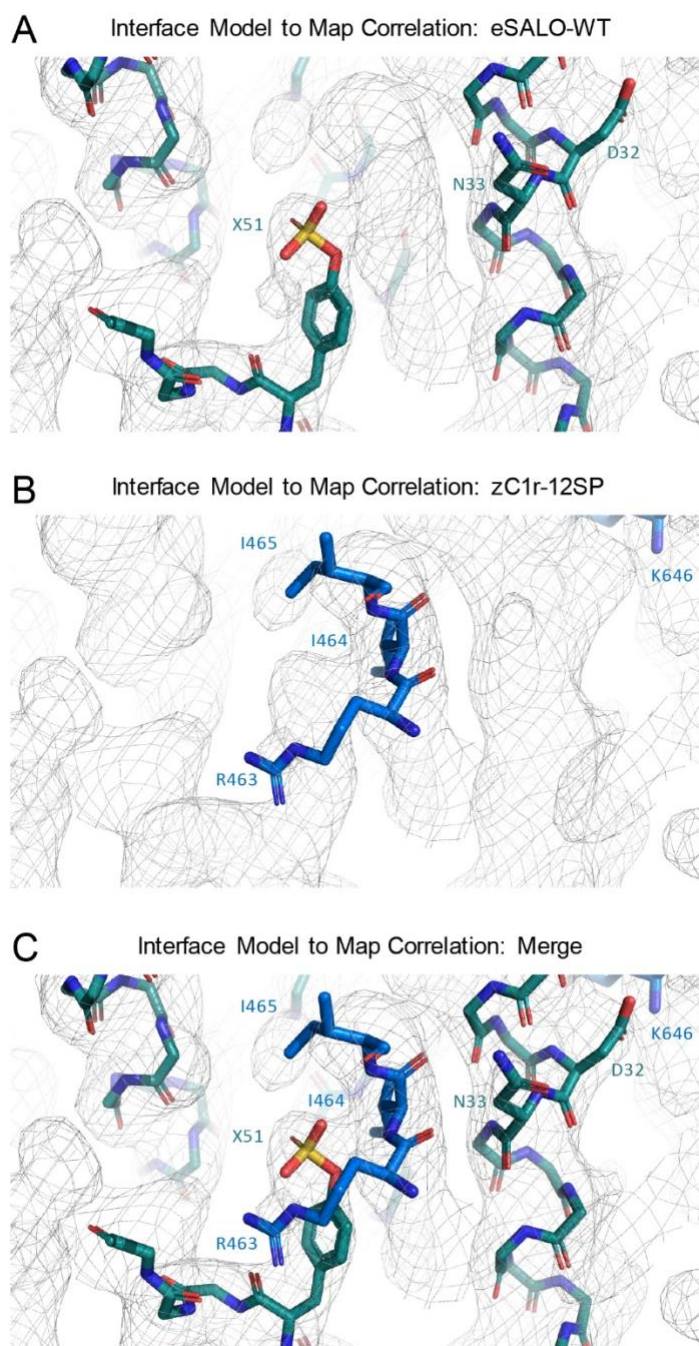


Figure S4.3. Supporting information pertaining to the interface of eSALO-WT/zC1r-12SP. (A) Representative model to map correlation at the interface of the eSALO-WT/zC1r-12SP complex represented by chains A and C in the PDB entry. The 2Fo-Fc map calculated at 3.3 Å limiting resolution and contoured at 1.2σ is drawn as a grey mesh, while eSALO-WT residues near the interface are drawn with their carbon atoms in teal. Sidechains of residues highlighted in Fig. S4.3 are shown in ball and stick convention. Note the central position of the sulfated tyrosine-51, X51. (B) Representative model to map correlation at the interface of the eSALO-WT/zC1r-12SP complex represented by chains A and C in the PDB entry. The 2Fo-Fc map calculated at 3.3 Å limiting resolution and contoured at 1.2σ is drawn as a grey mesh, while zC1r-12SP residues near the interface are drawn with their carbon atoms in blue. Sidechains of residues highlighted in Fig. S4.3 are shown in ball and stick convention. (C) A merged image of Panels A and B. Proteins are colored as described in the previous panels.

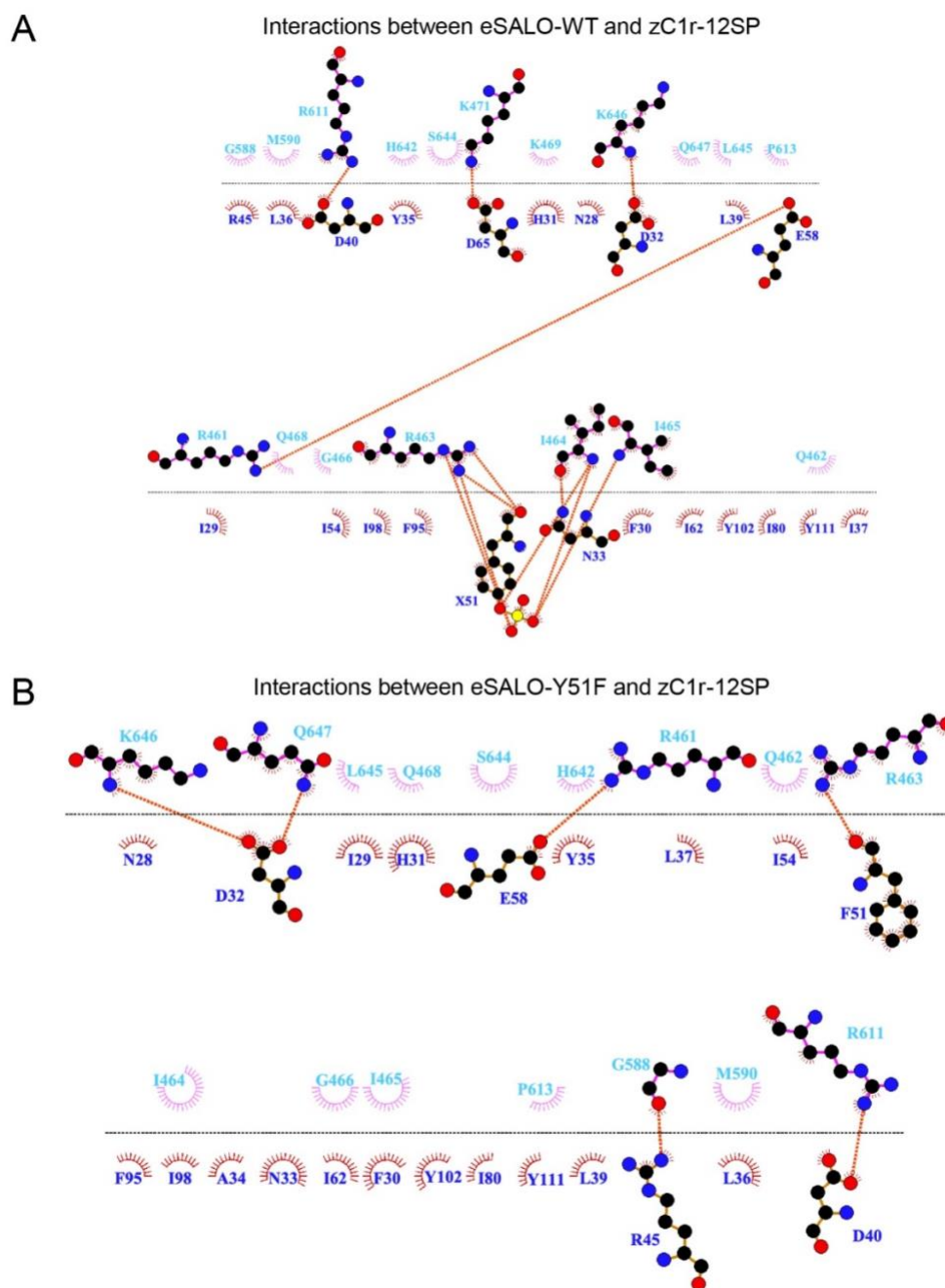


Figure S4.4. Supporting information pertaining to the interfaces of eSALO-WT/zC1r-12SP and eSALO-Y51F/zC1r-12SP. (A) Schematic representation of non-covalent interactions found at the eSALO-WT/zC1r-12SP interface. Hydrogen bonds are represented by dashed orange lines, while the toothed arcs represent packing/hydrophobic interactions. Residues derived from eSALO-WT are indicated with blue typeface and appear below the dashed black line, while residues derived from zC1r-12SP are indicated with cyan typeface and appear above the dashed black line. Note the sulfate group present on eSALO-WT residue X51. (B) Schematic representation of non-covalent interactions found at the eSALO-Y51F/zC1r-12SP interface. Hydrogen bonds are represented by dashed orange lines, while the toothed arcs represent packing/hydrophobic interactions. Residues derived from eSALO-Y51F are indicated with blue typeface and appear below the dashed black line, while residues derived from zC1r-12SP are indicated with cyan typeface and appear above the dashed black line.

**Chapter 5 - The C-terminal Domain of *S. aureus* Efb Affects
Synergistic Inhibition of the Terminal Complement Pathway By
recruiting FHR2 to C3b[#]**

This chapter supposed to be published as a journal article.

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Abstract

The extracellular fibrinogen-binding protein (Efb) is one of nearly a dozen proteins secreted by *Staphylococcus aureus* to inhibit complement activation or amplification. The C-terminal domain of Efb (Efb-C) forms a high-affinity interaction with the thioester-containing domain of C3b (TED/C3d), thereby blocking formation of the C3 proconvertase complex through an allosteric mechanism. However, further functional consequences of Efb-C binding to C3b remain unexplored. Here, we identified a previously unknown interaction between Efb-C, C3b, and the complement regulatory molecule, factor H-related protein 2 (FHR2). Since the FHR2/C3b interaction is centered upon the two C-terminal-most domains of FHR2 (FHR2(3-4)) and the TED/C3d domain of C3b, we tested whether Efb-C could influence the FHR2(3-4)/C3d interaction and observed a significant enhancement of FHR2(3-4)/C3d binding in the presence of Efb-C. We studied the FHR2(3-4)/C3d/Efb-C complex by X-ray crystallography and found that Efb-C forms few direct interactions with FHR2(3-4). Yet, the presence of Efb-C also enhanced binding of FHR2(3-4) and full-length FHR2 to C3b, suggesting that the effect of Efb-C on the FHR2/C3b interaction arises from increased accessibility of the FHR2-binding site. We found that enhanced FHR2 binding did not impact the rate of C3 convertase formation more than Efb-C alone, nor did it impart decay acceleration or cofactor activity. However, we observed potent, synergistic inhibition of complement downstream of C5 activation by Efb-C and FHR2 but not by Efb-C and FHR2(3-4). Our results show that Efb-C binding to C3b exerts additional inhibitory effects on the central complement components beyond blocking formation of the C3 proconvertase alone.

Introduction

The complement system is a cornerstone of innate immunity that is comprised of over 30 circulating and surface-bound proteins (1, 2). While complement fulfills many roles, it is perhaps best known as a front-line defender against invading microorganisms. Complement is activated through three canonical routes, known as the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP), respectively. The CP and LP serve as sensitive triggering mechanisms in response to various pathway-specific initiators. While the AP is a relatively inefficient initiating mechanism on its own (85), it is constitutively activated at low levels through spontaneous “tick-over” hydrolysis of the intramolecular thioester within its central component, C3. In this sequence, hydrolyzed C3 (i.e., C3(H₂O)) binds the pro-protease factor B (FB), which is then cleaved by factor D (FD) to form the fluid-phase AP C3 convertase (i.e., C3(H₂O)Bb). This initial convertase complex cleaves native C3 into C3a and C3b, the latter of which covalently attaches to reactive surfaces. Surface-bound C3b then binds FB, forming the AP C3 proconvertase complex (i.e., C3bB) that is also activated by FD to generate the fully-active AP C3 convertase (i.e., C3bBb). Although various aspects of this process are enhanced by the positive regulatory protein, properdin (FP) (81), the core components of the AP alone are sufficient to generate a self-amplifying loop that drives a majority of C3 activation under physiological conditions (84). In this manner, increasing levels of C3b deposited on the surface change the substrate specificity of the convertase from C3 to C5 (54-57). C5 activation releases the anaphylatoxin C5a, a potent chemoattractant for phagocytes, and also deposits C5b on the surface, thereby triggering assembly of the terminal pathway components (C6–C9) into the membrane attack complex (MAC). While the MAC directly lyses Gram-negative bacteria, the phagocytic cells recruited via C5a are needed engulf and kill MAC-resistant Gram-positive bacteria (153).

Despite its beneficial roles, complement activation must be tightly regulated through surface and fluid-phase regulators to prevent damaging self-cells (86). In this regard, Factor H (FH) serves as the primary negative regulator of the AP. FH is a large, ~155 kDa protein comprised of 20 sequential complement control protein (CCP) domains that are organized into discrete functional regions [Fig. 5.1A]. The N-terminal domains (CCPs 1-4) bind C3b, compete with FB to block C3 convertase formation and accelerate decay of existing C3 convertases. They also act as a cofactor for factor I (FI)-mediated degradation of C3b to iC3b, which itself can no longer form C3 convertases. The C-terminal domains (CCPs 19-20; hereafter FH(19-20)) bind to carbohydrates found on host cell surfaces that are absent from invading microorganisms (e.g., sialic acid), thus selectively protecting host cells from complement attack. FH(19-20) also binds the TED/C3d domain of C3b, thereby recruiting FH to sites of ongoing complement activation. Aside from FH, the FH-like protein 1 (FHL-1) and FH-related (FHR) proteins also modulate AP activity (86, 154). FHL-1, which arises from a splice variant of the *CFH* transcript encoding CCPs 1-7, retains the convertase-regulatory functions of its larger counterpart. By contrast, the FHR family consists of five distinct proteins that show structural similarities to FH, but which lack regions homologous to CCPs 1-4. The two C-terminal domains of all FHRs are homologous to FH(19-20) [Fig. 5.1B], however, and provide ligand (i.e., C3b) binding activity to FHR family members (154). In addition, the two N-terminal-most CCP domains of FHR1, FHR2, and FHR5 harbor a dimerization motif that allows them to form homo- or heterodimers with one another (155). Various studies have suggested distinct functional properties for FHRs (Reviewed in (154)).

Many pathogenic bacteria actively evade recognition and attack by the complement system to promote their survival within the host (6, 156). Although hijacking of host regulators is the most widespread of these strategies, certain other pathogens, like *Staphylococcus aureus*, express an

arsenal of unique immune evasion proteins that inhibit individual complement components or their assemblies at many points within the complement system. The C-terminal domain of the *S. aureus* extracellular fibrinogen-binding protein (Efb-C) binds to the thioester-containing domain (TED/C3d) of native C3 as well as C3b (53, 157). By stabilizing an open conformation of C3b that affects binding of several C3b ligands including FB (58), Efb-C greatly slows the rate of C3 pro-convertase assembly and therefore formation of the fully-active C3 convertase, thereby inhibiting the AP (117, 158). The *S. aureus* Efb homologous protein (Ehp) is 44% identical to Efb-C but contains two independent C3b-binding sites, making it a more potent AP inhibitor (158, 159). Previously, Amdahl et al. showed that Ehp forms a ternary complex with C3b and FH that is centered upon the TED/C3d domain and FH(19-20) regions, respectively (160). These authors suggested that Ehp and FH may enhance the complement inhibitory functions of one another, leading to more effective evasion of the complement system by *S. aureus*. While Efb-C leads to increased binding of both FH(19-20) and full-length FH to C3b (58, 117), the functional consequences of this interaction on complement activity remain unclear since Efb-C also blocks binding of FH(1-4) to C3b (117), presumably due to conformational effects on C3b (58). Furthermore, considering the sequence and structural homology between FH(19-20) and the C-terminal CCPs of FHRs, whether Efb-C exerts any effects on FHR binding remains unknown.

Here, we show that Efb-C binding leads to recruitment of FHR2 to C3b. Whereas FHR2 and its fragment FHR2(3-4) interacted comparatively weakly with Efb-C and C3d on their own, we found that their binding is collectively enhanced in the presence of Efb-C-bound C3d, highlighting a potential ternary interaction between FHR2, C3b, and Efb-C. We confirmed this ternary interaction by solving the crystal structure of the FHR2(3-4)/C3d/Efb-C complex, and showed that similarly enhanced interactions occur within C3b. Interestingly, we observed

synergistic inhibition of complement downstream of C5 activation in the presence of Efb-C and FHR2, but which was significantly weaker for Efb-C and FHR2(3-4), suggesting that this effect is potentiated by dimerization of FHR2. Overall, our findings unveil a cooperative immune evasion strategy in *S. aureus* mediated by the ternary complex of Efb-C, FHR2, and C3b.

Material and Methods

Protein Samples

Efb-C and C3d were expressed from pT7HMT-derived plasmids in *E. coli* BL21(DE3) (67). Efb-C was produced as polyhistidine-tagged fusion, while C3d was produced as a polyhistidine-tagged maltose-binding protein fusion. Cells grown in 1 L of Terrific Broth at 37 °C until the OD₆₀₀ reached 0.6-0.8, then 1 mM IPTG was used to induce protein expression in the soluble fraction by further incubation for 16 h at 18 °C. Following harvest by centrifugation, cells were resuspended and lysed using a microfluidizer in buffer of 20 mM Tris-HCl (pH 8.0), 20 mM imidazole, and 500 mM NaCl. After clarifying the lysate by centrifugation, the target proteins were purified by Ni-NTA affinity chromatography under native conditions. The fusion tag was removed from the target protein by digestion with Tobacco Etch Virus protease using a 100:1 mass ratio of target protein to protease and incubating overnight at room temperature. The digested sample was reapplied to a Ni-NTA affinity column and the unbound fraction was collected. The sample was subjected to a final purification by gel-filtration chromatography using a Superdex 75 26/60 column (Cytiva Life Sciences) and HBS as the running buffer. Fractions were analyzed by SDS-PAGE, and those containing the target proteins were pooled, concentrated, quantitated, and stored at either 4 or -80°C for later use.

FHR2(3-4) and FH(19-20) were expressed from pT7HMT-derived plasmids in *E. coli* BL21(DE3) cells as insoluble fusion proteins (67). Cells were grown in 1 L of Terrific Broth at 37 °C until the OD₆₀₀ reached 0.6-0.8, then 1 mM IPTG was used to induce protein expression for a further 16 h at 37 °C. Cells were harvested by centrifugation and lysed by resuspension with stirring for 30 min in 100 mM Tris-HCl (pH 8.0), 10 mM imidazole, and 6 M Guanidine-HCl. Following clarification of the lysate by centrifugation, the target protein was purified from the cell extract by Ni-NTA affinity chromatography under denaturing conditions (67). The purified, denatured protein was reduced with 1 mM TCEP for 30 min at 37 °C, then refolded overnight at 4 °C by diluting 10-fold into a buffer of 50 mM Tris-HCl (pH 8.3), 3 mM reduced Glutathione, 1 mM oxidized Glutathione, 5 mM EDTA, and 500 mM arginine. The diluted protein was then dialyzed for 24 h at room temperature against 4 L of HBS. The polyhistidine tag was removed using TEV cleavage, as described above. The cleaved protein was obtained following an additional affinity chromatography step where the unbound fraction was collected. The protein sample was subjected to a final purification by gel-filtration chromatography using a Superdex 75 26/60 column (Cytiva Life Sciences) and HBS as a running buffer. Fractions were analyzed by SDS-PAGE, and those containing the target protein were pooled, concentrated, quantitated, and stored at either 4 or -80 °C for later use.

Samples of C3b, iC3b, FB, FD, FH, and FI were purified from pooled human serum and were obtained from Complement Technology. A recombinant form of full-length human Complement Factor H-related 2 (CFHR2) protein expressed from mouse NS0 cells was obtained from R&D Systems.

Surface Plasmon Resonance Binding Studies

Direct binding studies were performed according to the general methods described previously (113). All experiments were conducted at 25 °C using a Biacore T-200 system (Cytiva Life Sciences) and a flow rate of 30 μ L/min. HBS-T (20 mM HEPES (pH 7.4), 140 mM NaCl, and 0.005% (v/v) Tween-20) was used as a running buffer. Surfaces were prepared by standard amine coupling to a CMD200M sensor chip (Xantec BioAnalytics). Flow cell 1 was used as a reference channel, while Efb-C was immobilized on both flow cell 2 (2456 RU) and flow cell 3 (1362 RU), and C3d was immobilized on flow cell 4 (1446 RU). For presaturation studies, 100 nM C3d was injected over flow cell 2 only prior to each sample injection. FHR2, FHR2(3-4), and FH(19-20) were then injected over the biosensor surface in reference subtraction mode, using a two-fold concentration series ranging from 50 nM to 800 nM.

For C3b binding and functional studies, a streptavidin-coated sensor chip SA (Cytiva Life Sciences) was used to capture C3b that had been site-specifically biotinylated at Cys1010 via EZ-link Maleimide-PEG2-Biotin (Thermo Fisher Scientific) according to a previously published protocol (113). A reference surface was prepared by injecting flow cell 1 with 10 μ M biotin. Experimental surfaces were subsequently prepared by injecting dilute solutions of C3b-biotin (8 ng/ μ L) across flow cell 3 and flow cell 4 to achieve final capture levels of 2168 RU and 2126 RU, respectively. For presaturation studies, 100 nM Efb-C was injected over flow cell 3 only prior to each sample injection. Samples of various analytes were injected over the biosensor surface in reference subtraction mode using a two-fold concentration series ranging from 62.5 nM to 1 μ M, with the exception that 125 nM to 2 μ M were used for SCIN-A.

Crystallization, X-ray Diffraction, Structure Solution, and Refinement

A sample of the C3d/FHR2(3-4) complex was prepared by mixing the individual monomers in a 1:1 molar ratio. The sample was then exchanged into 10 mM HEPES (pH 7.4), 50

mM NaCl and concentrated to 1.5 mg/mL. Initial crystallization conditions were determined through vapor diffusion of sitting drops prepared by a Crystal Gryphon liquid handling system (Art Robbins Instruments, LLC) using the Crystal Index HT and PEGRX screening kits (Hampton Research Corp). Individual crystals for diffraction studies were obtained at 18 °C by vapor diffusion of hanging drops, which were established by mixing 1 μ L of protein solution with 1 μ L of precipitant solution consisting of 75 mM HEPES (pH 7.5), 1.2 M (NH₄)₂SO₄, and equilibrating over 500 μ L of precipitant solution. Crystals were cryopreserved after a short incubation in a buffer consisting of the precipitant solution supplemented with 30% (v/v) glycerol, followed by rapid cooling in liquid N₂.

A sample of the FHR2(3-4)/C3d/Efb-C complex was likewise prepared by mixing the individual monomers in a 1:1:1 molar ratio. The sample was exchanged into 10 mM HEPES (pH 7.5), 50 mM NaCl, and concentrated to 5 mg/mL. Initial crystallization conditions were identified using the procedure described in the previous paragraph. Individual crystals for diffraction studies were obtained at 18 °C by vapor diffusion of hanging drops, which were established by mixing 1 μ L of protein solution with 1 μ L of precipitant solution consisting of 100 mM imidazole (pH 6.7), 12% (w/v) peg-20k, and equilibrating over 500 μ L of precipitant solution. Crystals were cryopreserved after a short incubation in a buffer consisting of the precipitant solution supplemented with 23% (v/v) glycerol, followed by rapid cooling in liquid N₂.

X-ray diffraction data on crystals of the FHR2(3-4)/C3d and FHR2(3-4)/C3d/Efb-C complexes were collected using beamline 22-ID and 22-BM of the Advanced Photon Source at Argonne National Laboratory, respectively. All X-ray diffraction data were indexed, integrated, and scaled using the HKL-2000 software package (69). PHASER (70) was used to solve both structures by molecular replacement using the structures of C3d (161), FHR2(3-4) (155), and Efb-

C (53) as search models where appropriate. Following placement of the respective models, structures were refined through a combination of reciprocal space positional, B-factor, and TLS refinement using PHENIX.REFINE (72) as well as automated and manual rebuilding using PHENIX.AUTOBUILD and COOT (72, 135).

The final model for FHR2(3-4)/C3d consists of two copies of the complex along with four sulfate anions and 363 ordered solvent molecules. Chain A consists of residues 992-1287 of the human C3 reference sequence (UniProt P01024), while Chain C consists of residues 994-998, 1001-1040, 1042-1132, 1134-1223, 1228-1262, and 1265-1285 of the same sequence. Chain B consists of residues 146-269 of the human FHR2 reference sequence (UniProt P36980), while Chain D consists of residues 146-224 and 228-269. The final model for FHR2(3-4)/C3d/Efb-C consists of a single copy of the complex. Chain A consists of residues 994-1287 of human C3, Chain B consists of residues 146-192, 194-224, and 227-270 of human FHR2, and Chain C consists of residues 101-165 of *S. aureus* Efb (UniProt P68799). Refined models for FHR2(3-4)/C3d and FHR2(3-4)/C3d/Efb-C have been deposited in the PDB under accession codes 9N1Z and 9N20, respectively. Additional details on X-ray diffraction data collection and refinement statistics are provided in the accompanying table (Table 5.1).

Functional Studies of C3 Convertase Formation and Decay by SPR

The effects of Efb-C, FHR2, and various combinations thereof on AP C3 convertase (C3bBb) formation and decay were assessed through real-time SPR using the general methods described in earlier publications (114, 162). All experiments used HBS-T buffer supplemented with 5 mM MgCl₂ as a running buffer and a flow rate of 10 μ L/min. Experimental flow cells containing surface-captured C3b-biotin were either left naïve or saturated with 100 nM Efb-C prior to sample injection series.

The convertase formation assay consisted of a single injection sequence. Briefly, the AP C3 convertase was generated on flow cells by injecting a mixture of 500 nM FB and 500 nM FD for 1 min. A 3 min dissociation period was used to monitor intrinsic stability of the AP C3 convertase on the surface. Thereafter, the surface was regenerated using an initial injection of 10 mM EDTA, 2 M NaCl, followed by two pulses of 0.1 M acetate buffer (pH 4.0).

The convertase decay acceleration assay consisted of two injection sequences within the same experimental cycle (114). First, the AP C3 convertase was generated by injected the FB/FD mixture described above for 2 min, followed by a 2 min dissociation phase. After a brief stabilization period, the second injection introduced various proteins, either alone or in combination, for 2 min, followed by a 3 min dissociation phase to monitor the AP C3 convertase on the surface. The surface was regenerated using the same sequence outlined above.

Assay of Cofactor Activity for FI-mediated Degradation of C3b and iC3b

The presence of cofactor activity for FI-mediated cleavage of C3b and iC3b was evaluated using an adaptation of a previously described method (163). Each reaction contained 4 µg C3b or iC3b, 1 µg FI, and various combinations of Efb-C, FHR-2(34), and FHR-2 in a final volume of 15 µL. All cleavage reactions were initially established on ice, but then shifted simultaneously to 37 °C for a 30 min incubation period. Reactions were terminated by adding SDS-PAGE sample buffer containing reducing agent, followed by heating at 95 °C for 5 min. The contents of each cleavage reaction were separated by SDS-PAGE and visualized by Coomassie staining.

Alternative pathway hemolysis assay

Assays that quantify lysis of rabbit erythrocytes (Er) under AP-specific conditions were used to investigate the complement inhibitory properties of Efb-C, FHR2, and various combinations thereof (164). Briefly, all assays were conducted in 100 µL total volume and

consisted of 12 μL of normal human serum (Creative Biolabs), 50 μL of 0.1% (w/v) gelatin veronal buffer (GVBo without Mg^{2+} and Ca^{2+} ; Complement Technologies), 10 μL of 0.1 M MgEGTA (Complement Technologies), 8 μL of each protein sample needed to achieve the desired final concentration prepared in GVBo buffer, and 20 μL of rabbit erythrocytes ($5 \times 10^8/\text{mL}$ exchanged into GVBo) that were resuspended immediately before beginning each assay. After incubating each reaction mixture at 37 °C for 30 min, all samples were centrifuged at 200g for 10 min and the supernatant was collected. The supernatants were diluted 4-fold in ddH₂O and the absorbance was measured at 412 nm. The percent inhibition for each sample was given by the following equation:

$$100 - [100 \times (\text{OD}_{412, \text{ sample}} - \text{OD}_{412, \text{ buffer}}) / (\text{OD}_{412, \text{ no inhibitor}} - \text{OD}_{412, \text{ buffer}})].$$

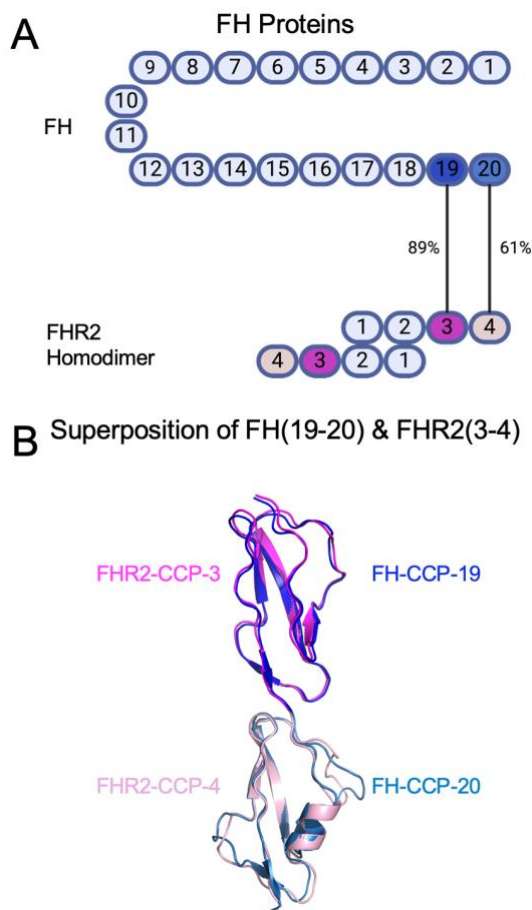


Figure 5.1 Structural Comparison of FH and FHR2.

(A) A schematic representation of the domain structure of FH is shown at the top of the panel. Each oval represents a CCP domain of FH, and is numbered sequentially from 1-20. CCP domains 19 and 20 bind to the C3d region of C3b, and are colored in dark blue. A schematic representation of the FHR2 homodimer is shown at the bottom of the panel. CCP domains 1 and 2 mediate dimerization, while CCP domains 3 and 4 mediate C3d binding and correspond to CCP domains 19 and 20 of FH. The percent identities between corresponding domains are shown. (B) Structural superposition for FH(19-20) (PDB code 2G7I; (165)) and FHR2(3-4) (PDB code 3ZD1; (155)) illustrated as ribbon diagrams. Similar to panel A, FHR2(3-4) is colored in shades of pink similar while FH(19-20) is colored in shades of dark blue.

Results

Efb-C Recruits FHR2 to C3d

In an earlier report, we showed that Efb-C recruits both FH(19-20) and full-length FH to C3b (58, 117). Given the sequence and structural similarities between the C-terminal domains of FH and FHR2 [Fig. 5.1A, B], we hypothesized that Efb-C might also recruit FHR2 to C3b. To test this idea, we immobilized Efb-C and C3d on two separate flow cells of a biosensor chip and examined their interactions with FHR2 [Fig. 5.2A-B]; since Efb-C forms a slowly dissociating complex with C3d (53, 58, 117, 158), we also immobilized Efb-C on a third flow cell of the sensor chip and subjected that flow cell to presaturation with 100 nM C3d to examine interactions of the C3d/Efb-C complex with FHR2 [Fig. 5.2C]. Although we observed relatively weak interactions between FHR2 and immobilized Efb-C [Fig. 5.2D] and C3d [Fig. 5.2E] in these experiments, we found strong evidence for an enhanced interaction between FHR2, C3d, and Efb-C across all concentrations we examined [Fig. 5.2F].

FH(19-20) carries a C3d binding site that helps recruit FH to C3b-opsonized surfaces (154). Since the two C-terminal CCPs of FHR2 (i.e., FHR2(3-4)) show 81% and 61% sequence identity to CCP 19 and CCP20 of FH [Fig. 5.1A] (154), respectively, we next tested whether FHR2(3-4) behaved similarly to full-length FHR2. Although FHR2(3-4) bound to Efb-C [Fig. 5.2G] and C3d [Fig. 5.2H] more weakly than full-length FHR2, we still observed strong evidence of an enhanced interaction between FHR2(3-4), C3d, and Efb-C across all concentrations we examined [Fig. 5.2I]. Under the conditions employed in this study, we noted that the previously reported enhanced effect between FH(19-20), C3d, and Efb-C was more pronounced than for FHR2(3-4) (58, 117) [Fig. 5.2J-L]. However, the dissociation rate of the ternary complex formed between C3d, Efb-C, and FHR2 was noticeably slower than those of either FHR2(3-4) or FH(19-20) [Fig. 5.2F, I, L].

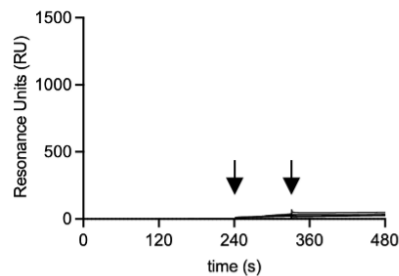
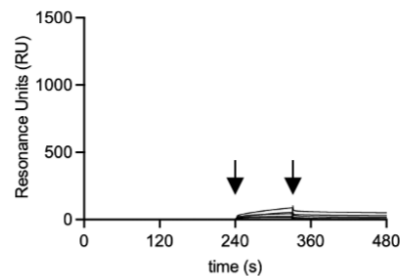
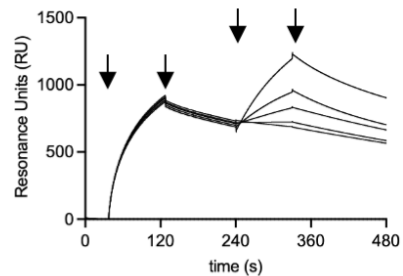
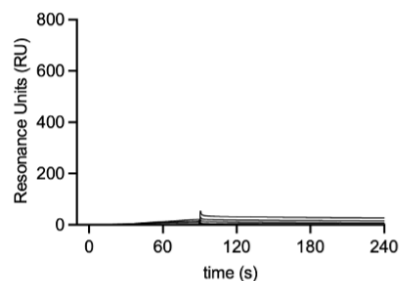
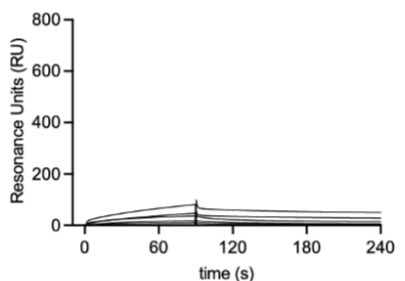
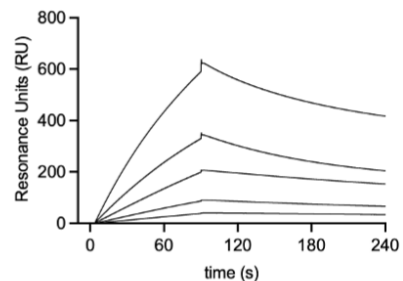
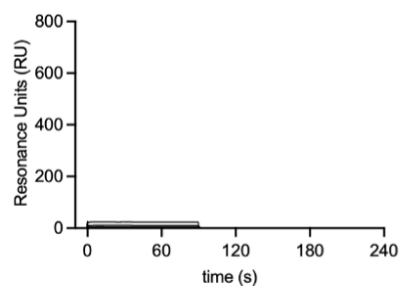
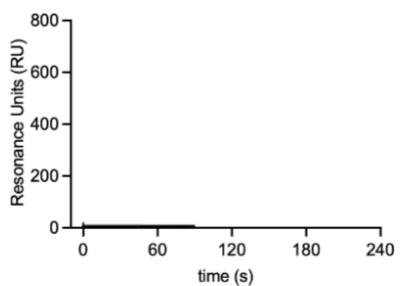
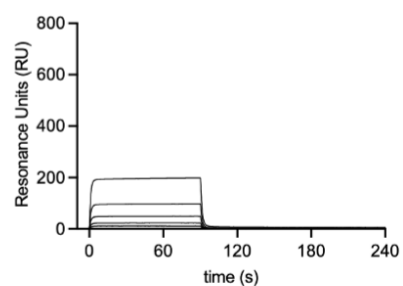
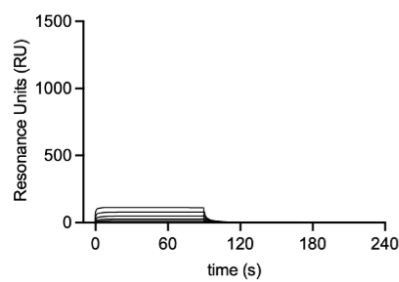
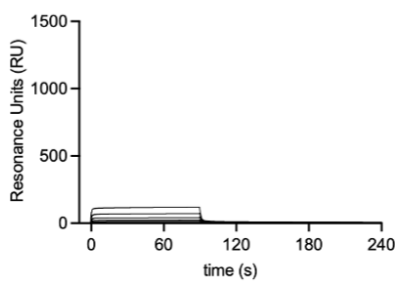
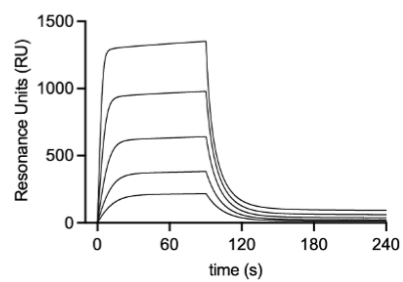
A Efb-C Surface**B** C3d Surface**C** Efb-C Surface with C3d pre-saturation**D** FHR2 over Efb-C**E** FHR2 over C3d**F** FHR2 over Efb-C with C3d pre-saturation**G** FHR2(3-4) over Efb-C**H** FHR2(3-4) over C3d**I** FHR2(3-4) over Efb-C with C3d pre-saturation**J** FH(19-20) over Efb-C**K** FH(19-20) over C3d**L** FH(19-20) over Efb-C with C3d pre-saturation

Figure 5.2 Efb-C Binding Recruits FHR2 to C3d.

Efb-C and C3d were immobilized on two separate experimental flow cells of a sensor chip, while C3d was immobilized on the third experimental flow cell and saturated with Efb-C to prepare the C3d/Efb-C complex. Thereafter, concentration series of various analytes were injected over the experimental surfaces in reference-subtraction mode. (A) Sensorgrams obtained by injecting a concentration series of FHR2 over the Efb-C surface. Arrows indicate analyte injection start and stop. (B) Sensorgrams obtained by injecting a concentration series of FHR2 over the C3d surface. Arrows indicate analyte injection start and stop. (C) Sensorgrams obtained by presaturating a C3d surface with Efb-C, then injecting a concentration series of FHR2. Arrows indicate presaturation injection start and stop, followed by analyte injection start and stop. (D) Identical to panel A, but only showing the time period corresponding to the FHR2 association and dissociation phase. (E) Identical to panel B, but only showing the time period corresponding to the FHR2 association and dissociation phase. (F) Identical to panel C, but only showing the time period corresponding to the FHR2 association and dissociation phase. (G) Identical to panel D, but with FHR2(3-4) as the analyte. (H) Identical to panel E, but with FHR2(3-4) as the analyte. (I) Identical to panel F, but with FHR2(3-4) as the analyte. (J) Identical to panel D, but with FH(19-20) as the analyte. (K) Identical to panel E, but with FH(19-20) as the analyte. (L) Identical to panel F, but with FH(19-20) as the analyte.

Structure of FHR2(3-4)/C3d Complex Reveals an FH(19-20)/C3d-like Interaction

Morgan et al. previously reported a crystal structure for FH(19-20)/C3d (18). In this complex, essentially all of the interface donated by FH(19-20) is derived from residues within CCP19 domain and the loop that connects it to CCP20. These positions are conserved between FH(19-20) and FHR2(3-4), which implied that the interaction between these two proteins and C3d should be largely the same. To obtain experimental evidence supporting this idea, we crystallized the FHR2(3-4)/C3d complex, collected X-ray diffraction data extending to 2.3 Å limiting resolution, and solved the structure by molecular replacement. Following refinement, the final model had R_{work} and R_{free} values of 19.4% and 22.7%, respectively (Table 5.1). Although the asymmetric unit of this crystal contained two copies of the FHR2(3-4)/C3d complex, we restricted our analysis to the complex represented by chains A and B in the final PDB model [Fig. 5.3A & Fig. S5.1A-B].

The FHR2(3-4)/C3d interaction buries 788 Å² of surface area, which is slightly greater than the 716 Å² of FH(19-20)/C3d. However, as suggested by the relatively high levels of

sequence conservation between these two regulators, we observed a globally similar C3d-binding mode between FHR2(3-4) and FH(19-20) [Fig. 5.3B]: superpositioning of the two structures showed that 300 of the 410 residues modeled aligned with an RMSD value of 0.372 Å. The interface between FHR2(3-4) and C3d is derived primarily of residues within the CCP3 domain, whereas the CCP4 domain appears to contribute little to the interaction. Not surprisingly, the main regions of divergence following structural superposition were found in the models of CCP4 and CCP20; consistent with this, 271 of the 350 residues modeled aligned with an RMSD value of 0.242 Å when CCP4 and CCP20 were removed from the superpositioning calculation. In summary, the main features of the FHR2(3-4)/C3d complex mirrored that of FH(19-20)/C3d (18). They also showed that neither of the regulators appeared to interfere with the previously characterized Efb-C binding site on C3d [Fig. 5.3B] (53).

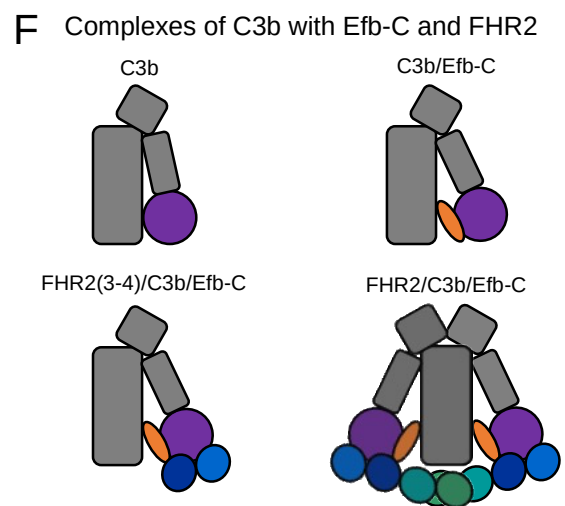
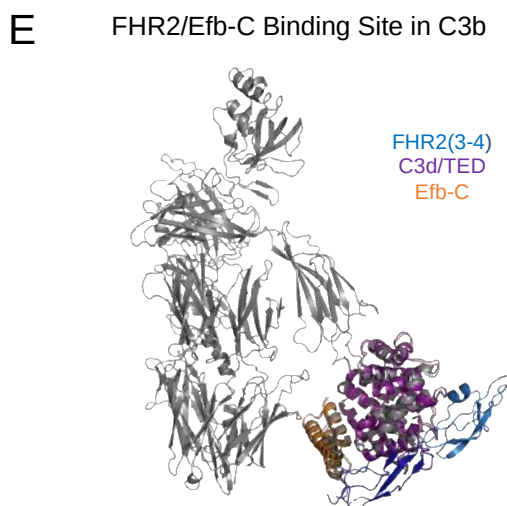
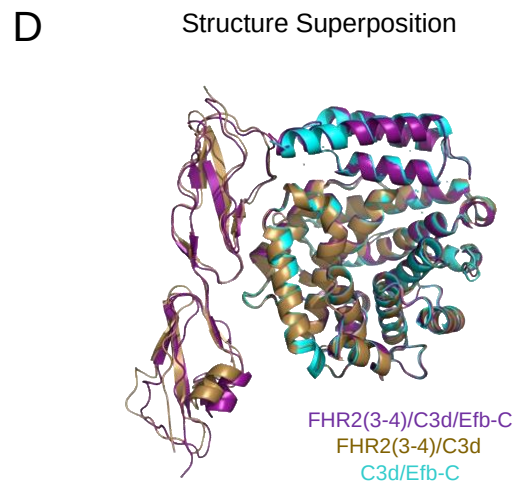
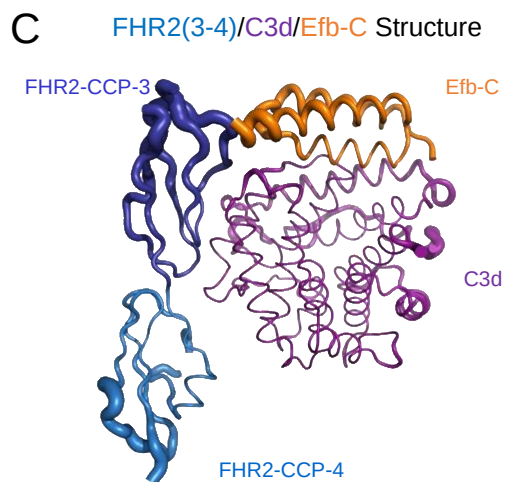
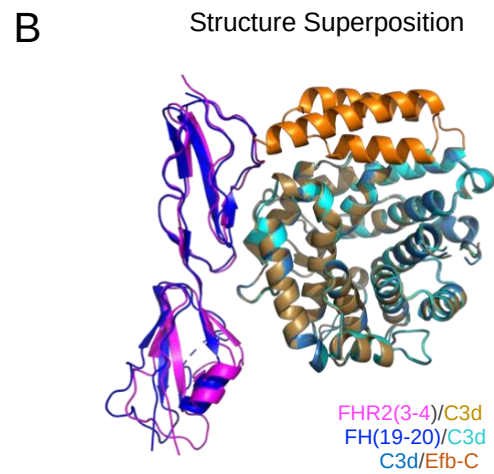
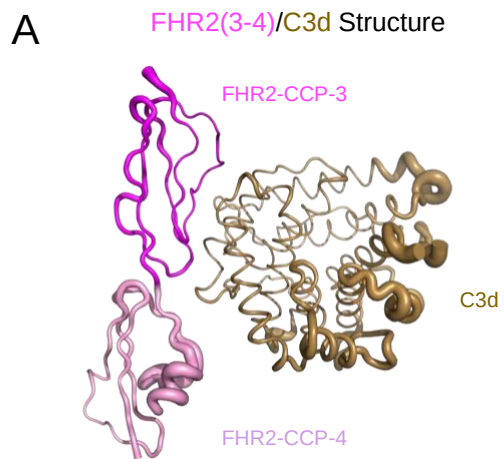


Figure 5.3 Structural Studies of the FHR2(3-4)/C3d and FHR2(3-4)/C3d/Efb-C Complexes.

(A) Structure of the FHR2(3-4)/C3d binary complex determined at 2.3 Å resolution. Note that only Chains A and B of the final PDB model are shown. FHR2(3-4) is drawn as a ribbon diagram with its CCP3 domain colored magenta and its CCP4 domain colored pink, while C3d is colored gold. For all proteins, the thickness of the tracing corresponds to the final B-factor for that region of the model, where thinner corresponds to lower and thicker to higher B-factors. Note the minimal contacts between the CCP4 domain of FHR2(3-4) and C3d. (B) Superpositioning of the FHR2(3-4)/C3d (PDB code 9N1Z), FH(19-20)/C3d (PDB code 3OXU; (18)), and C3d/Efb-C (PDB code 2GOX; (53)) structures suggests that FHR2(3-4) and FH(19-20) binding to C3d are compatible with Efb-C binding. (C) Structure of the FHR2(3-4)/C3d/Efb-C complex determined at 3.3 Å resolution. FHR2(3-4) is drawn as a ribbon diagram with its CCP3 domain in dark blue and its CCP4 domain in light blue, while C3d is colored purple and Efb-C is colored orange. The thickness of the tracing corresponds to the final B-factor for that region of the model, where thinner corresponds to lower and thicker to higher B-factors. (D) Superpositioning of the FHR2(3-4)/C3d/Efb-C (PDB code 9N20), FHR2(3-4)/C3d (PDB code 9N1Z), and C3d/Efb-C (PDB code 2GOX; (53)) structures reveals a lack of significant conformational changes between the binary and ternary complexes. (E) Mapping of the FHR2(3-4)/C3d/Efb-C structure within the context of C3b. The FHR2(3-4)/C3d/Efb-C structure (colored as above) was superimposed on a C3b/Efb-C model derived from SAXS data (117) using the C3d/TED domain as a basis. (F) Schematic representation of C3b and its complexes with Efb-C and FHR2. Although previous studies have shown that C3b must undergo a conformational change to accommodate Efb-C binding (53, 58, 117, 158), the location and orientation of the FHR2(3-4) binding site perpendicular to the long dimension of C3b is compatible with binding to FHR2 homodimers, as shown in the bottom right image.

Structure of FHR2(3-4)/C3d/Efb-C Complex Provides Insights into FHR2 Recruitment to C3b

To better understand how Efb-C influences FHR2 binding to C3b, we crystallized the FHR2(3-4)/C3d/Efb-C complex. We collected X-ray diffraction data extending to 3.3 Å limiting resolution, and solved the structure by molecular replacement using the structures of FHR2(3-4) (155), C3d (161), and Efb-C (53) as search models. We then refined the structure of the FHR2(3-4)/C3d/Efb-C complex to R_{work} and R_{free} values 17.8% and 21.4%, respectively (Table 5.1). Unlike the FHR2(3-4)/C3d crystal described above, the asymmetric unit of the FHR2(3-4)/C3d/Efb-C crystal contained a single copy of the complex [Fig. 5.3C & Fig. S5.1C-D].

We found that the FHR2(3-4)/C3d/Efb-C structure is an amalgamation of the individual binary complexes [Fig. 5.3D]. The ternary complex buries a total of 1,797 Å², of which 746 Å²

could be attributed to the FHR2(3-4)/C3d and 846 Å² to the C3d/Efb-C interfaces. These values compare favorably to those observed in the structures of the FHR2(3-4)/C3d [Fig. 5.3A] and C3d/Efb-C (53) binary complexes at 788 Å² and 829 Å², respectively. By contrast, the FHR2(3-4)/Efb-C interface buries only 205 Å². It also lacks any likely hydrogen bonds or salt-bridges that can be identified by EBI-PISA (78). Together, these observations account for the relatively weak interactions we observed between Efb-C and either FHR2 [Fig. 5.2D] or FHR2(3-4) [Fig. 5.2G] in the absence of C3d [Fig. 5.2F, I].

Table 5.1 X-ray Diffraction Data, Structure Solution, and Refinement Statistics

X-ray Diffraction Data, Structure Solution, and Refinement Statistics^a		
	FHR2(3-4)/C3d	FHR2(3-4)/C3d/Efb-C
PDB Accession Code	9N1Z	9N20
<i>Data Collection</i>		
Beamline	APS 22-ID	APS 22-BM
Space Group	C222 ₁	P6 ₁
Wavelength (Å)	1.000	1.000
Cell Dimensions		
a, b, c (Å)	113.30, 116.23, 165.58	145.58, 145.58, 76.86
Resolution (Å)	50.00-2.30	50.00-3.30
Wilson B-Factor (Å ²)	35.7	87.6
Completeness (%)	99.2 (96.9)	100.0 (99.6)
I / σI	10.6 (2.3)	15.4 (1.6)
R _{pim}	0.079 (0.356)	0.048 (0.472)
CC _{1/2}	0.946 (0.598)	1.000 (0.507)
Redundancy	11.2 (7.6)	20.8 (10.7)
<i>Refinement</i>		
Resolution (Å)	47.57-2.31	48.74-3.30
Number of Reflections	47,435	13,333
R _{work} / R _{free} (%)	19.4 / 22.7	17.8 / 21.4
Atoms Modeled	6,876	3,808
Ramachandran Plot		
Favored/Allowed (%)	97.38 / 2.50	96.82 / 2.76
Average B-Factors (Å ²)	59.0	106.0
R.M.S. Deviations		
Bond Lengths (Å)	0.003	0.002
Bond Angles (°)	0.56	0.46

^aValues in parentheses are for the highest-resolution shell.

We previously reported that Efb-C binding to the TED domain of C3b is incompatible with several C3b structures determined by X-ray crystallography (45, 53, 74). This led us to predict that C3b must adopt an alternative conformation compatible with Efb-C binding, which we detected biochemically (53) and characterized structurally using both small angle X-ray scattering and hydrogen/deuterium exchange mass spectrometry (117). In this open conformation (58), the TED domain of C3b moves away from the remainder of the macroglobulin-like core region, providing increased access to the Efb-C binding site. Such increased accessibility of the Efb-C binding site could also influence accessibility of the FHR2 binding site in C3b, and would be required to accommodate the FHR2(3-4)/C3d/Efb-C structure described here [Fig. 5.3E-F].

Efb-C Recruits FHR2 to C3b

We next investigated whether the Efb-C-dependent recruitment of FHR2 observed for C3d and illustrated by the FHR2(3-4)/C3d/Efb-C structure also occurred in C3b. To test this hypothesis, we used an SPR approach wherein C3b that had been site-specifically biotinylated at its thioester cysteine was captured at essentially equivalent levels (i.e. 2168 RU vs 2126 RU) on two streptavidin-derivatized flow cells (162); then, as described above, we presaturated one flow cell with 100 nM Efb-C prior to every experimental injection to compare differences between the two flow cells. Consistent with earlier studies (53, 58, 117), we found that injection of Efb-C generated a slowly-dissociating C3b/Efb-C complex on the surface that could be used to characterize differences in binding between C3b alone and the C3b/Efb-C complex [Fig. 5.4A, B] (58, 117): whereas binding of FB was significantly diminished by Efb-C [Fig. S5.2A, B], binding of both full-length FH [Fig. S5.2C, D] and FH(19-20) [Fig. S5.2E, F] was mildly enhanced, while binding of the *S. aureus* complement inhibitor, SCIN, was not affected [Fig. S5.2G, H].

Subsequently, we examined the interactions of FHR2 with C3b and compared them with C3b/Efb-C. We found that the overall response of FHR2 binding in the presence of Efb-C increased by ~3-fold across all concentrations examined [Fig. 5.4C, D]. As with our earlier studies using C3d/Efb-C, we noted that FHR2 itself formed a relatively slowly dissociating complex with Efb-C in these experiments. This stood in contrast to FHR2(3-4), which showed clear evidence of enhanced C3b binding in the presence of Efb-C, but which also decayed rapidly [Fig. 5.4E, F]. We observed a similar ~3-fold increase in binding of FH(19-20) to C3b in the presence of Efb-C, albeit with a fast decay rate similar to FHR2(3-4) [Fig. S5.2E, F]. In summary, these results showed that Efb-C leads to enhanced FHR2 binding to C3b, forming a slowly dissociating complex when the N-terminal, oligomerization domains of FHR2 are present.

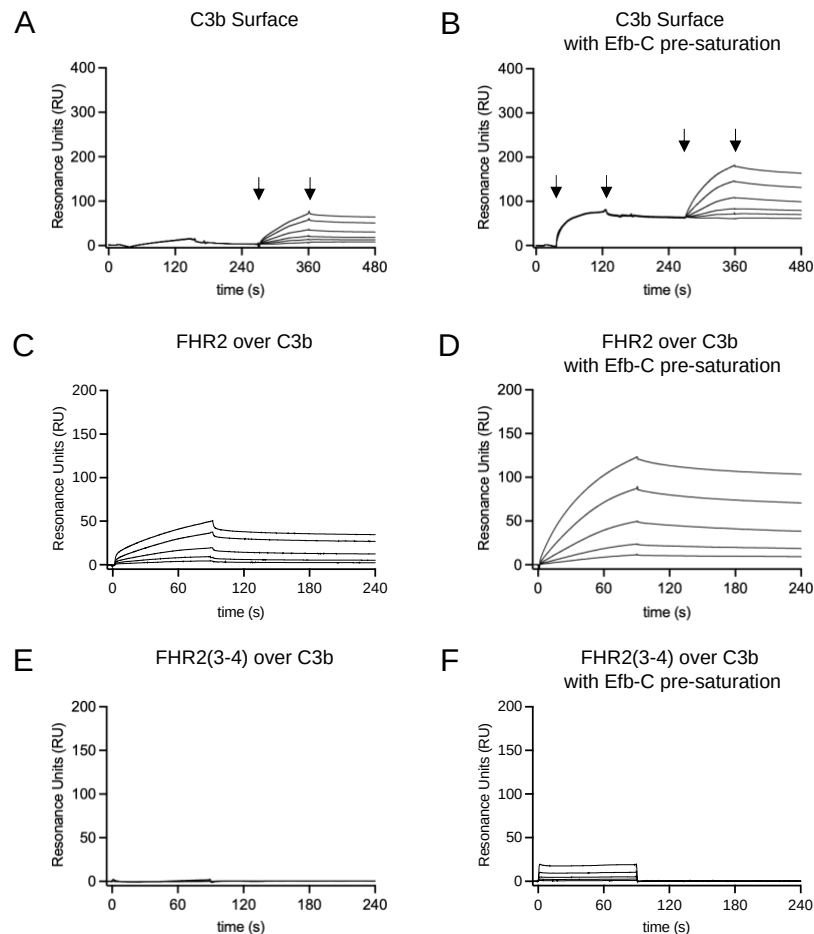


Figure 5.4 Efb-C Binding Recruits FHR2 to C3b.

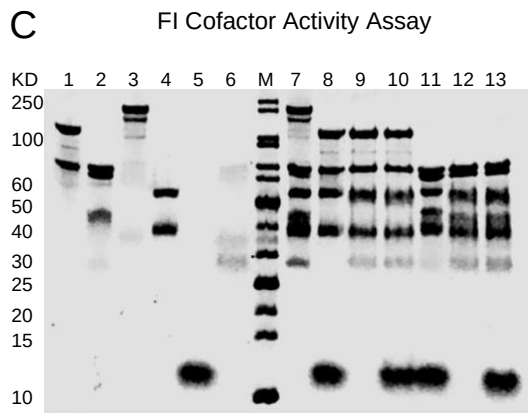
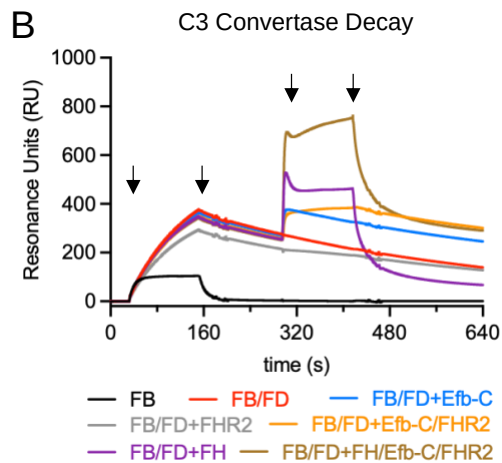
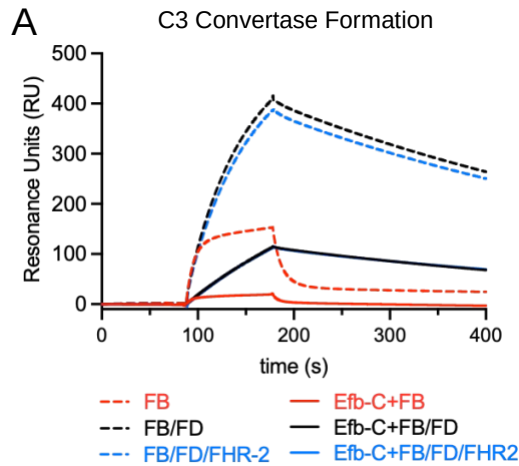
Site-specifically biotinylated C3b was captured on two experimental flow cells of a streptavidin-coated biosensor. Efb-C was injected at the beginning of each cycle over one of the flow cells to prepare the C3b/Efb-C complex. Thereafter, concentration series of various analytes were injected over the experimental surfaces in reference-subtraction mode. (A) Sensorgrams obtained by injecting a concentration series of FHR2 over the surface with C3b alone. Arrows indicate analyte injection start and stop. (B) Sensorgrams obtained by presaturating a C3b surface with Efb-C, then injecting a concentration series of FHR2. Arrows indicate presaturation injection start and stop, followed by analyte injection start and stop. (C) Identical to panel A, but only showing the time period corresponding to the FHR2 association and dissociation phase. (D) Identical to panel B, but only showing the time period corresponding to the FHR2 association and dissociation phase. (E) Identical to panel A, but only showing the time period corresponding to the FHR2(3-4) association and dissociation phase. (F) Identical to panel B, but only showing the time period corresponding to the FHR2(3-4) association and dissociation phase.

Efb-C and FHR2 Affect Synergistic Inhibition of Complement Downstream of C5

To understand the consequences of FHR2 recruitment to C3b in the presence of Efb-C, we employed a series of assays that investigate aspects of complement function which are C3b-dependent. This included analysis of AP C3 convertase formation and decay, examination of C3b and iC3b stability in the presence of FI, and measurements of hemolytic activity to assess function of terminal complement events. In these experiments, we used Efb-C and FHR2, truncations thereof (e.g. FHR2(3-4), or its analogs (e.g. FH(19-20))), to test the effects of these inhibitors either alone or in combination with one another.

We first tested the effects of Efb-C and FHR2 on formation of the AP C3 convertase. We used an identical SPR approach to that described in previous publications (114, 162), and which is based upon capture of site-specifically biotinylated C3b as presented above. Whereas Efb-C greatly diminished binding of FB to C3b (i.e. formation of the AP C3 proconvertase) and subsequent formation of the convertase, we observed no effect on convertase formation for FHR2 alone, nor any further effect when FHR2 was included along with EfbC [Fig. 5.5A]. We next tested the effects of Efb-C and FHR2 on decay of the AP C3 convertase. Although C3 convertases are intrinsically labile and decay spontaneously and irreversibly, the rate at which they decay can be

enhanced by regulatory molecules with so-called “decay accelerating activity”, including FH. Whereas injection of FH following convertase formation resulted in decay of the convertase surface as expected, we observed no decay accelerating activity following injection of either Efb-C



C or FHR2 on their own, or in combination with one another [Fig. 5.5B]. We then tested whether Efb-C, FHR2, or the combination thereof exhibited cofactor activity for C3b cleavage by FI. Although FI helps regulate the AP by degrading the C3b scaffold for convertase assembly, it requires a molecule with so-called “cofactor” activity (e.g. FH) to bind and cleave its C3b substrate. Whereas FH provided cofactor activity for cleavage of C3b to iC3b by FI, we observed no cofactor activity when Efb-C, FHR2, or both proteins together were added along with FI to C3b [Fig. 5.5C]. Furthermore, we found that neither Efb-C, FHR2, nor both proteins together provided any cofactor activity for FI-mediated cleavage of iC3b into C3c [Fig. 5.5C].

Figure 5.5 Efb-C Mediated Recruitment of FHR2 to C3b Does Not Influence AP C3 Convertase Formation, Decay, or C3b Turnover.

(A) Site-specifically biotinylated C3b was captured on a streptavidin-coated biosensor and used to assess formation of AP C3 convertase (i.e., C3bBb).

Representative reference-corrected sensorgrams illustrating formation of the AP C3 convertase in the absence (dashed lines) or presence (solid lines) of Efb-C presaturation. Sensorgrams corresponding to injections of FB alone (red traces), an FB/FD mixture (black traces), and an FB/FD/FHR2 mixture (blue traces) are shown. (B) Representative reference-corrected sensorgrams illustrating the formation (first injection phase) and decay (second injection phase) of the AP C3 convertase. Control injections of FB alone (black trace) are presented along with injections of an FB/FD mixture (red trace), while the decay-acceleration activity of FH is highlighted by injections of FB/FD followed by FH (purple trace). Injections of FB/FD followed by Efb-C (blue trace), FHR-2 (grey trace), an Efb-C/FHR2 mixture (orange trace), or an FH/Efb-C/FHR2 mixture (brown trace) yielded an increased SPR response when compared with FH alone (purple trace), but the decay rate was unaffected. (C) Potential FI cofactor activity was assessed by detecting the proteolytic degradation of C3b or iC3b and visualized by Coomassie blue staining. Purified proteins were included as controls and appear at the left-hand side of the image. Proteolytic reactions containing 4 µg of C3b or iC3b, 1 µg of FI with 1 µg of either FH, Efb-C, FHR2 or an Efb-C/FHR2 mixture appear at the right side of image. Legend is as follows; M, molecular mass marker; 1, C3b; 2, iC3b; 3, FH; 4, FI; 5, Efb-C; 6, FHR2; 7, C3b+FI+FH; 8, C3b+FI+Efb-C; 9, C3b+FI+FHR2; 10, C3b+FI+Efb-C+FHR-2; 11, iC3b+FI+Efb-C; 12, iC3b+FI+FHR2; 13, iC3b+FI+Efb-C+FHR2.

Hemolytic assays are a sensitive means of assessing function of the terminal events in the complement system. They provide a wholistic view of all reactions downstream of C5 activation, since disruption at any point along this pathway will impact generation of the MAC. Therefore, we used the lysis of rabbit erythrocytes in the presence of 12% (v/v) normal human serum and under conditions specific for the AP to test for effects of Efb-C, FHR2, or both proteins together on C5 activation. Since Efb-C is a potent inhibitor of the AP on its own (53), we initially determined the IC₅₀ value for Efb-C under these experimental conditions; we obtained a value of ~0.51 µM, which corresponded well to the expected concentration of C3 in these assays (i.e. ~0.78 µM) [Fig. 5.6A]. We then examined the effects of including FHR2 or FHR2(3-4) in hemolytic assays that contained sub-optimal levels of Efb-C [Fig. 5.6B]. When we performed hemolytic assays in the presence of 0.25 µM Efb-C, FHR2, or FHR2(3-4) alone, we observed little inhibition of hemolytic activity and no statistically significant differences between groups when Efb-C was combined with other proteins in the experiment. However, when we repeated these hemolytic

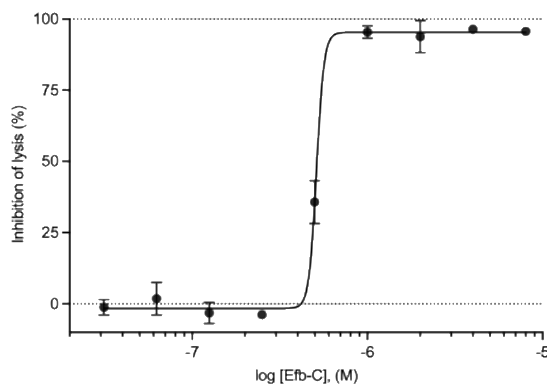
assays in the presence of 0.5 μ M Efb-C, FHR2, or FHR2(3-4), either alone or in combination, we observed a synergistic effect between Efb-C and FHR2. Whereas Efb-C yielded blocked hemolysis by $25.6 \pm 1.8\%$ and FHR2 by $6.5 \pm 1.4\%$, including both Efb-C and FHR2 resulted in a potent increase in inhibition to $81.0 \pm 3.1\%$ that was highly statistically significant (i.e., $p < 0.0001$) when compared to either protein alone. We also noted synergistic effects for FHR2(3-4), which resulted in $6.4 \pm 3.8\%$ inhibition on its own but which rose to $34.3 \pm 4.0\%$ when combined with Efb-C, as well as FH(19-20), which yielded $3.6 \pm 2.0\%$ and $38.3 \pm 3.5\%$ inhibition, respectively. Nevertheless, the overall inhibitory efficacy for both of these truncations in the presence of Efb-C was noticeably

lower than for full-length FHR2. This strongly suggests that the synergistic effect between Efb-C and FHR2 is potentiated by dimerization of the endogenous AP regulator.

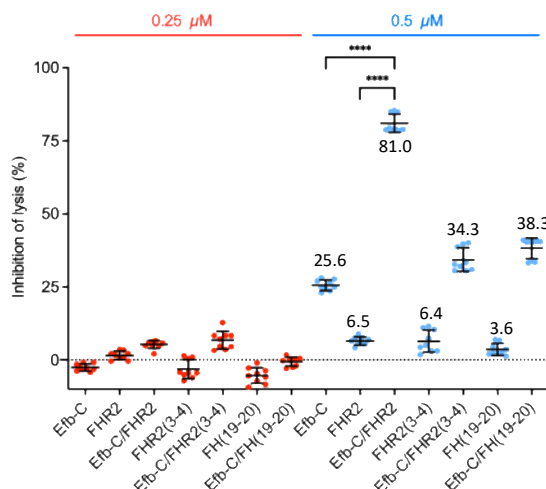
Figure 5.6 Efb-C Mediated Recruitment of FHR2 to C3b Synergistically Inhibits Complement Downstream of C5 Activation.

(A) Rabbit erythrocytes were washed and resuspended in GVBo + Mg-EGTA, then combined with various concentrations of Efb-C in the presence of 12% (v/v) normal human serum as a source of complement components. Following a 30 min incubation at 37 °C, hemolysis was assessed spectrophotometrically according to the procedures described in Materials and Methods. Normalized values from six technical replicates ($n=6$) were expressed as a function of Efb-C concentration and were fit to a four-parameter dose-response curve. Error bars represent the standard deviation. The apparent IC_{50} for Efb-C was 0.51 μ M under these conditions. (B) Identical hemolytic

A Hemolysis Inhibition – Dose-Response



B Hemolysis Inhibition – Fixed Concentration



assays to those shown in panel A were used to interrogate terminal complement pathway activity in the presence of various inhibitors. Trials were conducted for FHR2, FHR2(3-4), and FH(19-20) either in the absence or presence of an equal concentration of Efb-C. Note that two different concentrations were used (i.e., 0.25 μ M and 0.5 μ M) that give incomplete inhibition for Efb-C alone. Nine technical replicates (n=9) were performed for each experimental series, prior to plotting the data. Error bars show the mean plus and minus one standard deviation, while the mean percent inhibition is listed above each experimental series for the 0.5 μ M group. Measures of statistical significance were determined by one-way ANOVA, using Graph Pad Prism. ****, $p<0.0001$.

Discussion

FH is a potent negative regulator of AP activity and is relatively abundant in human blood (~0.5 mg/mL; (166)). Thus, it is not surprising that over a hundred different immune evasion proteins from various bacteria, fungi, viruses, protzoa, and parasites have been reported to bind FH and recruit it to the pathogen surface (167). Yet, it seems that there is more to this phenomenon than simply camouflaging the pathogen with a complement inhibitory shield. In certain cases, evasion proteins like *S. pneumoniae* PspC have been shown to augment the functional properties of the FH they capture (168). In other cases, evasion proteins like *S. aureus* Efb (53, 58, 117, 158) have been shown to stabilize a conformational state in C3b that is associated with enhanced FH binding activity (117). Considering this, and owing to the sequence similarities between FH and FHR family members [Fig. 5.1], we wondered whether Efb-C-mediated conformational effects on C3b might also influence its binding to FHRs. Here we found that Efb-C binding to the TED/C3d domain recruits FHR2 to C3b [Figs 5.2-5.4]. Although this recruitment effect arises from interactions between Efb-C, C3d, and FHR2(3-4) [Figs. 5.2-5.3], its influence on complement activity requires FHR2 dimerization via FHR2(1-2) to manifest full potency [Fig. 5.6]. The ternary complex between FHR2, C3b, and Efb-C does not slow the rate of C3 convertase formation more than Efb-C on its own, nor does it impart any FH-like complement regulatory properties that act at the level of C3b [Fig. 5.5]. However, it does result in potent, synergistic inhibition of the terminal

complement pathway downstream of C5 activation [Fig. 5.6]. Since *S. aureus* is a Gram-positive bacterium and resistant to lysis by the MAC (153), we suspect this mechanism actually serves to block C5a generation, thereby impairing neutrophil recruitment to sites of complement activation. The fact that *S. aureus* secretes at least eight other proteins that impair neutrophil chemotaxis and activation serves to emphasize how important evasion of neutrophil-mediated killing is to *S. aureus* survival (52).

In this study, we characterized both the FHR2(3-4)/C3d and FHR2(3-4)/C3d/Efb-C complexes through biochemical and structural approaches [Figs. 5.2-5.4]. Despite the clear recruitment effect of Efb-C on FHR2(3-4) binding to C3d [Fig. 5.2], we found few direct interactions and little buried surface between Efb-C and FHR2(3-4) in the ternary complex structure that could explain this recruitment [Fig. 5.3]. It is possible that electrostatic effects could somehow contribute to FHR2(3-4) recruitment when C3d is bound by Efb-C, which is a highly positively charged molecule on its own (pI = 10.5); however, this seems unlikely since the recruitment effect is ~6-fold greater for FH(19-20) than FHR2(3-4), and FH(19-20) has a much higher isoelectric point than does FHR2(3-4) (c.f. pI = 9.1 versus 6.9). Alternatively, unstructured residues at the N-terminus of Efb-C could help promote FHR2(3-4) binding to C3d through transient interactions; indeed, contacts of this sort are known to occur between the N-terminal residues of Efb-C and C3d, as has been shown through protein footprinting approaches (169). In this regard, it is worth noting that the N-terminal region of Efb is protease-sensitive and intrinsically-disordered in the absence of its ligand, fibrinogen (53, 157). Whether proteolytic degradation of this region, or the presence of Efb-bound fibrinogen influences recruitment of full-length FHR2 to C3b will require further investigation.

Although Efb-C mediated recruitment of FHR2 to C3b arises from effects on the FHR2(3-4) region [Fig. 5.2-5.4], our results show that dimerization of FHR2 is required for maximal functional outcome [Fig. 5.6]. Indeed, while inclusion of FHR2(3-4) along with Efb-C in hemolytic assays resulted in a ~1.3-fold increase in inhibition that was highly statistically significant when compared to Efb-C alone, inclusion of full-length FHR2 in identical experiments resulted in a much greater ~3.2-fold increase in inhibition. The most obvious structural difference between FHR2(3-4) and FHR2 is the presence of the FHR2(1-2) region, which is known to mediate dimerization of FHR2 (154, 155). Both FHR1 and FHR5 have regions nearly identical to FHR2(1-2) at their respective N-termini (154, 155), which allows them to form both homo- and heterodimers of amongst each other. Whether homodimers of FHR1 and FHR5 display synergistic inhibition of complement in the presence of Efb-C remains unknown. Moreover, certain combinations of FHR1, FHR2, and FHR5 heterodimers exist in human serum (154), and whether these synergistically inhibit complement along with Efb-C is also unknown at this time. Both of these subjects should be explored through future studies.

Previously, Amdahl et al. reported that the *S. aureus* evasion protein Ehp and FH enhance the complement inhibitory functions of one another (160). Although no crystal structure was provided in their study, the high levels of conservation between Efb-C and Ehp (159) strongly suggest that the effects Amdahl et al. reported arise through a ternary complex analogous in many ways to the FHR2(3-4)/C3d/Efb-C structure presented here [Fig. 5.3]. Nevertheless, a noteworthy exception between Efb-C and Ehp is that the latter contains a second high-affinity C3d-binding site (158, 159), and can potentially bind two C3b molecules. Because of this bivalency, Ehp could conceivably trigger assembly of FH/C3b dimers through an alternative mechanism to those formed by FHR2/C3b/Efb-C [Fig. 5.3F], which forms dimers due to the intrinsic behavior of FHR2. Over

two decades ago, a series of pioneering studies by Rawal and Pangburn showed that C5 binding to convertases becomes tighter with increasing surface densities of C3b (54-57). Thus, it seems that the high surface densities of C3b required for efficient C5 activation would also favor formation of dimeric C3b structures via these *S. aureus* immune evasion proteins. Unraveling the interplay between Efb, Ehp, C5 convertases and other complement components during *S. aureus* infection would be an attractive subject for future work.

Earlier studies on the structure, function, and mechanism of Efb-C resulted in the identification of two conformational states in C3b (53, 58, 117, 158). By binding to the TED domain and selectively stabilizing an open conformational state in C3b, Efb-C changes how C3b interacts with multiple ligands including FB, FH, and C5 (58). Inhibiting FB binding (i.e., forming the AP C3 pro-convertase) is the earliest acting of these effects and has a clearly defined role in blocking AP activity (53, 58, 117, 158). However, the functional consequences of altered FH binding have remained elusive due to their seemingly opposing effects: whereas Efb-C enhances binding of full-length FH and FH(19-20) to C3b, it completely blocks binding of the complement regulatory region, FH(1-4), to C3b (58, 117). Here, we showed the Efb-C binding to C3b recruits the complement regulatory protein, FHR2, and results in synergistic inhibition of the complement system downstream of C5 activation. This observation shows that selective stabilization of a conformational substate in C3b can result in unexpected inhibitory effects arising via endogenous complement regulatory proteins, above and beyond impairment of convertase formation. In the future, it is possible that phenomena such as these could be leveraged for development of new complement targeted therapeutics (58).

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Abbreviations

AP; alternative pathway of complement
C3; complement component C3
C3b; complement component C3b
iC3b; inactivated complement component C3b
C3d; complement component C3d
C4b; complement component C4b
C5; complement component C5
CCP; complement control protein domain
CP; classical pathway of complement
Efb; extracellular fibrinogen-binding protein from *S. aureus*
Efb-C; C-terminal domain of the *S. aureus* extracellular fibrinogen-binding protein
Ehp; Efb homolog protein
FB; complement factor B
FD; complement factor D
FH; complement factor H
FHR; complement factor H related
FI; complement factor I
FP; properdin
HBS-T; HEPES buffered saline with tween-20
HBS; HEPES buffered saline
LP; lectin pathway of complement
MAC; membrane attack complex
PEG; polyethylene glycol
RCA; regulator of complement activity
SP; serine protease domain
SPR; surface plasmon resonance
TED; thioester-containing domain

Supplementary information

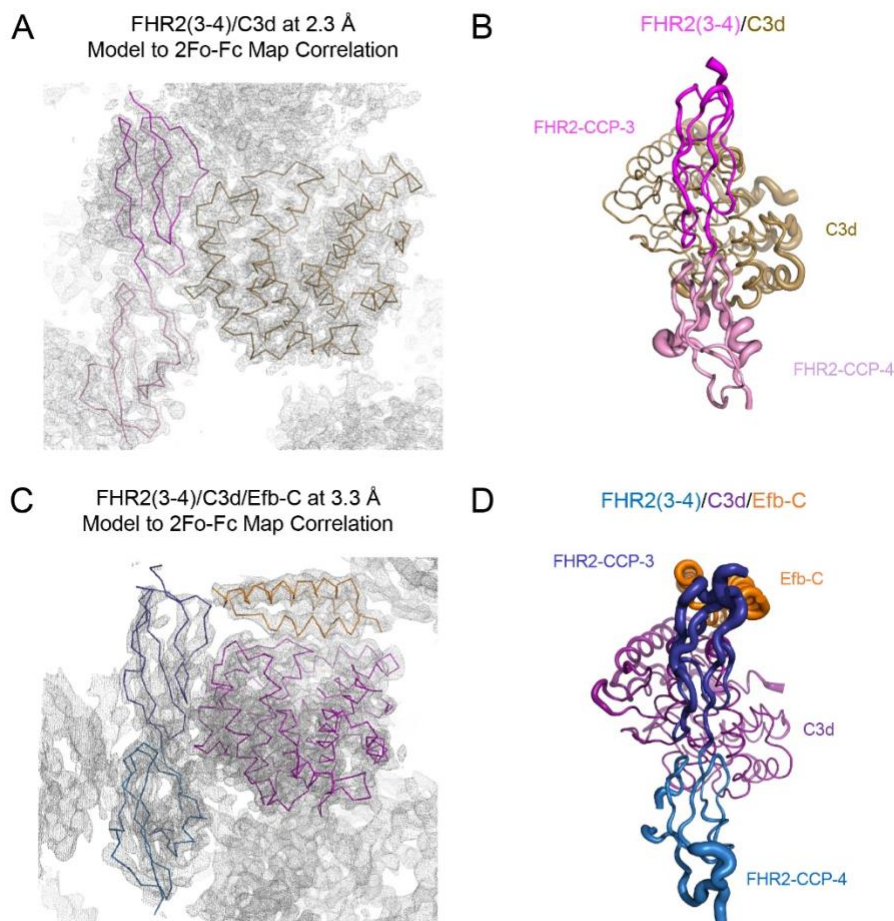


Figure S5.1. Supporting Information Pertaining to Crystal Structure Determinations. (A) Representative model to map correlation for the FHR2(3-4)/C3d complex represented by chains A and B in the PDB entry 9N1Z. The 2Fo-Fc map calculated at 2.3 Å limiting resolution and contoured at 1.2σ is presented as a grey mesh. Proteins are drawn as Cα wire diagrams, with FHR2(3-4) in pink and C3d in gold. (B) An orthogonal view of the FHR2(3-4)/C3d complex determined at 2.3 Å resolution is shown. FHR2(3-4) is drawn as a ribbon diagram with its CCP3 domain colored magenta and its CCP4 domain colored pink, while C3d is colored gold. (C) Representative model to map correlation for the FHR2(3-4)/C3d/Efb-C complex in the PDB entry 9N20. The 2Fo-Fc map calculated at 3.3 Å limiting resolution and contoured at 1.2σ is presented as a grey mesh. Proteins are again drawn as Cα wire diagrams, with FHR2(3-4) in blue, C3d in purple, and Efb-C in orange. (D) An orthogonal view of the FHR2(3-4)/C3d/Efb-C complex determined at 3.3 Å resolution is shown. FHR2(3-4) is drawn as a ribbon diagram with its CCP3 domain in dark blue and its CCP4 domain in light blue, while C3d is colored purple and Efb-C is colored orange. Note that the FHR2(3-4) and Efb-C proteins meet at roughly a right angle to one another.

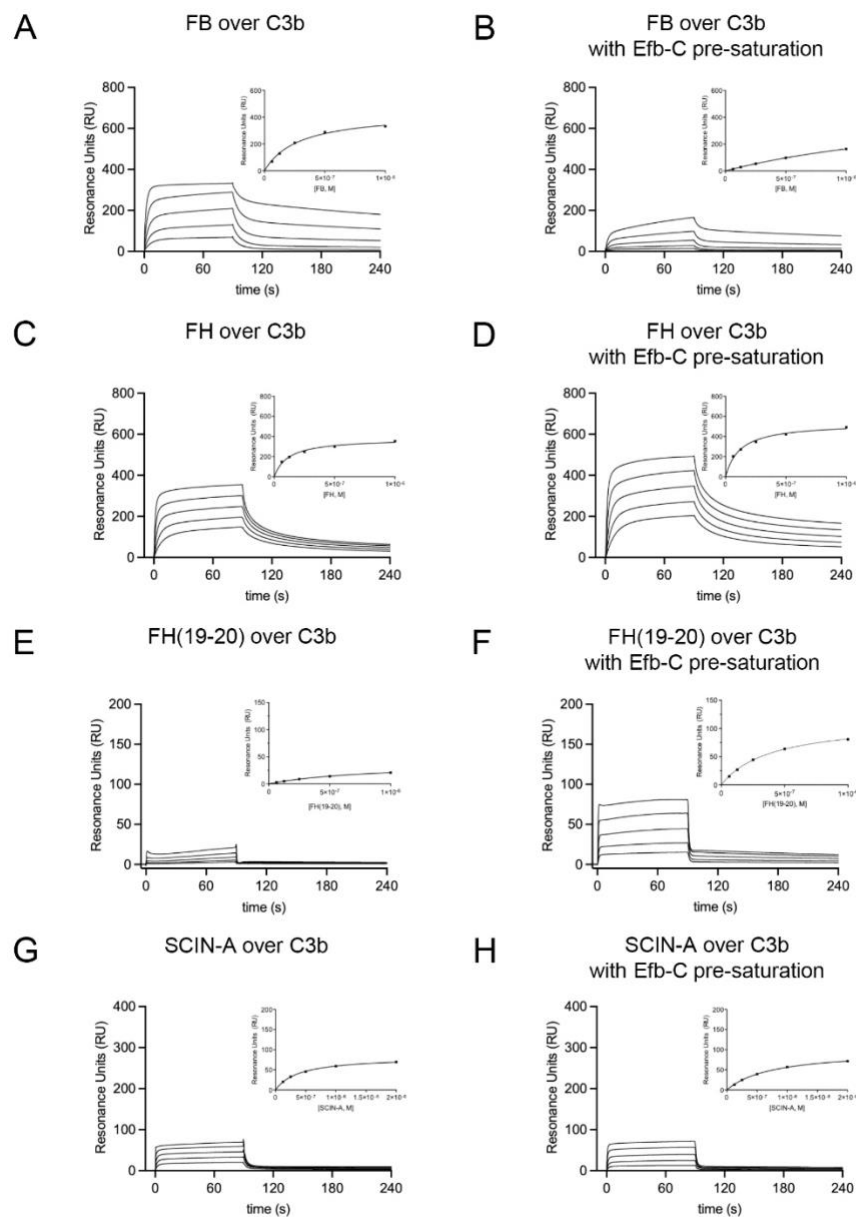


Figure S5.2. Validation of Differential Ligand Binding for C3b versus C3b/Efb-C. Site-specifically biotinylated C3b was captured at nearly equivalent densities on two experimental flow cells of a streptavidin-coated sensor chip. Prior to each injection series, a single C3b-modified flow cell was presaturated with Efb-C as described in Materials and Methods. Thereafter, concentration series of various analytes were injected over the experimental surfaces in reference-subtraction mode. Simple dose-response fits illustrating the differences between C3b and C3b/Efb-C are inset. (A, B) Sensorgrams obtained by injecting a concentration series of FB over the surface of C3b alone or C3b/Efb-C, respectively (C, D) Sensorgrams obtained by injecting a concentration series of FH over the surface of C3b alone or C3b/Efb-C, respectively. (E, F) Sensorgrams obtained by injecting a concentration series of FH(19-20) over the surface of C3b alone or C3b/Efb-C, respectively. (G, H) Sensorgrams obtained by injecting a concentration series of SCIN-A over the surface of C3b alone or C3b/Efb-C, respectively. Note that binding of C3b ligands may be affected negatively, positively, or not at all by the presence of Efb-C.

Chapter 6 - Conclusion

The complement system, a cornerstone of innate immunity, is a double-edged sword: while essential for host defense, its dysregulation leads to pathologies ranging from thrombotic microangiopathies to chronic inflammatory disorders (10). Inhibiting the complement system is the most effective strategy for treating complement-mediated diseases. Although several complement inhibitors have been developed and approved, limitations such as reduced efficiency and specificity underscore the need for novel complement inhibitors (27). This thesis focuses on the fundamental processes of complement inhibition, exploring four distinct strategies, each offering unique insights into complement inhibition.

First, we evaluated the binding affinity of CRIg to C3 cleavage products (C3b, iC3b, and C3c) and explored CRIg-based alternative pathway (AP) inhibitors through mutagenesis and chemical modification. Contrary to earlier reports of weak C3b binding ($K_D = 1.1 \mu\text{M}$), our results reveal significantly stronger affinities (10–100 nM) for wild-type CRIg and lysine-depleted variants (CRIg-Mut-1/2). CRIg exhibited progressively weaker binding to downstream C3 products ($\text{C3b} > \text{iC3b} > \text{C3c}$), indicating a biological preference for surface-bound C3b/iC3b over inactive C3c. To enhance its inhibitory activity, lysine-depleted CRIg mutants were engineered for site-specific crosslinking. Although these mutants displayed modest (~2–3-fold) functional reductions, crosslinking with a short PEG12 spacer substantially improved AP inhibition. A longer PEG25 spacer reduced activity, likely owing to steric hindrance in targeting C5 convertases (C3bBbC3b), highlighting the importance of crosslinker design. CRIg thus emerges as a promising AP-specific therapeutic scaffold, with future work potentially involving polyvalent dendrimers or lysine-free mutants to optimize efficacy, as well as providing tools for studying C5 convertase dynamics.

Second, we demonstrated that synthetic DNA aptamers targeting factor B (FB) effectively inhibit AP hyperactivity induced by atypical hemolytic uremic syndrome (aHUS)–associated FB variants. aHUS is a severe disease marked by hemolytic anemia, thrombocytopenia, and renal failure, driven primarily by dysregulated AP activity (103). While many cases stem from loss-of-function mutations in complement regulators, some involve gain-of-function mutations in AP components, notably FB (29). Here, a DNA aptamer specifically designed to interrupt the FB–C3b interaction provided proof-of-concept for reducing excessive AP activity caused by mutant FB variants. Systematic analyses of multiple aHUS-linked FB variants uncovered differences in C3b affinity and proconvertase formation kinetics compared to previous reports, with enhanced stability against factor H–mediated decay as a common feature. Interestingly, one mutation (F286L) showed reduced hemolytic activity at high concentrations, suggesting alternative binding events might diminish the availability of catalytically competent complexes. The aptamer retained its inhibitory effect even in the presence of properdin, underscoring its capacity to prevent AP overactivation. Although measured affinity fell in the nanomolar range, rather than the originally reported picomolar range, this did not compromise potency given the high plasma concentration of FB. Future directions include optimizing aptamer pharmacokinetics via chemical modifications that enhance serum half-life and stability. Despite current therapies for aHUS, FB-binding aptamers may offer an additional therapeutic modality for complement-mediated diseases driven by AP dysfunction, leveraging their synthetic accessibility, high solubility, and low immunogenicity, while demanding further engineering to ensure robust in vivo performance.

Third, we illustrated the mechanism of SALO, a classical pathway (CP) inhibitor identified nearly a decade ago. SALO binds the zymogen form of C1r with nanomolar affinity and blocks its autoactivation by shielding the activation loop, thereby preventing downstream C1r and C1s

functions. Structural studies showed that sulfation of SALO at Y51, though not strictly required, markedly boosts its affinity and inhibitory potency. This “zymogen-shielding” mechanism distinguishes SALO from other immune evasion proteins or therapeutic inhibitors, which typically target active proteases or substrate-binding sites. By inhibiting C1 at an earlier step than current monoclonal anti-C1s therapies, SALO could achieve more comprehensive CP suppression, though its antigenicity in humans remains uncertain. Future work will need to address possible immunogenicity, investigate the presence of SALO-like molecules in other hematophagous species or pathogens, and evaluate whether this zymogen-blocking approach can be developed into viable therapeutics for CP-driven diseases. More broadly, SALO’s novel mode of action may spur new strategies for selectively controlling other protease zymogens in diverse biological pathways.

Fourth, we defined a novel immune evasion strategy of *S. aureus*, which employs its virulence factor EfbC to hijack the complement regulator FHR-2. While EfbC alone binds C3b and prevents C3 convertase assembly, it also recruits FHR-2 to form a ternary complex with C3b. Although FHR-2 alone does not accelerate convertase decay or exhibit cofactor activity, this enhanced interaction with C3b, mediated by EfbC, yields stronger inhibition of the terminal complement pathway than either protein alone. This mechanism mirrors how other pathogens recruit FHR proteins to evade complement. From a therapeutic perspective, understanding EfbC–FHR-2 complex formation could inspire novel approaches for selectively inhibiting late-stage AP events, potentially mitigating inflammation or tissue damage stemming from excessive complement activation. Future investigations might explore whether other members of the FHR family can be similarly hijacked, further clarifying this sophisticated immune-evasion strategy and informing the design of new terminal pathway inhibitors.

Overall, our investigations reinforce the importance of complement regulation and provide diverse models for how interference at different levels of the cascade can be harnessed as potential therapeutics. From CRiG-based designs and DNA aptamers to SALO's unique zymogen-shielding mechanism and the ternary complexes formed by EfbC, these studies underscore that highly targeted approaches offer the possibility of effective and precise inhibition. Moving forward, more detailed pharmacological characterization, combined with advanced structural and functional assays, will guide rational improvements to these inhibitors and foster new discoveries that push the boundaries of complement-targeted interventions.

Chapter 7 - Reference

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Chapter 2-New Insights into the Complement Receptor of the Ig Superfamily (CRIg) Obtained from Structural and Functional Studies on Two Mutants

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