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Domain Swapping Reveals Complement Control Protein Modules Critical for Imparting Cofactor and Decay-Accelerating Activities in Vaccinia Virus Complement Control Protein

Muzammil Ahmad,* Sunil Raut,* Kalyani Pyaram,* Ashish Kamble,* Jayati Mullick,† and Arvind Sahu*

Vaccinia virus encodes a structural and functional homolog of human complement regulators named vaccinia virus complement control protein (VCP). This four-complement control protein domain containing secretory protein is known to inhibit complement activation by supporting the factor I-mediated inactivation of complement proteins, proteolytically cleaved form of C3 (C3b) and proteolytically cleaved form of C4 (C4b) (termed cofactor activity), and by accelerating the irreversible decay of the classical and alternative pathway C3 convertases (termed decay-accelerating activity [DAA]). In this study, we have mapped the VCP domains important for its cofactor activity and DAA by swapping its individual domains with those of human decay-accelerating factor (CD55) and membrane cofactor protein (MCP; CD46). Our data indicate the following: 1) swapping of VCP domain 2 or 3, but not 1, with homologous domains of decay-accelerating factor results in loss in its C3b and C4b cofactor activities; 2) swapping of VCP domain 1, but not 2, 3, or 4 with corresponding domains of MCP results in abrogation in its classical pathway DAA; and 3) swapping of VCP domain 1, 2, or 3, but not 4, with homologous MCP domains have marked effect on its alternative pathway DAA. These functional data together with binding studies with C3b and C4b suggest that in VCP, domains 2 and 3 provide binding surface for factor I interaction, whereas domain 1 mediates dissociation of C2a and Bb from the classical and alternative pathway C3 convertases, respectively. The Journal of Immunology, 2010, 185: 6128–6137.

The complement system is one of the vital barriers of the innate immune system that serves as a key defense against diverse microbes, including viruses (1, 2). It recognizes viruses as a foreign body by various mechanisms leading to their neutralization. These include opsonization by complement components, phagocytosis through complement receptors, aggregation by complement components, and lysis due to the formation of membrane attack complex (3, 4). In addition to these direct assaults on viruses, the complement system is also known to recruit inflammatory cells at the site of infection and boost virus-specific Ab responses (5–8). Because viruses encounter this hostile surveillance system of complement during infection, they have developed an array of evasion mechanisms to elude the host complement system, which involve the following: 1) encoding structural and/or functional homologs of host complement regulatory proteins (9–14); 2) acquiring host complement regulatory proteins such as decay-accelerating factor (DAF; CD55), membrane cofactor protein (MCP; CD46), CD59, and factor H (3, 15–17); and 3) making cellular entry through host complement receptors such as complement receptor 2, MCP, and DAF (3, 4, 18).

Vaccinia virus (VACV), the most thoroughly studied member of the genus Orthopoxvirus, is a cytoplasmic dsDNA virus with a cluster of immunomodulatory genes at the terminal region of its genome (19–21). One among these is C21L gene that encodes the vaccinia virus complement control protein (VCP), a homolog of the human regulator of complement activation proteins (11). Complement regulators homologous to VCP are also encoded by many other Orthopoxviruses, including variola (13) and monkeypox virus (12, 22). Apart from encoding VCP, VACV is also known to evade the complement attack by acquiring host complement regulators MCP, DAF, and CD59 while budding (16).

VCP is encoded as a 244-aa polypeptide with a 19-aa signal peptide (23). It folds into four compact six β-strand structures termed as complement control protein (CCP) domains separated by four amino acid linkers (24, 25). Initial studies performed using culture medium of infected cells containing secreted VCP showed that VCP inhibits activation of the classical pathway (CP) as well as the alternative pathway (AP) of the complement system (11). Later, detailed mechanistic studies revealed that it inhibits complement by binding to proteolytically cleaved form of C3 (C3b) and proteolytically cleaved form of C4 (C4b), and supporting their inac-

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The online version of this article contains supplemental material.

Abbreviations used in this paper: AP, alternative pathway; C3b, proteolytically cleaved form of C3; C4b, proteolytically cleaved form of C4; CCP, complement control protein; CFA, cofactor activity; CP, classical pathway; DAA, decay-accelerating activity; DAF, decay-accelerating factor; DQVB, dextrose gelatin veronal buffered saline; GVB, gelatin veronal buffered saline; HS1, heparin binding site 1; HS2, heparin binding site 2; HS3, heparin binding site 3; MCP, membrane cofactor protein; RUs, response units; SPICE, smallpox inhibitor of complement enzymes; SPR, surface plasmon resonance; VACV, vaccinia virus; VBS, veronal buffered saline; VCP, vaccinia virus complement control protein; vWFA, von Willebrand factor type A.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1001617
tivation by factor I (termed as cofactor activity [CFA]) as well as by accelerating irreversible decay of the CP, and to a limited extent of the AP C3 convertases (termed as decay-accelerating activity [DAA]) (26, 27). Although VCP is a soluble protein, it has also been shown to anchor to the cell surface by interacting with heparan sulfate proteoglycans (28) and the viral protein A56 (29), suggesting that the protein has an ability to protect infected cells from complement assault. The importance of VCP in protecting VACV against complement-mediated neutralization and in VACV pathogenesis has also been examined. Data revealed that VCP protects VACV virions from Ab-dependent complement-enhanced neutralization, and plays an important role in the pathogenesis, as evidenced by attenuation of virus that does not express VCP (30). The mapping of CCP domains of VCP in interaction with C3b and C4b was investigated earlier by four different groups. Rosenberg et al. (31) used VCP-CR2 (CD21) chimeric mutants expressed on the cell surface to identify the interacting domains, whereas Smith et al. (32) used soluble deletion mutants. Both these studies suggested that all the four domains of VCP are important for binding to C3b. Later, Isaacs et al. (33) used neutralizing mAbs to address this question and concluded that structural elements important for binding to C3b and C4b are located within CCP modules 2–4, because mAbs that blocked the interaction of VCP with C3b/C4b bound to these domains. Because of these conflicting reports, our group revisited this issue and attempted to identify the C3b/C4b interacting domains using soluble deletion mutants. In addition, we also investigated which domains contribute to its CFAs and DAAas. Our data (34) indicated that CCP modules 1–3 are dispensable for binding to C3b and C4b and for imparting C3b and C4b CFAs, whereas CCP modules 1–2 and 2–4 are the minimum domains necessary for displaying CP and AP DAAas, respectively. All the four CCP domains, however, were found to contribute to its optimal binding and functional activities.

Although the minimum essential domains for various functional activities in VCP were mapped in our previous study (34), what principally remained unanswered is the following: what is the relative contribution of each of the individual CCP modules in DAA and CFA, and which domains are vital for the interaction with factor I during CFA, and for dissociation of the C3 convertases during DAA? In the current study, we therefore swapped VCP modules with homologous modules of DAF and MCP to identify the critical modules of VCP. We reasoned that because DAF possesses only DAA and is devoid of CFA (35), whereas MCP possesses only CFA and is devoid of DAA (36), swapping of the VCP domains with homologous DAF or MCP domains would allow the identification of VCP domain(s) critical for CFA and DAA as well as those central for factor I interaction and decay of C2a/Bb. Our findings suggest that the middle two domains are critical for interaction with factor I, whereas domain I is vital for dissociation of the protease subunits from the CP and AP C3 convertases.

Materials and Methods

Purified complement proteins, reagents, and buffers

The complement proteins C3 (34) and factor B (37) were purified from human plasma, as described before. Native C3 was separated from C3H (HLO) by running the sample on a Mono S column (Amersham Pharmacia Biotech, Uppsala, Sweden) (38). C3b was generated by limited tryptic cleavage of C3 and purified by running the cleavage mixture on a Mono Q column (Amersham Pharmacia Biotech) (27). The complement proteins C1, C2, C4, C4b, and factor I were purchased from Calbiochem (La Jolla, CA). Factor D was a gift of M. Pangburn (University of Texas Health Centre, Tyler, TX). Purity of all the purified proteins exceeded 95% as judged by SDS-PAGE analysis. Ab-sensitized sheep erythrocytes were generated by incubating the sheep erythrocytes with anti-sheep erythrocyte Ab purchased from ICN Biomedical Inc. (Irvine, CA). Veronal buffered saline (VBS) contained 5 mM barbital and 145 mM NaCl (pH 7.4). Gelatin VBS (GVB) was VBS containing 0.1% gelatin. GVB EDTA (GVB E) was GVB containing 10 mM EDTA. Dextrose gelatin VBS (DGVB) was half ionic strength GVB with 2.5% dextrose (pH 7.4), and DGVB 5% was DGVB containing 0.5 mM MgCl2 and 0.15 mM CaCl2. PBS contained 10 mM sodium phosphate and 145 mM sodium chloride (pH 7.4).

Construction of domain swap mutants

The full-length VCP and CCP modules 1–4 of DAF and MCP cloned in pPICZa (34, 37) were used as a template for generation of VCP-DAF and VCP-MCP domain swap mutants. The construction of these mutants was achieved by gene splicing and overlap extension method (39). In brief, cDNA region corresponding to an individual CCP module or modules of interest was PCR amplified in such a way that it included a short stretch of the 5’ and/or 3’ region of the neighboring CCP module/linker region to be connected. The primer sets used for amplifying the specific regions are listed in Table I. The desired PCR products were then annealed, amplified by PCR, and cloned into pGEM-T easy vector (Promega, Madison, WI). These constructs were then subcloned into the yeast expression vector pPICZa (Invitrogen, Carlsbad, CA) at EcoRI and XbaI sites downstream of the AOX1 methanol-inducible promoter and then integrated into Pichia pastoris, as per the manufacturer’s protocol. The validity of all the constructs was confirmed by automated DNA sequencing, and integration of the mutants into Pichia was authenticated by amplifying the respective genomic DNA using AOX1 and gene-specific primers.

Expression and purification of domain swap mutants

Expression and purification of VCP, DAF, MCP, and the domain swap mutants were performed, as described (34, 37). For purification, the supernatants containing the expressed mutants were concentrated by ultrafiltration, precipitated with 80% ammonium sulfate at 0°C, and then dissolved and dialyzed in PBS. To further purify V1M2V34 and V1D3V34 mutants, they were loaded onto heparin-agarose in 10 mM sodium phosphate (pH 7.4) and eluted with 250 mM NaCl, and then loaded onto Mono Q column after exchanging into 20 mM sodium phosphate (pH 7.4), eluted with 250 mM NaCl. Fractions containing the mutants were then exchanged into 20 mM sodium phosphate, pH 7.0 (for V1M2V34) or 6.0 (for V1D3V34), loaded onto a Mono S column, and eluted with a linear gradient of 0–500 mM NaCl. For purification of V12M3V4, V1-3M4, and V12D4V4, the samples were passed through heparin-agarose in 10 mM sodium phosphate (pH 7.4), eluted with 250 mM NaCl, and then loaded onto Mono Q column after exchanging into 20 mM Tris (pH 8.0). The bound proteins were eluted with a linear salt gradient from 0 to 500 mM NaCl. Purification of M1V2-4 and D2V2-4 was achieved by passing the samples through DEAE-Sephacel (Sigma-Aldrich, St. Louis, MO) in 10 mM sodium phosphate and eluting with 500 mM NaCl, and then further loading the fractions of interest onto a Mono Q column in 20 mM Tris (pH 8.0) and eluting with a linear gradient of 0–500 mM NaCl. In all of the above purifications, eluted fractions were analyzed by SDS-PAGE and Western blotting using anti-VCP and anti-MCP (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-DAF (Santa Cruz Biotechnology) Abs. The fractions containing purified mutants were pooled, dialyzed into PBS, concentrated using ultrafiltration, and then subjected to SDS-PAGE and circular dichroism analyses (37, 40).

Factor I CFA assay

Factor I CFA of Pichia expressed VCP, DAF, MCP, and VCP-DAF, and VCP-MCP chimeras were analyzed in PBS (pH 7.4), as previously described (41).

CP and AP C3 convertase DAA assay

The CP DAA and AP DAA of VCP, DAF, and VCP-DAF and VCP-MCP chimeras were determined by forming the CP (C4b,2a) and AP (C3b,Bb) C3 convertase enzymes on sheep and rabbit erythrocytes, respectively, using purified complement components, as described (34, 42, 43).

Surface plasmon resonance measurements

The kinetics of binding of VCP and the domain swap mutants to C3b and C4b were determined using surface plasmon resonance (SPR)-based bio-sensor Biacore 2000 (Biacore AB, Uppsala, Sweden), as previously described (41). In brief, C3b (~1300 response units [RUs]) and C4b (~1900 RUs), labeled through their free thiol group with biotin, were oriented on the sensor Biacore 2000 (Biacore AB, Uppsala, Sweden), as previously described (41). In brief, C3b (~1300 response units [RUs]) and C4b (~1900 RUs), labeled through their free thiol group with biotin, were oriented on the sensor Biacore 2000 (Biacore AB, Uppsala, Sweden), as previously described (41).
performed at 25°C in PBS containing 0.05% Tween 20 at 50 μl/min flow rate to avoid mass transport effect. Association was measured by injecting 1 μM VCP or the mutants for 120 s, and dissociation of the complex was indicated in boldface.

Hemolytic assays

The inhibitory effect of VCP and the domain swap mutants on activation of the CP and AP was assessed by using hemolytic assays, as described (27).

Results

Design, expression, and purification of domain swap mutants of VCP

In this study, we generated seven domain swap mutants to delineate the role of individual modules of VCP in CFA and DAA using primers listed in Table I. We considered each CCP module as the sequence between and inclusive of the first and the fourth conserved cysteines. To dissect the role of individual domains in CFA, we swapped VCP domains (denoted as V) with those of DAF (denoted as D). The CCP modules 1–3 of VCP are homologous to the CP and AP was assessed by using hemolytic assays, as described (27).

FIGURE 1. Schematic representation, SDS-PAGE, and mass analysis of VCP, DAF, and VCP-DAF domain swap mutants. A, Cartoon illustration of VCP, DAF, and the VCP-DAF domain swap mutants. V, the CCP domains of VCP, and D, the CCP domains of DAF, are numbered. B, Purified VCP, DAF, and the mutants were electrophoresed on a 10% SDS-PAGE under reducing conditions and visualized by staining with Coomassie blue. C, The apparent molecular mass of VCP, DAF, and the mutants were determined by SDS-PAGE.

Table I. Oligonucleotides used for constructing the domain swap mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Annealing Targeta</th>
<th>Primer</th>
<th>Sequenceb</th>
</tr>
</thead>
<tbody>
<tr>
<td>D2V2–4</td>
<td>D2 Forward 5'-ggaATTCTgCgAggTggCCAACAggC-3'</td>
<td>V1 Reverse 5'-gTTAAACAgATTCTgCACAACAggTC-3'</td>
<td></td>
</tr>
<tr>
<td>D2</td>
<td>Reverse 5'-gCCTTTAATACAAATCgACTgCgTggAC-3'</td>
<td>V1 Reverse 5'-gTTAAACAgATTCTgCACAACAggTC-3'</td>
<td></td>
</tr>
<tr>
<td>V2</td>
<td>Forward 5'-gAAATTCTgTgATTAAACAggTggCCAACAggC-3'</td>
<td>V1 Reverse 5'-gTTAAACAgATTCTgCACAACAggTC-3'</td>
<td></td>
</tr>
<tr>
<td>V4</td>
<td>Reverse 5'-gCTCTAgATAgCgTgACACATTTgCgTgACAC-3'</td>
<td>V1 Reverse 5'-gTTAAACAgATTCTgCACAACAggTC-3'</td>
<td></td>
</tr>
<tr>
<td>V1D3V34</td>
<td>V1 Forward 5'-gAAATTCTgTgTgATTAAACAggTggCCAACAggC-3'</td>
<td>V1 Reverse 5'-gTTAAACAgATTCTgCACAACAggTC-3'</td>
<td></td>
</tr>
<tr>
<td>D3</td>
<td>Reverse 5'-gTTAAACAgATTCTgCACAACAggTC-3'</td>
<td>V1 Reverse 5'-gTTAAACAgATTCTgCACAACAggTC-3'</td>
<td></td>
</tr>
<tr>
<td>D3</td>
<td>Reverse 5'-TTAACAAGAttCTgCATACTgTgCC-3'</td>
<td>V1 Reverse 5'-gTTAAACAgATTCTgCACAACAggTC-3'</td>
<td></td>
</tr>
<tr>
<td>V3</td>
<td>Forward 5'-gAAATTCTgCgAggTggCCAACAggC-3'</td>
<td>V1 Reverse 5'-gTTAAACAgATTCTgCACAACAggTC-3'</td>
<td></td>
</tr>
<tr>
<td>V4</td>
<td>Reverse 5'-gCTCTAgATAgCgTgACACATTTgCgTgACAC-3'</td>
<td>V1 Reverse 5'-gTTAAACAgATTCTgCACAACAggTC-3'</td>
<td></td>
</tr>
</tbody>
</table>

aVCP, DAF, and MCP domains are denoted as V, D, and M, respectively, and numbers denote the domain number of the respective regulator.
bEcoRI and XbaI restriction sites in the primers are indicated by underlines, and 5' and/or 3' regions of the neighboring domains/linker region are indicated in boldface.

Design, expression, and purification of domain swap mutants of VCP

In this study, we generated seven domain swap mutants to delineate the role of individual modules of VCP in CFA and DAA using primers listed in Table I. We considered each CCP module as the sequence between and inclusive of the first and the fourth conserved cysteines. To dissect the role of individual domains in CFA, we swapped VCP domains (denoted as V) with those of DAF (denoted as D). The CCP modules 1–3 of VCP are homologous to CCP modules 2–4 of DAF; thus, we swapped the respective
modules of these proteins generating mutants D2V2-4, V1D3V34, and V12D4V4 (Fig. 1). Next, to dissect the role of individual CCP modules in DAA, we swapped VCP domains with MCP domains (denoted as M). Because VCP modules 1–4 are homologous to MCP modules 1–4, we swapped the homologous modules of VCP and MCP generating the mutants M1V2-4, V1M2V34, V12M3V4, and V1-3M4 (Fig. 2). These mutants along with VCP, DAF, and MCP were then expressed using the *Pichia* expression system as secretory proteins and purified using a series of chromatographic procedures, as described in Materials and Methods. The purified DAF and VCP-DAF chimeras migrated as single bands on SDS-PAGE (Fig. 1), whereas purified MCP and VCP-MCP chimeras, except M1V2-4 and V12M3V4, migrated as broad diffuse bands reflecting glycosylation (Fig. 2). All the proteins demonstrated reactivity to the respective polyclonal Abs in Western blot analysis, and yielded a peak at ≈230 nm upon circular dichroism analysis, validating proper conformation (40) (data not shown).

**Characterization of CFAs of VCP-DAF domain swap mutants**

VCP inactivates C3b as well as C4b by acting as a cofactor for the serine protease factor I, whereas DAF is devoid of this activity. Thus, replacing VCP domains that are critical for its CFA with those of DAF is expected to result in abrogation of this activity. In this study, to identify the VCP module critical for CFA, the VCP-DAF chimeras generated were analyzed for their CFAs using a fluid-phase assay wherein VCP, DAF, or each of the mutants was incubated with C3b or C4b and factor I for varying time periods, and inactivation of C3b or C4b was assessed by quantitating C3b/C4b cleavage. Data presented in Fig. 3 depict that of the three VCP-DAF chimeras, two mutants, V1D3V34 and V12D4V4, displayed a total loss in both C3b and C4b CFAs, indicating that CCP modules 2 and 3 of VCP are critical for the CFAs of the molecule. The mutant D2V2-4, wherein VCP domain 1 was replaced with DAF domain 2, though retained C3b and C4b CFAs, displayed lower C3b CFA than VCP (Fig. 3). We thus performed a time course analysis of C3b and C4b CFAs of this mutant and compared it with VCP (Supplemental Fig. 1). The D2V2-4 mutant displayed 7-fold less C3b CFA compared with VCP, but its C4b CFA was similar to that of VCP (Fig. 3, Table II). These data therefore suggest that although CCP module 1 of VCP is important for C3b CFA, it is not indispensable.

**Characterization of DAAs of VCP-DAF domain swap mutants**

Because DAF possesses DAA, it is expected that VCP-DAF chimeras would selectively lose CFA and retain DAA. In addition, given that DAF possesses significantly higher DAA compared with VCP, it is expected that chimeras containing DAF modules vital for DAA would show gain-in-function. To measure CP and AP DAA of the domain swap mutants, the CP (C4b,2a) and AP (C3b,Bb) C3 convertases were formed on erythrocytes using purified complement components, and their decay was determined by incubating the enzyme-coated cells with increasing concentrations of VCP, DAF, and VCP-DAF chimeras. Data presented in Fig. 4 depict that of the three VCP-DAF chimeras, two mutants, V1D3V34 and V12D4V4, displayed a total loss in both CP and AP CFAs, indicating that CCP modules 2 and 3 of VCP are critical for the CFAs of the molecule. The mutant D2V2-4, wherein VCP domain 1 was replaced with DAF domain 2, though retained CP and AP CFAs, displayed lower CP and AP CFAs than VCP (Fig. 4). We thus performed a time course analysis of CP and AP CFAs of this mutant and compared it with VCP (Supplemental Fig. 1). The D2V2-4 mutant displayed 7-fold less CP CFA compared with VCP, but its AP CFA was similar to that of VCP (Fig. 4, Table II). These data therefore suggest that although CCP module 1 of VCP is important for C3b CFA, it is not indispensable.
DAF, or the VCP-DAF chimeras and then measuring hemolysis after addition of EDTA sera (C3–C9 source).

As expected, all the chimeras retained the CP DAA, but intriguingly, the mutant D2V2-4 demonstrated a 55-fold increase in CP DAA compared with VCP. Among the other two mutants, V1D3V34 showed no change in the activity, whereas V12D4V4 displayed a 7-fold decrease in CP DAA (Fig. 4, Table II). A comparison of CP DAA of VCP and DAF showed that DAF is 81-fold more potent than VCP in decaying the CP C3 convertase (Fig. 4, Table II). As expected, all the chimeras retained the CP DAA, but interestingly, there was a 670- and 106-fold increase in AP DAA of V1D3V34 and V12D4V4, respectively, compared with VCP. The activity of D2V2-4 was comparable to VCP (Fig. 4, Table II). The large increase in AP DAA of D3 and D4 domain swap mutants indicates that these domains are critical for the decay of the AP C3 convertase.

Characterization of DAAs of VCP-MCP domain swap mutants

In the VCP-MCP series of chimeras (Fig. 2), it is anticipated that swapping of VCP domains critical for its DAA with those of MCP would result in reduction/loss in DAAs. The measurement of CP DAA of the four VCP-MCP domain swap mutants revealed that the mutant M1V2-4 lacked this activity, whereas V1M2V34 and V1-3M4 had no noticeable difference in their CP DAA. V12M3V4 showed 5-fold increase compared with VCP (Fig. 5, Table II). Clearly, these data point toward the utmost importance of CCP1 module in begetting the CP DAA in VCP. These data are similar to our findings on VCP-DAF chimeras described above that indicate the central role of CCP2 module of DAF in CP DAA, which is homologous to the CCP1 module of VCP.

The AP DAA data of these chimeras revealed that the first three CCP modules of VCP are important for this activity, as mutants M1V2-4, V1M2V34, and V12M3V4 demonstrated abrogation in their ability to decay the AP C3 convertase (Fig. 5, Table II). However, the most puzzling finding was the AP DAA of V1-3M4 mutant, which portrayed ∼270-fold enhancement in the activity (Fig. 5, Table II). Because MCP does not possess DAA, the increase in the AP DAA of this mutant could possibly be owing to increase in its affinity for C3b. Binding data (described below) verified that indeed there was substantial increase in binding of this mutant to C3b.

Characterization of CFAs of VCP-MCP domain swap mutants

Like the DAF chimeras retained the DAAs, it is expected that the MCP chimeras would retain CFAs against C3b and C4b. To validate this premise, we assessed CFAs of the VCP-MCP chimeras. As predicted, all the mutants retained CFA against C3b and C4b (Fig. 6). A comparison of C3b CFA of the mutants with VCP exhibited a 7-fold decrease in CP DAA of the four VCP-MCP domain swap mutants revealed that the mutant M1V2-4 lacked this activity, whereas V1M2V34 and V1-3M4 had no noticeable difference in their CP DAA. V12M3V4 showed 5-fold increase compared with VCP (Fig. 5, Table II). Clearly, these data point toward the utmost importance of CCP1 module in begetting the CP DAA in VCP. These data are similar to our findings on VCP-DAF chimeras described above that indicate the central role of CCP2 module of DAF in CP DAA, which is homologous to the CCP1 module of VCP. The AP DAA data of these chimeras revealed that the first three CCP modules of VCP are important for this activity, as mutants M1V2-4, V1M2V34, and V12M3V4 demonstrated abrogation in their ability to decay the AP C3 convertase (Fig. 5, Table II). However, the most puzzling finding was the AP DAA of V1-3M4 mutant, which portrayed ∼270-fold enhancement in the activity (Fig. 5, Table II). Because MCP does possess DAA, the increase in the AP DAA of this mutant could possibly be owing to increase in its affinity for C3b. Binding data (described below) verified that indeed there was substantial increase in binding of this mutant to C3b.

Like the DAF chimeras retained the DAAs, it is expected that the MCP chimeras would retain CFAs against C3b and C4b. To validate this premise, we assessed CFAs of the VCP-MCP chimeras. As predicted, all the mutants retained CFA against C3b and C4b (Fig. 6). A comparison of C3b CFA of the mutants with VCP exhibited that swapping of the second and fourth CCP modules of VCP with those of MCP did not alter the C3b CFA, whereas swapping of the first and third CCP module led to ∼4- and 6-fold loss in the activity (Fig. 6, Supplemental Fig. 2, Table II). Furthermore, examination of...
of the C4b CFA of the mutants showed that like C3b CFA, swapping of the second and fourth domains had no effect on the activity compared with VCP, whereas swapping of the first and third CCP modules led to 5-fold reduction in the activity (Fig. 6, Supplemental Fig. 2, Table II). Whether loss in CFAs of the mutants is due to reduction in their binding to C3b and C4b is addressed below.

Characterization of binding of VCP-DAF and VCP-MCP domain swap mutants to C3b and C4b

The DAA and CFA are a result of trimolecular interactions. DAA involves binding of the viral complement regulator to C3b or C4b and to Bb or C2a (44, 45), whereas CFA requires interaction of the viral complement regulator with C3b or C4b and the complement regulator (46). To determine whether loss or gain in the activity of the first domain swap mutant could be, in part, due to its reduced binding to C3b, whereas loss in the activity of the other two mutants could be solely due to their reduced affinity for C3b. The fourth domain swap mutant V1-3M4, which showed 270-fold increase in binding to C4b, increased CP DAA of this mutant was not due to enhancement of its affinity for C4b. As stated above, binding of V1D3V4 for C3b was moderately lower than VCP, but binding of V12D4V4 was substantially increased (Fig. 7A). It could therefore be concluded that increase in AP DAA of V1D3V4, but not of V12D4V3, was due to its higher affinity for C3b compared with VCP.

We next measured binding of VCP-MCP domain swap mutants to C3b and C4b. The measurement of CP DAA of these mutants revealed that M1V2-4 was largely inactive, whereas the other three mutants either had comparable (V1M2V34 and V1-3M4) or enhanced (V12M3V4) activities to that of VCP (Fig. 5). The M1V2-4 mutant showed attenuated binding to C4b (Fig. 7B); therefore, the observed loss in CP DAA of this mutant could be attributed to its reduced affinity for C4b. The V12M3V4 mutant displayed moderate increase in binding to C4b; thus, better CP DAA could be attributed to its increased binding to C4b (Fig. 7B). Unlike CP DAA, the AP DAA activity was lost by the three following domain swap mutants: M1V2-4, V1M2V34, and V12M3V4 (Fig. 5). Binding of the first domain swap mutant to C3b was moderately decreased compared with VCP, whereas that of the second and third domain swap mutants was highly attenuated compared with VCP (Fig. 7A). These data therefore suggest that of these three mutants, loss in the activity of the first domain swap mutant could be, in part, due to its reduced binding to C3b, whereas loss in the activities of the other two mutants could be solely due to their reduced affinity for C3b. The fourth domain swap mutant V1-3M4, which showed 270-fold increase in AP DAA, also exhibited substantial increase in its binding to C3b (Fig. 7A).

Examination of the CFAs of VCP-MCP domain swap mutants showed that M1V2-4 and V1M2V34 mutants had ~4- to 6-fold lower C3b and C4b CFAs compared with VCP (Fig. 6, Table II). Consistent with this, in comparison with VCP, the M1V2-4 mutant displayed lower binding to C3b and C4b and V1M2V34 mutant displayed attenuated binding to C3b. However, binding of V12M3V4 for C4b did not correlate with its C4b CFA (Fig. 7, Table II); it displayed moderate increase in binding to C4b compared with VCP (Fig. 7B).

Characterization of CP and AP inhibitory activity of VCP-DAF and VCP-MCP domain swap mutants

In the experiments discussed above, we evaluated the effect of VCP and its mutants on the C3 convertases and its components (C3b and C4b) using purified complement components. We next determined whether the increase/decrease in activities of these mutants on the convertases is also reflected in their ability to inhibit CP- and AP-mediated lysis of erythrocytes using human serum as a source of complement.

Examination of the VCP-DAF domain swap mutants for inhibition of CP-mediated lysis showed that mutant D2V2-4, which exhibited a 7-fold decrease in C3b CFA compared with VCP, showed a significant decrease in binding to C3b (Fig. 7A); thus, the observed reduction in its C3b CFA could be attributed to its reduced affinity for C3b.

Interestingly, the D2V2-4 showed 55-fold increase in CP DAA, whereas V1D3V34 and V12D4V4 displayed 670- and 106-fold increase in AP DAA in comparison with VCP (Fig. 4, Table II). Assessment of binding of D2V2-4 to C4b showed that, if any, its binding was lower than VCP (Fig. 7B), suggesting thereby that the increased CP DAA of this mutant was not due to enhancement of its affinity for C4b. As stated above, binding of V1D3V4 for C3b was moderately lower than VCP, but binding of V12D4V4 was substantially increased (Fig. 7A). It could therefore be concluded that increased in AP DAA of V1D4V4, but not of V12D4V3, was due to its higher affinity for C3b compared with VCP.
inhibitory activity. The other three mutants showed no effect to limited increase in CP inhibitory activities; these mutants had either no effect or limited effect on CP DAA and C4b CFA. Thus, overall, the CP inhibitory activities of the mutants correlated with their CP DAA, but not with the C4b CFA (Fig. 8, Table II).

VCP is known to possess a weak AP inhibitory activity (26, 27). Analysis of inhibition of AP-mediated lysis by VCP-DAF swap mutants showed that mutants V1D3V34 and V12D4V4, which demonstrated 670- and 106-fold increase in AP DAA (Fig. 4), also showed 137- and 67-fold enhanced AP inhibition, respectively (Fig. 8, Table II). Among the VCP-MCP mutants, V1-3M4 showed noticeable gain in AP DAA, and consistent with this, it also showed a 76-fold increase in AP inhibitory activity. Apart from these domain swap mutants, none of the other mutants showed any inhibition of AP activity up to 10 \( \mu M \) concentration. It is difficult to infer about the reduced AP activities of these mutants because the maximum concentration used in this study was <2-fold higher compared with the IC\(_{50}\) of VCP for AP inhibition (Fig. 8, Table II).

Together, the above data revealed that increased DAA of the domain swap mutants was associated with superior AP and CP inhibitory activities.

**Discussion**

In the current study, we have employed a domain-swapping approach to further map the functional domains in VCP and understand the relative contribution of its individual domains in the functional activities. The advantage of this approach as opposed to the deletion mutagenesis strategy that we performed earlier (34) is that this allowed delineation of the functional CCP modules in the context of the whole molecule. In addition, because DAF does not interact with factor I and MCP lacks decay activity for the C3 convertases, swapping of VCP domains with homologous MCP
Furthermore, during characterization of VCP domains 1–4 are structurally and functionally similar to factor H complement C1r-C1s, UEGF, BMP1 domains of C3b. The VCP sites formed by the modules 1–3 of factor H, and C345C and (51) and previous studies (50, 52), it was proposed that factor I molecule in a discontinuous manner. On the basis of the structure showed that all the four modules of factor H interact with the C3b complex with C3b has recently been solved (51). The structure of module 1 resulted in 7-fold decrease in C3b CFA and no change in C4b CFA (Fig. 3, Table II). A comparison of CFAs with their binding to C3b and C4b indicated that decrease in the C3b CFA of module 1 swap mutant was associated with the decrease in its binding to C3b, whereas abrogation in C3b and C4b CFAs of module 2 and 3 swap mutants was not consistent with loss in binding to C3b and C4b (Fig. 7, Table II). Because CFA entails interaction of the complement regulator with the target protein (C3b or C4b) as well as factor I, we attribute the decrease in CFAs of module 1 swap mutant to decrease in its binding to C3b, and loss in the CFAs of module 2 and 3 swap mutants to loss in their binding to factor I. In our previous studies on Kaposica (Kaposi’s sarcoma-associated herpesvirus complement regulator) (48) and soluble complement control protein homolog of herpesvirus saimiri (49), we found that domains 2 and 3 are enough to display CFAs against C3b and C4b. Similarly, in case of smallpox inhibitor of complement enzymes (SPICE; variola virus complement regulator), it was found that the putative factor I site is located in domain 2 (50). Thus, it seems that factor I interaction site is conserved at collinear positions in pox as well as herpes viral complement regulators.

The structure of the N-terminal four CCP modules of factor H in complex with C3b has recently been solved (51). The structure showed that all the four modules of factor H interact with the C3b molecule in a discontinuous manner. On the basis of the structure (51) and previous studies (50, 52), it was proposed that factor I inactivates C3b by interacting with the C3b-factor H complex at sites formed by the modules 1–3 of factor H, and C345C and complement C1r-C1s, UEGF, BMP1 domains of C3b. The VCP domains 1–4 are structurally and functionally similar to factor H modules 1–4. Furthermore, during characterization of VCP deletion mutants, we noted that all of the four domains are required for its optimal binding to C3b and C4b (34). Thus, it is likely that VCP interacts with C3b in a manner similar to factor H. Our data described in this work point out that VCP modules 2 and 3 are critical for interaction with factor I (Figs. 3, 7). We therefore suggest that like factor H, the four domains in VCP interact with the C3b molecule and modules 2–3 provide a docking surface for factor I (Fig. 9). A similar mechanism could also be operative for the interaction of VCP with C4b and factor I. Because domain requirements for ligand binding and CFAs in viral (34, 48, 49, 54) and human complement regulators (55–62) are conserved, it could be inferred that the recognition sites for C3b/C4b and factor I are spatially conserved in both human and viral complement regulators, and that they employ a common mechanism to inactivate C3b and C4b.

VCP domains critical for DAAs

Previous examination of decay of the CP C3 convertase utilizing various VCP deletion mutants revealed that the mutant consisting of modules 1 and 2 meditated the decay acceleration, albeit with much lesser efficiency compared with the full-length molecule (34). In this study, analysis of CP DAA of the VCP-MCP domain swap mutants showed that swapping of module 1, but not 2, 3, or 4, with the homologous domains of MCP resulted in abrogation of this activity (Fig. 5), suggesting a functional role for module 1 in CP DAA. Earlier examination of CP DAA by human complement regulators suggested that dissociation of the protease subunit from the convertase is a result of binding of the regulators to C4b and C2a, followed by a conformational change in the von Willebrand factor type A (vWFA) domain (45, 47, 63, 64). More recently, it has been suggested that dissociation of the convertases could also be a result of displacement of the protease subunit by the regulator owing to a competition posed by the regulator for the protease interaction site on the noncatalytic subunit of the convertase (51). Our data presented in this study show that binding of module 1 swap mutant (M1V2-4) to C4b was significantly reduced compared with VCP (Fig. 7B). Thus, the inability of the mutant to accelerate the decay is largely due to its decreased binding ability to C4b. It is likely, therefore, that similar to human regulators, viral complement regulators employ a common mechanism to inactivate C4b.
domain 1 in VCP participates in dissociation of C2a (Fig. 9) by competing for the C2a interaction site on C4b. Alternatively, the dissociation of C2a by the domain 1 could be a result of a conformational change in the vWFA domain. Previous observations on domain mapping of other viral complement regulators have shown that domains 1–2 are the minimum domains important for CP DAA in Kaposia (48) and soluble complement control protein homolog of herpesvirus saimiri (49). It is therefore possible that like VCP, domain 1 in these proteins too contributes to dissociation of C2a from the CP C3 convertase and other domains play a supportive role in binding to C4b. Furthermore, SPICE domain 1 is identical to VCP domain 1; consequently, their CP DAA are expected to be comparable. Consistent with this premise, it should be pointed out in this work that both VCP and SPICE exhibit similar CP DAA (50).

VCP possesses only a residual AP DAA compared with human complement regulators (26, 34), and therefore, its role in vaccinia virus pathogenesis is uncertain. Nevertheless, examination of AP DAA of VCP-MCP chimeras revealed that swapping of the three N-terminal domains of VCP resulted in considerable decrease in AP DAA, suggesting their involvement in decay acceleration of the AP C3 convertase (Fig. 5, Table II). The binding studies demonstrated that there was a large decrease in binding of domain 2 and 3 swap mutants, and moderate decrease in binding of domain 1 swap mutant to C3b (Fig. 7). Based on our earlier (34) and present data, and the past proposals on decay acceleration of AP C3 convertase by DAF (63, 64) and factor H (51), we suggest that whereas all the domains in VCP contribute to its optimal binding to C3b (41), domain 1 also contributes in destabilization of AP C3 convertase either by inducing a conformational change in vWFA domain or by competing for the Bb interaction site on C3b. Having said the above, structural data on VCP in complex with C3b and C4b, and more relevantly in complex with the convertases, are needed to understand the precise mechanism of VCP-mediated C3 convertase decay.

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Disclosures
The authors have no financial conflicts of interest.

References


