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Decay-accelerating factor (DAF; CD55) inhibits the complement (C) cascade by dissociating the multimolecular C3 convertase enzymes central to amplification. We have previously demonstrated using surface plasmon resonance (Biacore International) that DAF mediates decay of the alternative pathway C3 convertase, C3bBb, but not of the inactive proenzyme, C3bB, and have shown that the major site of interaction is with the larger cleavage subunit factor B (Bb) subunit. In this study, we dissect these interactions and demonstrate that the second short consensus repeat (SCR) domain of DAF (SCR2) interacts only with Bb, whereas SCR4 interacts with C3b. Despite earlier studies that found SCR3 to be critical to DAF activity, we find that SCR3 does not directly interact with either subunit. Furthermore, we demonstrate that properdin, a positive regulator of the alternative pathway, does not directly interact with DAF. Extending from studies of binding to decay-accelerating activity, we show that truncated forms of DAF consisting of SCRs 2 and 3 bind the convertase stably via SCR2-Bb interactions but have little functional activity. In contrast, an SCR34 construct mediates decay acceleration, presumably due to SCR4-C3b interactions demonstrated above, because SCR3 alone has no binding or functional effect. We propose that DAF interacts with C3bBb through major sites in SCR2 and SCR4. Binding to Bb via SCR2 increases avidity of binding, concentrating DAF on the active convertase, whereas more transient interactions through SCR4 with C3b directly mediate decay acceleration. These data provide new insights into the mechanisms involved in C3 convertase decay by DAF.


The central enzyme of the AP amplification loop is the C3 convertase, C3bBb. The proenzyme, C3bB, is formed following Mg2+−dependent binding of factor B (B) to the activated fragment of C3, C3b. Bound B is cleaved by the plasma serine protease factor D (D), resulting in release of Ba (smaller cleavage subunit of B) and activation of the serine protease domain within Bb (larger cleavage subunit of B) to form the active convertase (4). The convertase then cleaves many molecules of C3 to C3b, each of which can form a convertase, thus amplifying the pathway. The enzyme is naturally labile with a half-life of ~90 s; association of the complex with properdin extends the half-life to ~30 min (5). Following cleavage of C3 to nascent C3b, nucleophilic attack on the internal thioester bond in C3b results in covalent binding to the activating surface (6). Foreign surfaces are usually devoid of membrane regulatory proteins that inactivate the convertase, this lack of regulation thus leads to amplification. Host cells, however, are protected from the damaging effects of C by cell surface-associated convertase regulatory proteins. In the human, these are decay-accelerating factor (DAF; CD55) and membrane cofactor protein (MCP; CD46). These proteins cooperate to prevent opsonisation and damage to cells at a site of C activation by inactivating the convertase enzymes (7–9). DAF accelerates decay of the convertase, releasing Bb (or C2a in the CP), whereas MCP binds to C3b (or C4b) on the surface and acts as a cofactor for its irreversible proteolytic inactivation by the plasma protease factor I. DAF and MCP are encoded by genes located in the “regulators of C activation” gene cluster and are assembled from similar functional domains, termed short consensus repeats (SCRs), found in many C-regulatory proteins that bind C3b and C4b (10). DAF is composed from the amino terminus of four SCR domains followed by a short “stalk” that is heavily glycosylated and a GPI anchor (11).

We have previously used surface plasmon resonance (SPR) to investigate formation and regulation of the AP C3 convertase and
have demonstrated that kinetics of formation of the proenzyme and the activated convertase were quite different; activation of the proenzyme produced a more stable enzyme with a slower rate of decay (12). The two complexes also differed in their susceptibility to decay using EDTA and soluble DAF. The proenzyme was rapidly broken apart by Mg$^{2+}$ chelation with EDTA whereas the activated enzyme was not, presumably reflecting differences in proenzyme conformation and coordination around the Mg$^{2+}$ ion. Only the activated enzyme was subject to decay acceleration mediated by soluble DAF. Using SPR, we analyzed the way in which DAF interacted with individual components of the AP convertase enzyme (12). DAF bound weakly to C3b ($K_D$ $\sim 10^{-5}$ M), very weakly to fB ($K_D$ $\sim 4 \times 10^{-5}$ M), but much more tightly to the cleaved fragment Bb ($K_D$ $\sim 10^{-6}$ M). DAF bound Bb via the von Willebrand factor type A (vWFA) domain rather than the serine protease domain. Functional studies with recombiant C2 and fB mutated within this domain indicated that DAF mediated its regulatory action via interactions with vWFA, and our kinetic analyses demonstrated that the Bb fragment and the vWF domain bind DAF with the same kinetic profiles and affinities (12–14). The specificity of DAF for the active convertase may be a consequence of its higher affinity for Bb compared with fB, and suggests that the regulatory function of DAF on the cell membrane is focused entirely on inhibition of activated enzymes rather than on binding to inactive proenzymes.

The mechanism of decay acceleration is still incompletely understood. It is clear from work described above that DAF interacts with both subunits of the active C3 convertase and that spacing of DAF above the membrane is crucial to function (12, 15, 16). Structure/function studies of human DAF using SCR-deletion mutants indicated that the first SCR was not required for regulatory function, whereas SCRs 2 and 3 were required for inhibition of either the CP or AP (15, 17). Deletion of SCR4 had a critical effect on regulation of the AP. In support of a key role for SCRs 2 and 3, AB-blocking studies demonstrated that Abs directed against these SCRs blocked DAF function, with Abs to SCR3 having a particularly profound effect (15, 18). To further define which domains of DAF are involved in the interaction with components of the convertase, we have generated soluble recombiant mutants of DAF lacking specific SCR domains. These were used for SPR analysis to assess the affinity of interactions between specific domains of DAF and individual components of the AP convertase. Functional assays, including real-time analyses, were used to determine which domains of DAF were required for decay acceleration of the intact enzyme. These data shed new light on the mechanisms involved in decay acceleration of the AP C3 convertase.

Materials and Methods

Materials

C3, properdin, and fB were prepared by classical column chromatography using established methods (19). fD was purchased from Complement Technology. C3b and Bb were prepared from these reagents by activation of the AP as described previously (12). Soluble recombiant C receptor 1 (CR1) was a gift from T Cell Sciences (Needham, MA).

Expression and purification of soluble DAF constructs

Recombiant human DAF comprising the four SCRs (DAF1234) was isolated and refolded from Escherichia coli as described previously (20, 21). The structure and function of DAF1234 has been previously studied, and the purified, refolded protein is known to consist of four correctly folded inclusion bodies using an identical protocol to that of DAF1234. These mutants have been described previously and have been shown to be functional with respect to echovirus binding (22).

Hemolysis assays

To assess regulation in the CP, sheep E (TCS Microbiology) were sensitized by incubating 1 volume of 4% E (v/v) with 1 volume of 1/250 rabbit anti-sheep E (Amboceptor; Behring Diagnostics) for 30 min at 37°C. Sensitized cells were washed three times in complement fixation diluent (Oxoid) supplemented with 0.1% gelatin and resuspended to 2%. To assess regulation in the AP, rabbit E were washed into AP buffer (5 mM sodium barbitone (pH 7.4), 150 mM NaCl, 7 mM MgCl$_2$, 10 mM EGTA, and 0.1% gelatin) and resuspended to 2%. For both assays, a 50 µl aliquot was incubated with 50 µl of normal human serum and 50 µl of a dilution of test protein or a control protein. All dilutions were made in complement fixative essentially as described previously (13, 23). In brief, a very small nidius of C3b ($\sim$50 unit) was coupled to the chip surface using amine coupling. Subsequently, a mix of fB and fD was flowed over the surface to form the convertase followed by C3. Cleavage of C3 to C3b close to the chip surface enabled coupling through the native thioester group. Affinity measurements were taken on surfaces with different amounts of C3b immobilized. It was not possible to immobilize Bb on the surface and retain binding activity; therefore, all Bb interactions were studied with soluble recombiant DAF mutants (20) on the chip surface, these were immobilized using amine coupling according to the manufacturer’s instructions (amine coupling kit; Biacore International). Properdin was also immobilized on the surface of a CM5 chip using amine coupling as described previously (24). For all kinetic analyses a CM5 chip (carboxymethylated dextran surface; Biacore International) was used and data collected at 25°C, the flow rate was maintained at 30 µl/min, and data from a reference cell was subtracted to control for bulk flow effects. Specific activity of the C3 convertase was expressed as percent lysis in the absence of inhibitor. Cells were incubated at 37°C for 30 min, centrifuged to pellet cells, and 50 µl of supernatant was removed for measurement of absorbance at 415 nm (hemoglobin release). Control incubations included cells incubated in buffer only (0%) or in 0.01% Triton X-100 (100%). Percentage of lysis was calculated as follows: percentage of lysis = 100 × (A$_{415}$ test sample − A$_{415}$ 0% control)/(A$_{415}$ 100% control − A$_{415}$ 0% control). A noninhibitory protein was used as a control, and inhibitory activity of the test protein was calculated: percentage of inhibition = 100 × (percentage of lysis with negative control protein − percentage of lysis with test protein)/percentage of lysis with control protein.

Surface plasmon resonance

All analyses were conducted on a Biacore 3000 (Biacore International) other than that in Fig. 4, which was conducted on a Biacore T100. C3b was coupled to the chip surface using its native thioester bond as described previously (12, 23). In brief, a very small nidius of C3b ($\sim$50 unit) was coupled to the chip surface using amine coupling. Subsequently, a mix of fB and fD was flowed over the surface to form the convertase followed by C3. Cleavage of C3 to C3b close to the chip surface enabled coupling through the native thioester group. Affinity measurements were taken on surfaces with different amounts of C3b immobilized. It was not possible to immobilize Bb on the surface and retain binding activity; therefore, all Bb interactions were studied with soluble recombiant DAF mutants (20) on the chip surface, these were immobilized using amine coupling according to the manufacturer’s instructions (amine coupling kit; Biacore International). Properdin was also immobilized on the surface of a CM5 chip using amine coupling as described previously (24). For all kinetic analyses a CM5 chip (carboxymethylated dextran surface; Biacore International) was used and data collected at 25°C, the flow rate was maintained at 30 µl/min, and data from a reference cell was subtracted to control for bulk flow effects. Specific activity of the C3 convertase was expressed as percent lysis in the absence of inhibitor. Cells were incubated at 37°C for 30 min, centrifuged to pellet cells, and 50 µl of supernatant was removed for measurement of absorbance at 415 nm (hemoglobin release). Control incubations included cells incubated in buffer only (0%) or in 0.01% Triton X-100 (100%). Percentage of lysis was calculated as follows: percentage of lysis = 100 × (A$_{415}$ test sample − A$_{415}$ 0% control)/(A$_{415}$ 100% control − A$_{415}$ 0% control). A noninhibitory protein was used as a control, and inhibitory activity of the test protein was calculated: percentage of inhibition = 100 × (percentage of lysis with negative control protein − percentage of lysis with test protein)/percentage of lysis with control protein.

Decay assays

Surface plasmon resonance. The ability of DAF mutants to decay the AP C3 convertase was assessed in real time using SPR. A total of 3800 RU of C3b was deposited on the surface of a CM5 chip as described above. Active convertase was formed by flowing 20 µl of a mix of IB (3.8 µM) and fD (0.07 µM) over the C3b surface at 20 µl/min. DAF mutants (20 µl) at specified concentrations were then flowed across the convertase, and decay was visualized in real time. To regenerate back to baseline C3b, 10 µl of DAF1234 (0.5 µM) was injected over the chip surface.

Plate assay. The ability of DAF mutants to decay the AP C3 convertase was assessed using an ELISA-based protocol essentially as described previously (15, 25). All steps were for 1 h at 37°C unless otherwise stated in 2.5 mM sodium barbitone (pH 7.4), 71 mM NaCl, 1 mM NiSO$_4$, and 0.1% Tween 20. ELISA plates (Medisorb plates; Nunc) were coated with
C3b at 3 μg/ml and blocked with buffer containing 1% BSA. Convertase was formed by incubating plates for 30 min with 0.5 μg/ml fB and 50 ng/ml fD. Plates were washed and incubated with DAF, soluble CR1, or buffer only for 20 min at room temperature. Plates were washed, and residual fB in the convertase was detected using polyclonal sheep anti-fB (Binding Site) followed by HRP-conjugated anti-sheep Ig (Binding Site). Color was developed using o-phenylenediamine dihydrochloride (Dako-Cytomation). Background values were obtained by omitting fB and fD. Absorbance was measured at 490 nm, and percentage of convertase remaining was calculated as follows: percentage of convertase remaining = 100 × (A490inh – A490Bgd)/(A490buffer – A490bnd).

Results

Assessment of C inhibitory activity by hemolysis assay

DAF1234 and deletion mutants DAF3, DAF34, and DAF23 were tested for their ability to prevent CP-mediated lysis of Ab-coated sheep EA or AP-mediated lysis of rabbit E (Fig. 1). In the CP assay, DAF1234 and DAF23 were able to inhibit lysis, and the activity of DAF23 was reduced by 4-fold compared with DAF1234. In the AP assay, DAF1234 was the only active reagent. These data are in agreement with previous reports that describe an essential role for SCR4 in the regulation of the AP.

Binding affinity between DAF, C3b, and Bb

We have previously demonstrated that DAF binds components of the AP convertase (12). In the presence of Mg2+, DAF binds weakly to C3b and fB and much more tightly to the Bb fragment. The binding affinities of the DAF SCR-deletion mutants to C3b and Bb were analyzed in this study using SPR. To analyze the binding of C3b to DAF, C3b was immobilized on the chip surface via its internal thioester group. Interaction with DAF34 (a) or DAF1234 (b) was analyzed in HBS/1 mM Mg2+ at various concentrations and sensorgram traces were obtained. Interaction experiments were performed multiple times, a typical example is illustrated here for each form of DAF. The affinity of the interaction was analyzed by steady-state analysis (see inset); Req refers to the RU of DAF specifically bound to C3b at equilibrium. c, DAF23 and DAF3 were flowed across a surface coated with high levels of C3b, no interaction was evident.

**FIGURE 1.** Hemolysis assays to assess inhibition of C by DAF mutants. a, CP: Ab-coated sheep E were attacked with human C, and the ability of recombinant soluble DAF to prevent hemolysis was determined. Percentage of inhibition of lysis was calculated as described in Materials and Methods by comparison with a noninhibitory protein. DAF1234 ( ), DAF23 ( ), DAF34 ( ), and DAF3 (x). b, AP: Rabbit E were attacked with human C and inhibitory activities determined as described for the CP. Results are means of three determinations and vertical bars represent SD.

**FIGURE 2.** Equilibrium analysis of recombinant soluble DAF mutants binding to C3b. C3b was immobilized on the chip surface via its internal thioester group. Interaction with DAF34 (a) or DAF1234 (b) was analyzed in HBS/1 mM Mg2+ at various concentrations and sensorgram traces were obtained. Interaction experiments were performed multiple times, a typical example is illustrated here for each form of DAF. The affinity of the interaction was analyzed by steady-state analysis (see inset); Req refers to the RU of DAF specifically bound to C3b at equilibrium. c, DAF23 and DAF3 were flowed across a surface coated with high levels of C3b, no interaction was evident.
when the density of C3b on the chip surface was increased to 2700 RU. To analyze binding of the Bb fragment to DAF, the various DAF mutants were immobilized on to the chip surface and Bb was flowed across the surface. It was not possible to flow DAF across a Bb-immobilized surface because amine coupling of Bb abolished the binding interaction, presumably due to the conformational sensitivity of Bb. The binding interaction between DAF1234 and Bb demonstrated a kinetic profile similar to that which we previously reported, with a $K_D$ of 0.77 μM (Table I) (12). The data generated during the association/dissociation phases fit a Langmuir 1:1 model tightly; the rapid change in signal at the inject start/stop may suggest the presence of a small proportion of fB with an altered conformation in the vWFA domain and very low affinity for DAF.

In contrast to the C3b binding described above, DAF34 did not bind Bb, whereas DAF23 bound to Bb with the same affinity and kinetic profile as DAF1234 (Fig. 3). Surface density of DAF34 was increased such that the theoretical $R_{max}$ was 2850 RU; however, binding of Bb was not evident. When DAF3 was immobilized on the chip surface no interaction with Bb was evident, even when 1000 RU of protein was immobilized (data not shown).

### Interaction of DAF with properdin

In vivo, the active AP convertase is likely to be associated with properdin. To investigate whether properdin bound to DAF, properdin was immobilized on to the dextran chip surface and DAF1234 was flowed across it. No binding interaction was evident at 48.2 μM DAF1234, even when the density of properdin on the surface was increased to 2900 RU (Fig. 4a). The integrity of the surface was confirmed by forming the proenzyme C3bB (gray line) or C3bBb (black line) on immobilized properdin (b). Soluble DAF1234 was able to decay the active convertase C3bBbP.

### Table I. Kinetic constants for binding of DAF mutants to the ligands C3b and Bb and ability to decay AP convertase

<table>
<thead>
<tr>
<th>Construct</th>
<th>C3b-Binding $K_D$ (μM)</th>
<th>Bb-Binding $K_D$ (μM)</th>
<th>Ability to Decay C3bBb</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAF1234</td>
<td>10.8 ± 1.0 (n = 5)</td>
<td>0.77 ± 0.18 (n = 2)</td>
<td>++</td>
</tr>
<tr>
<td>DAF23</td>
<td>No interaction</td>
<td>0.92 ± 0.40 (n = 3)</td>
<td>–</td>
</tr>
<tr>
<td>DAF34/C3b</td>
<td>8.7 ± 1.1 (n = 4)</td>
<td>No interaction</td>
<td>+</td>
</tr>
<tr>
<td>DAF3/C3b</td>
<td>No interaction</td>
<td>No interaction</td>
<td>–</td>
</tr>
</tbody>
</table>

Values of $K_D$ given are mean ± SD of multiple experiments (n). Ability to cause decay acceleration of the AP C3 convertase was assessed as follows: +++, powerful decay acceleration; +, decay acceleration evident; –, no decay acceleration.

Interaction of DAF with properdin

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rapid release of Bb from the convertase followed by a slower release of C3b from properdin.

**DAF-mediated decay of C3bBb monitored using SPR**

The AP convertase, C3bBb, was assembled on a Biacore chip surface by covalently binding C3b to the chip surface and forming convertase by flowing fB and fD. The ability of DAF to decay the constituent proteins was analyzed by SPR. DAF1234 was flowed across the surface at different concentrations. DAF1234 decayed the convertase very efficiently; at 5 μM and 0.5 μM baseline levels were reached almost immediately (Fig. 5, a and b), and at 0.05 μM DAF1234, decay was still evident, albeit less efficient (Fig. 5c). A binding interaction between the convertase (C3bBb) and DAF1234 was not visible because the drop in RU from accelerated decay occurred too rapidly for DAF interaction to be visualized or quantitated. At very high concentrations of DAF1234 (5 μM), a residual DAF/C3b binding event characterized by very rapid dissociation kinetics was seen (Fig. 5a).

The ability of the DAF deletion mutants to accelerate decay of the convertase was similarly analyzed. When DAF34 was flowed across the convertase surface at 5 μM, accelerated decay was evident (Fig. 5a, second inject); and at 0.05 μM DAF34, decay was still evident, albeit less efficient (Fig. 5c). A binding interaction between the convertase (C3bBb) and DAF1234 was not visible because the drop in RU from accelerated decay occurred too rapidly for DAF interaction to be visualized or quantitated. At very high concentrations of DAF1234 (5 μM), a residual DAF/C3b binding event characterized by very rapid dissociation kinetics was seen (Fig. 5a).

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barely visible (Fig. 5b). These data illustrate that SCRs 3 and 4 together contain functional sites that accelerate decay of the convertase, although ~100-fold more DAF34 was required to decay the convertase compared with DAF1234. In contrast, DAF23 bound to the convertase but accelerated decay was not apparent, even at the highest concentration (Fig. 5a, third inject). Because decay was not accelerated, it was possible to see DAF23 binding to C3bBb; the binding profile resembled the interaction of DAF with Bb (Fig. 3). DAF3 had no decay-accelerating ability at 5 μM (Fig. 5a, fourth inject). No binding of DAF3 to the convertase was evident, confirming that the observed binding of DAF23 was through SCR2.

DAF-mediated decay of C3bBb monitored using an ELISA

A second assay was used to monitor the ability of the DAF deletion mutants to decay the AP convertase. A microtiter plate was coated with C3b, and the AP convertase was assembled on the surface using fB and fD. In early experiments, plates were coated with either C3b monomer or dimer to differentiate between functional decay of the C3 and C5 convertase; however, the data were identical (data not shown) and all subsequent assays used C3b monomer on the plate surface. Following assembly of the convertase, different concentrations of DAF were incubated in wells of the plate to accelerate decay of the enzyme. Residual Bb was detected using a polyclonal anti-fB Ab, and decay was quantitated by comparing to control wells that had contained just buffer during the decay period. These data confirmed the SPR results: DAF1234 was very efficient at decaying the AP convertase with an activity comparable to soluble CR1 (Φ), and residual Bb bound to C3b was detected using anti-fB Ab as described in Materials and Methods. Results are means of three determinations and vertical bars represent SD.

Discussion

To further investigate the mechanism by which DAF accelerates decay of the AP C3 convertase, we have generated recombinant soluble constructs of DAF with deletions of SCR domains and have assessed in this study their binding interactions and functional activities in the AP. Together, these mutants covered and overlapped the proposed C-regulatory domains and were centered on SCR3, a domain suggested from various studies to be important in regulation of both the AP and CP (15, 26, 27). We used hemolysis assays to study C inhibitory function of these DAF mutants and confirmed previous observations that SCR4 was an essential requirement for regulation of the AP but not the CP (Fig. 1). To assess direct binding interactions between the DAF constructs and the AP C3 convertase components, C3b and Bb, we used SPR. The data implicated SCR2 in binding to the Bb portion of the convertase, whereas binding to C3b was mediated by SCR4. Neither the DAF23 nor the DAF3 construct bound C3b (Figs. 2 and 3). The data demonstrate two primary sites of interaction between DAF and the AP C3 convertase, but do not rule out the possibility of other weaker interactions, particularly in the context of the whole molecule where the presence of other domains may have subtle effects on the overall tertiary structure of the molecule and orientation of the domains with respect to each other. It is important to note, however, that the binding affinity of DAF1234 for C3b (~10 μM) was equal to that of DAF34 (Fig. 2; Table I), and affinity of DAF1234 for Bb (~0.9 μM) was equal to that of DAF23 (Fig. 3; Table I). This result demonstrates that these two sites of interaction can function independently and are not compromised in the smaller constructs. Because both of these interactions can take place simultaneously when DAF binds C3bBb, avidity effects will enhance overall binding affinity to the active convertase.

To further address the relative importance of the C3b/SCR4 and Bb/SCR2 interactions in regulation of the AP convertase, we used two different assays that assessed the ability of constructs to decay the convertase. Decay of the enzyme was monitored using SPR (Fig. 5). By visualizing binding and decay in real time, we found that the construct consisting of SCRs 3 and 4 mediated decay of C3bBb, albeit less efficiently than the construct containing all four SCRs. DAF23 bound the convertase (binding affinity for Bb is ~0.9 μM) but binding did not mediate accelerated decay, in contrast to binding of SCR34 that accelerated release of Bb. These activities were confirmed using ELISA-based assays to monitor release of Bb from C3b on the plate surface (Fig. 6). One interpretation of these data is that decay of C3bBb occurs as a consequence of the C3b/SCR4 interaction. A possible sequence of events is as follows: DAF binds to the active convertase, C3bBb, through interactions in SCR2 and SCR4. Efficient binding to Bb (but not fB) targets DAF to the active enzyme. Because binding between SCR4 and C3b is likely to be weak (Kd of DAF34/C3b interaction is ~10^-5 M), we suggest that the primary role of the Bb/SCR2 interaction in regulation of the AP is to focus DAF on the active convertase and to hold the regulator in position, thereby aiding the SCR4/C3b interaction. Following binding of SCR4, accelerated decay is induced, possibly through a conformational transition in C3b that results in destabilization of the interaction with Bb and its release. Because C3b can rebind fB, any transition in C3b must be reversible following dissociation of DAF and Bb from C3b. It is noteworthy that the conformation of Bb once released from the convertase is likely to differ from that in the C3bBb complex or in free fB because released Bb does not rebind to C3b. Binding of the mutants DAF34 and DAF23 to the convertase (Fig. 5) can be explained as follows: DAF 34 binds to the C3b fragment of the convertase; there is no avidity of binding through Bb; therefore, 100-fold more DAF is required for binding (Fig. 5a, gray trace, compared with Fig. 5c, black trace). Destabilization of the convertase proceeds via the SCR4 interaction with C3b and Bb is dissociated. In the case of DAF23, the mutant binds to Bb, and this interaction can be visualized by SPR (Fig. 5a, black dashed trace); however, there is no interaction with C3b, the enzyme does not break apart, and Bb remains bound to C3b.

We propose that the role of SCR3 is as a crucial spacing domain enabling correct orientation and presentation of SCR2 to Bb and SCR4 to C3b. The data summarized above contradict the perceived
view of convertase regulation in that they do not reveal any binding interaction between SCR3 and the convertase. SCR3 has been implicated in regulatory function in various studies including Ab-blocking and mutation studies. Molecular modeling has highlighted two regions that might interact with convertases, a stretch of positively charged amino acids (KKK125–127) predicted to lie in the groove between SCRs 2 and 3, and a hydrophobic patch (L147–P148) located on the surface of SCR3 (28). Indeed, these amino acids are highly conserved across species, and disruption of either of these patches impaired ability of DAF to regulate C (29, 30). Point mutation studies have identified numerous amino acids whose substitution affects C regulation, with some of these appearing to be key to function (26, 27). Residues whose mutation affects regulation of both pathways are located in SCRs 2 and 3 and include R69, R96, R100, L171 (SCR3), and two residues in the previously identified regions K127 and F148 (SCR3). Other residues are specific to regulation of a particular pathway such as E134 in the CP and K126, F169 (SCR3), and E206 (SCR4) in the AP.

Resolution of the solution structure of pairs of DAF domains (SCR23) and the crystal structure of the entire molecule have enabled location of residues on the DAF surface and surface charge to be mapped. Residues highlighted from point mutation studies are located on both “faces” of DAF, implying that DAF may fit into a groove on the convertase with contacts on both sides of the molecule. The solution structure of SCR23 indicated a flexible hinge between the two domains with a positively charged interface encompassing the KKK residues highlighted above (31). The crystal structure of SCR1234 demonstrated a near linear molecule with a rigid junction between SCR2 and SCR3, a band of positive charge encircling the upper portion of this junction, and a band of uncharged residues encircling the amino-terminal end of SCR3, including F148, F169, and L171 (20, 27). This hydrophobic patch was prominent only in the crystal structure, a consequence of differences in side-chain orientations between this and the solution structure. It was proposed that the energy of binding between DAF and the convertase was provided in part by association between this hydrophobic domain and a similar hydrophobic patch on the convertase (20). It is clear that SCR3 is indeed key to function; however, we demonstrate no direct binding with the convertase, C3bBb, or with properdin (Fig. 4). The role of SCR3 in maintaining SCR2 and SCR4 in the correct orientation crucial for efficient decay acceleration is supported by the crystal structure showing the SCR2–3 and SCR3–4 junctions to be rigid. Alteration of residues, such as KKK125–127 at the SCR2–3 join or hydrophobic residues at the amino terminus of SCR3, will impact on DAF function by altering this crucial domain orientation. Mutations in SCR2 or its deletion weaken regulatory function due to loss of interaction with Bb. Mutations in SCR3 affect the relative orientations of SCR2 and SCR4, whereas deletion of SCR3 will destroy the correct spacing between two crucial domains, effectively removing the cooperative binding between SCR2 and SCR4. The observed function blocking effects of Abs against SCR3 are explained by steric hindrance because this SCR is located between the two SCR that interface with the convertase.

In summary, we have used sensitive techniques to study the interactions of individual domains of DAF with components of the AP C3 convertase and have identified SCR2 and SCR4 as primarily responsible for binding the active enzyme. Furthermore, we have interrogated the mechanism by which DAF mediates decay. The ability of DAF to “lock” in to an active enzyme, but not to the proenzyme is due to the comparatively tight interaction between SCR2 and the Bb domain. Once held in place, SCR4 interacts efficiently with C3b and decay acceleration follows.

Interaction between SCR4 and C3b is prevented in the proenzyme C3bB because the Ba domain (comprised from three SCR domains) is still juxtaposed with C3b. The data presented in this study shed new light on the mechanisms underlying the remarkable activity of DAF and show that multiple mechanisms ensure that the activities of DAF are focused on regulating the active enzyme.

Disclosures
The authors have no financial conflict of interest.

References


