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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 to a limited extent of the 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

as well as cell-mediated immune 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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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; DGVB, 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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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. Rosen-gard 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 DAAs. Our data (34) indicated that CCP modules 1–3 are indispensable 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 DAAs, 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 1 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 C3 (H₂O) 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 (GVBE) was GVB containing 10 mM EDTA. Dextrose gelatin VBS (DGVBS) was half ionic strength GVB with 2.5% dextrose (pH 7.4), and DGVBS²⁺ was DGVBS 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 pPICZα (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 pPICZα (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. 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)/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 biosensor 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 a streptavidin chip (Sensor Chip SA; Biacore AB). In case of C3b, additional molecules (~4500 RUs) were coupled onto the C3b-immobilized flow cell by forming AP C3 convertase (C3b,Bb) onto the chip and then flowing native C3 (37). Binding studies for all the interactions were

Table I. Oligonucleotides used for constructing the domain swap mutants

Mutant	Annealing Target ^a	Primer	Sequence ^b
D2V2-4	D2	Forward	5'-ggAATTCTgCgAggTgCCAACAaggC-3'
	D2	Reverse	5'-CCgTTTAATACAAAATTCgACTgCTgTggAC-3'
	V2	Forward	5'-gAATTTTgTATTAACgAgATgCCCATCgC-3'
	V4	Reverse	5'-gCTCTAgATTAgCgTACACATTTggAagTTCCg-3'
V1D3V34	V1	Forward	5'-ggAATTCTgCTgTACTATTCCgTCACgACCC-3'
	V1	Reverse	5'-AgggCATCTCCgTTTAATACATTgATTAAAg-3'
	D3	Forward	5'-AAACgAgATgCCCTAATCCgggAgAAATAC-3'
	D3	Reverse	5'-TTTAACAgATTCgCACTCTggCAACgggTCAC-3'
	V3	Forward	5'-gAgTgCgAATCTgTTAAATgCCAATCCCC-3'
	V4	Reverse	5'-gCTCTAgATTAgCgTACACATTTggAagTTCCg-3'
V12D4V4	V1	Forward	5'-ggAATTCTgCTgTACTATTCCgTCACgACCC-3'
	V2	Reverse	5'-TggACATTTAACAgATTACAAATAggTgCC-3'
	D4	Forward	5'-TCTgTTAAATgTCCAgCACCACCACAAATg-3'
	D4	Reverse	5'-TTTAACAATCTggCATTCAGgTggTgggCCAC-3'
	V4	Forward	5'-gAATgCCAgATTgTTAAATgTCCACATCC-3'
	V4	Reverse	5'-gCTCTAgATTAgCgTACACATTTggAagTTCCg-3'
M1V2-4	M1	Forward	5'-ggAATTCTgCCTgTgAggAgCCACCAAC-3'
	M1	Reverse	5'-CTCCgTTTAATACAggCgTCATCTgAgACAgg-3'
	V2	Forward	5'-CTgTATTAACgAgATgCCCATCgC-3'
	V4	Reverse	5'-gCTCTAgATTAgCgTACACATTTggAagTTCCg-3'
V1M2V34	V1	Forward	5'-ggAATTCTgCTgTACTATTCCgTCACgACCC-3'
	V1	Reverse	5'-ggACATCTCCgTTTAATACATTgATTAAAgAg-3'
	M2	Forward	5'-gTATTAACgAgATgTCCATATATACgggATCCTTT-3'
	M2	Reverse	5'-CATTTAACAgATTACATATTgggggCTTACCgCTC-3'
	V3	Forward	5'-gTgAATCTgTTAAATgCCAATCCCC-3'
	V4	Reverse	5'-gCTCTAgATTAgCgTACACATTTggAagTTCCg-3'
V12M3V4	V1	Forward	5'-ggAATTCTgCTgTACTATTCCgTCACgACCC-3'
	V2	Reverse	5'-gTACATTTAACAgATTACAAATAggTgCCTC-3'
	M3	Forward	5'-gAATCTgTTAAATgTACACCCTCCAAAAATAAAAAATg-3'
	M3	Reverse	5'-CATTTAACATCTgACACTCTggAgCAGCAGACTCC-3'
	V4	Forward	5'-gTgTCAGATTgTTAAATgTCCACATCC-3'
	V4	Reverse	5'-gCTCTAgATTAgCgTACACATTTggAagTTCCg-3'
V1-3M4	V1	Forward	5'-ggAATTCTgCTgTACTATTCCgTCACgACCC-3'
	V3	Reverse	5'-CgACATTTAACAATCTgACACgTgggTgg-3'
	M4	Forward	5'-gATTgTTAAATgTCgATTTCAGTAgTCgAAAAATg-3'
	M4	Reverse	5'-gCTCTAgATTAAAgACACTTTggAACTggggg-3'

^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.

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 measured for 180 s by replacing sample with the buffer. The sensor chip was regenerated by 30-s pulses of 0.2 M sodium carbonate (pH 9.5). Biosensor data obtained for the control flow cell (immobilized with BSA biotin) were then subtracted from those obtained for the flow cell immobilized with C3b or C4b to obtain the specific binding response.

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 CCP modules 2–4 of DAF; thus, we swapped the respective

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.

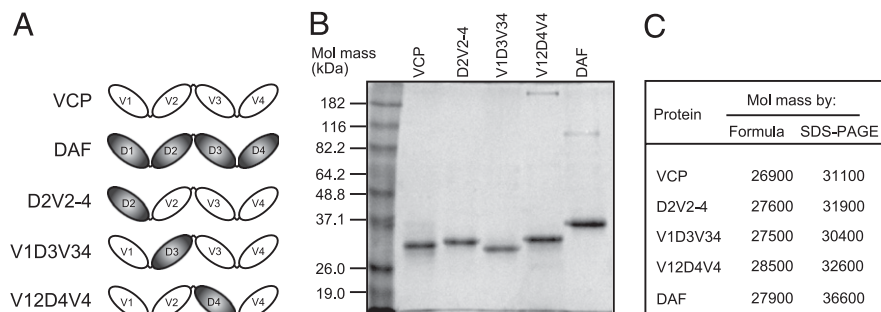
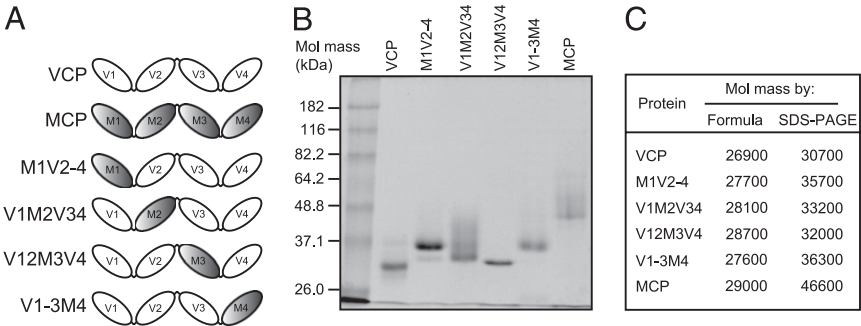


FIGURE 2. Schematic representation, SDS-PAGE, and mass analysis of VCP, MCP, and VCP-MCP domain swap mutants. *A*, Cartoon illustration of VCP, MCP, and the VCP-MCP domain swap mutants. *B*, Purified VCP, MCP, and the mutants were electrophoresed on a 9% SDS-PAGE under reducing conditions and visualized by staining with Coomassie blue. *C*, The apparent molecular mass of VCP, MCP, and the mutants were determined by SDS-PAGE.



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,

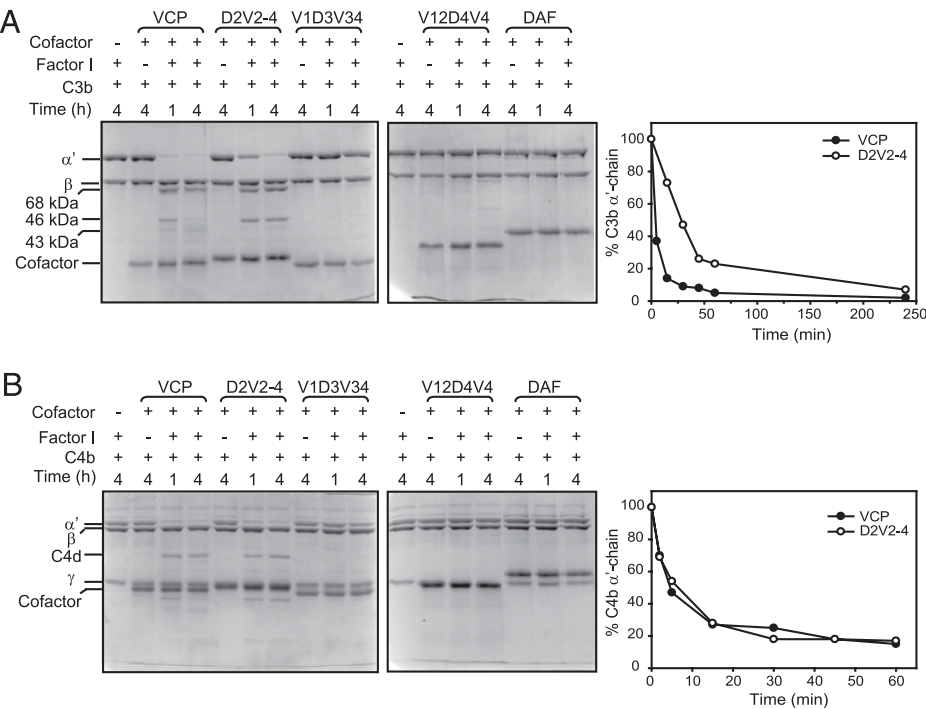


FIGURE 3. Factor I CFA of VCP, DAF, and VCP-DAF domain swap mutants for C3b and C4b. *Left and middle panels*, The CFA of VCP, DAF, and the mutants was measured by incubating VCP, DAF, or each of the mutants with C3b (*A*) or C4b (*B*) and factor I in PBS at 37°C. The reactions were stopped at the indicated time by adding sample buffer containing DTT. The cleavage products were resolved on 9% (for C3b) or 10.5% (for C4b) SDS-PAGE and visualized by staining with Coomassie blue. C3b cleavages: factor I-mediated cleavage of α' -chain of C3b results in generation of N-terminal 68-kDa and C-terminal 46-kDa fragments. The 46-kDa fragment is then cleaved into 43-kDa fragment. C4b cleavages: factor I-mediated cleavage of α' -chain of C4b results in generation of N-terminal 27-kDa fragment, a central C4d fragment, and C-terminal 16-kDa fragment (not visualized). *Right panels*, During time course analysis (Supplemental Fig. 1), the intensity of α' -chains of C3b or C4b was quantitated by densitometric analysis and graphed against time.

Table II. Summary of functional activities of VCP, DAF, and the domain swap mutants of VCP

Wild Type/ Chimeras	Time (min) for 50% Cleavage of C3b α' -Chain	Relative Cleavage of C3b CFA ^a	Time (min) for 50% Cleavage of C4b α' -Chain	Relative Cleavage of C4b CFA ^a	CP DAA IC ₅₀ (μ M)	Relative CP DAA ^a	AP DAA IC ₅₀ (μ M)	Relative AP DAA ^a	CP Lysis IC ₅₀ (nM)	Relative CP Activity ^a	AP Lysis IC ₅₀ (μ M)	Relative AP Activity ^a
VCP	4	1	4	1.0	0.26	1	22.8	1	96	1	7.4	1
D2V2-4	28	0.14	6.5	0.61	0.0047	55	27.5	0.83	3.6	26.7	>10 ^c	<0.74
V1D3V34	>240 ^b	<0.017	>240 ^b	<0.017	0.16	1.6	0.034	670	22.5	4.3	0.054	137
V12D4V4	>240 ^b	<0.017	>240 ^b	<0.017	1.8	0.14	0.215	106	78	1.2	0.11	67.3
M1V2-4	15	0.27	21	0.19	>10 ^c	< 0.026	>50 ^c	<0.46	5000	0.019	>10 ^c	<0.74
V1M2V34	1.8	2.2	1.7	2.4	0.34	0.76	>50 ^c	<0.46	104	0.92	>10 ^c	<0.74
V12M3V4	25	0.16	19	0.21	0.052	5	>40 ^c	<0.57	27	3.6	>10 ^c	<0.74
V1-3M4	5	0.80	5	0.80	0.16	1.6	0.085	268	14	6.9	0.098	75.5
DAF	>240 ^b	<0.017	>240 ^b	<0.017	0.0032	81.2	0.002	11400	ND	ND	ND	ND

^aRelative activity compared with VCP: <3-fold, no effect; 3- to 6-fold, limited effect; 6- to 20-fold, considerable effect; >20-fold, enormous effect.

^bNo cleavage was observed even after 4-h incubation.

^cHighest concentration of mutant tested in the assay.

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). Thus, from these data it is clear that CCP2 module of DAF primarily dictates the decay of the CP C3 convertase.

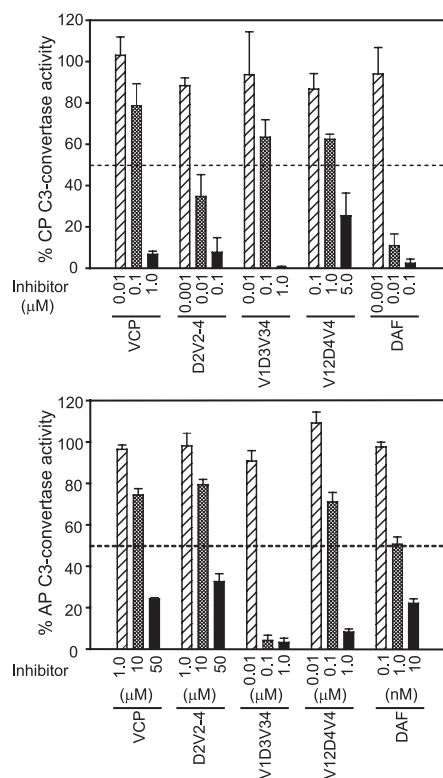


FIGURE 4. CP and AP C3 convertase DAAs of VCP, DAF, and VCP-DAF domain swap mutants. *Upper panel*, CP DAA of VCP, DAF, and the mutants was measured by forming the CP C3 convertase (C4b,2a) on Ab-coated sheep erythrocytes. *Lower panel*, AP DAA of VCP, DAF, and the mutants was measured by forming the AP C3 convertase (C3b,Bb) on rabbit erythrocytes. Data obtained were normalized by considering 100% C3 convertase activity to be equal to the average activity in the absence of inhibitor. The dotted line denotes 50% activity.

Unlike CP DAA, VCP is known to possess a residual DAA against the AP C3 convertase (26, 34). This is also clearly evident from its ~11,000-fold lower AP DAA compared with DAF (Fig. 4, Table II). The results showed that all the VCP-DAF chimeras retained AP DAA activities, 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

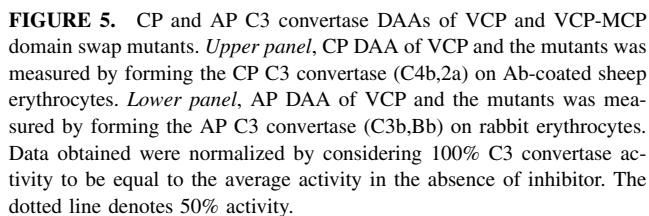
In the VCP-MCP series of chimeras (Fig. 2), it is anticipated that swapping of VCP domains critical for its DAA with those of MCP would result in reduction/loss in DAAs. The measurement of CP DAA of the four VCP-MCP domain swap mutants revealed that the mutant M1V2-4 lacked this activity, whereas V1M2V34 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 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



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

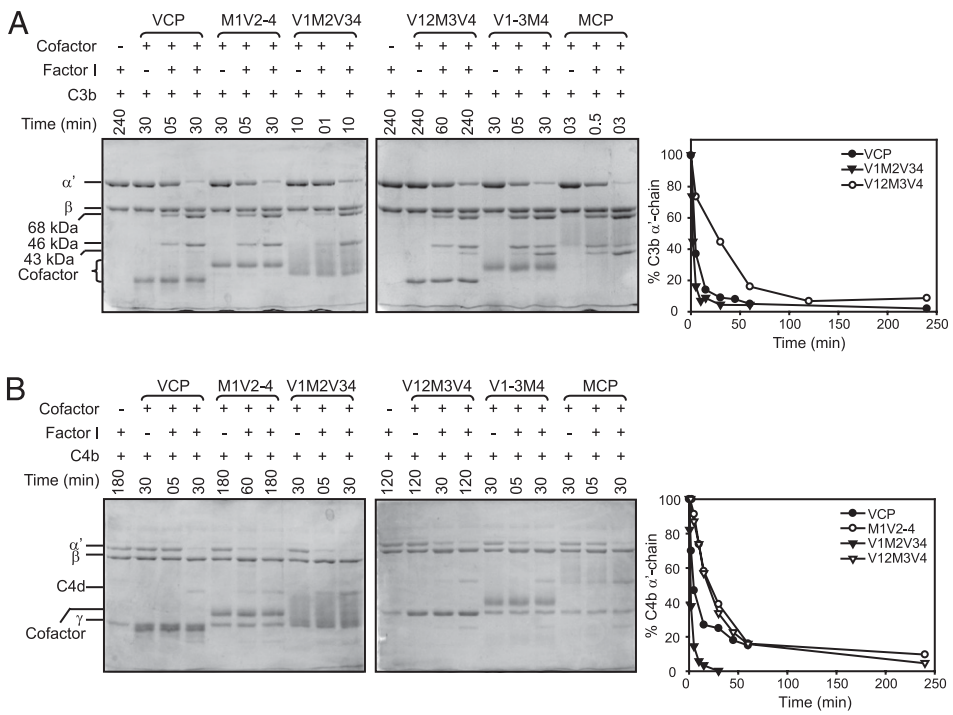
Our results presented above demonstrated that VCP-DAF domain swap mutants V1D3V34 and V12D4V4 were devoid of C3b and C4b CFAs (Fig. 3). Measurement of binding of V1D3V34 to C3b and C4b showed that its binding to C3b was moderately reduced compared with that of VCP, whereas its binding to C4b was higher than that of VCP. Interestingly, opposite results were obtained in case of V12D4V4; there was a substantial increase in its binding to C3b in comparison with VCP, but its binding to C4b was significantly decreased (Fig. 7). Thus, there was no correlation between the loss in the CFAs of these mutants and binding. The mutant

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).

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

Examination of the VCP-DAF swap mutants for inhibition of CP-mediated lysis showed that mutant D2V2-4, which had 55-fold increase in CP DAA compared with VCP (Fig. 4), also showed 27-fold higher CP inhibitory activity (Fig. 8, Table II). The other two VCP-DAF domain swap mutants that depicted loss in C4b CFA and no to limited effect on CP DAA (Table II) had limited effect on CP inhibitory activity (Fig. 8). Among the VCP-MCP swap mutants, M1V2-4 that lacked CP DAA displayed ~52-fold decrease in CP

FIGURE 6. Factor I CFA of VCP, MCP, and VCP-MCP domain swap mutants for C3b and C4b. *Left and middle panels.* The CFA of VCP, MCP, and the mutants was assayed by incubating VCP, MCP, or each of the mutants with C3b (A) or C4b (B) and factor I in PBS at 37°C for the indicated time. The reactions were stopped by adding sample buffer containing DTT, and the amount of C3b or C4b cleaved was visualized by running the samples on 9 or 10.5% SDS-PAGE, respectively, and staining with Coomassie blue. *Right panels.* The intensity of α' -chains of C3b or C4b remained during time course analysis (Supplemental Fig. 2) was quantitated by densitometric analysis and graphed against time.



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 μ 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

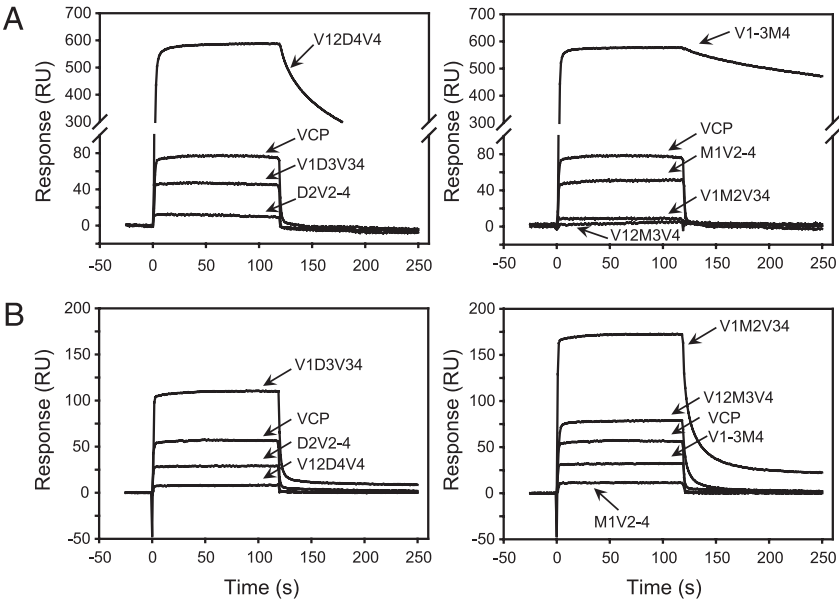


FIGURE 7. SPR analysis of binding of VCP and its domain swap mutants to C3b and C4b. *A*, Sensogram overlays for the interactions between VCP and its domain swap mutants with C3b. C3b was oriented on the streptavidin chip (see *Materials and Methods*), and binding was analyzed by injecting VCP or its mutants (1 μ M) onto the chip in PBS containing 0.05% Tween 20. *B*, Sensogram overlays for the interactions between VCP and its domain swap mutants with C4b. C4b was oriented on the streptavidin chip (see *Materials and Methods*), and binding was analyzed by injecting VCP or its mutants (1 μ M) onto the chip in PBS containing 0.05% Tween 20.

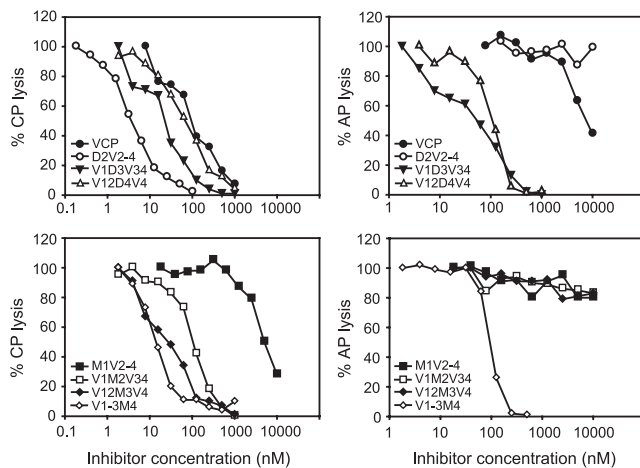


FIGURE 8. Inhibition of CP- and AP-mediated lysis of erythrocytes by VCP and domain swap mutants. The relative effect of VCP and the domain swap mutants on CP (left panels) and AP (right panels) was measured using hemolytic assays.

and DAF domains directed identification of domains central to factor I interaction and dissociation of the protease subunits from the C3 convertases.

VCP domains critical for factor I CFAs

Earlier, using deletion mutagenesis approach, we reported that CCP modules 1–3 of VCP are the minimum domains important for its binding to C3b and C4b and the CFAs, because further deletion of either CCP module 1 or 3 abolished ligand binding and the CFAs (34). In the current study, we observed that swapping of modules 2 and 3 of VCP with homologous domains of DAF resulted in complete loss in C3b and C4b CFAs, whereas substitution 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 de-

letion 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 mediated 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,

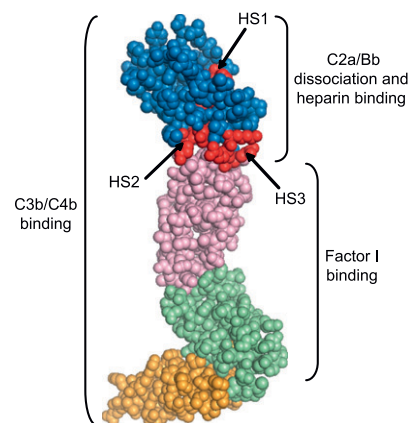


FIGURE 9. Summary of VCP domains and determinants critical for its functions. Each of the CCP modules of VCP molecule [1G40 (25)] is colored differently, and the domain(s) important for C3b and C4b binding, C2a and Bb dissociation, factor I interaction, and heparin binding is identified. Identification of domains important for various activities, except for C3b and C4b binding (34) and heparin binding (34), is based on data presented in this study. HS1, HS2, and HS3 (indicated in red) denote the three heparin binding sites identified earlier (53). Residues forming these sites also play a role in complement regulation (53). HS1, heparin binding site 1; HS2, heparin binding site 2; HS3, heparin binding site 3.

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 Kaposica (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

- Lachmann, P. J. 2002. Microbial subversion of the immune response. *Proc. Natl. Acad. Sci. USA* 99: 8461–8462.
- Mullick, J., A. Kadam, and A. Sahu. 2003. Herpes and pox viral complement control proteins: 'the mask of self.' *Trends Immunol.* 24: 500–507.
- Cooper, N. R. 1998. Complement and viruses. In *The Human Complement System in Health and Disease*. J. E. Volanakis, and M. M. Frank, eds. Marcel Dekker, Inc., New York, p. 393–407.
- Bernet, J., J. Mullick, A. K. Singh, and A. Sahu. 2003. Viral mimicry of the complement system. *J. Biosci.* 28: 249–264.
- Carroll, M. C. 2004. The complement system in regulation of adaptive immunity. *Nat. Immunol.* 5: 981–986.
- Köhl, J. 2006. The role of complement in danger sensing and transmission. *Immunol. Res.* 34: 157–176.
- Lambris, J. D., D. Ricklin, and B. V. Geisbrecht. 2008. Complement evasion by human pathogens. *Nat. Rev. Microbiol.* 6: 132–142.
- Dunkelberger, J. R., and W. C. Song. 2010. Complement and its role in innate and adaptive immune responses. *Cell Res.* 20: 34–50.
- Judson, K. A., J. M. Lubinski, M. Jiang, Y. Chang, R. J. Eisenberg, G. H. Cohen, and H. M. Friedman. 2003. Blocking immune evasion as a novel approach for prevention and treatment of herpes simplex virus infection. *J. Virol.* 77: 12639–12645.
- Means, R. E., J. K. Choi, H. Nakamura, Y. H. Chung, S. Ishido, and J. U. Jung. 2002. Immune evasion strategies of Kaposi's sarcoma-associated herpesvirus. *Curr. Top. Microbiol. Immunol.* 269: 187–201.
- Kotwal, G. J., S. N. Isaacs, R. McKenzie, M. M. Frank, and B. Moss. 1990. Inhibition of the complement cascade by the major secretory protein of vaccinia virus. *Science* 250: 827–830.
- Liszewski, M. K., M. K. Leung, R. Hauhart, R. M. Buller, P. Bertram, X. Wang, A. M. Rosengard, G. J. Kotwal, and J. P. Atkinson. 2006. Structure and regulatory profile of the monkeypox inhibitor of complement: comparison to homologs in vaccinia and variola and evidence for dimer formation. *J. Immunol.* 176: 3725–3734.
- Rosengard, A. M., Y. Liu, Z. Nie, and R. Jimenez. 2002. Variola virus immune evasion design: expression of a highly efficient inhibitor of human complement. *Proc. Natl. Acad. Sci. USA* 99: 8808–8813.
- Mullick, J., J. Bernet, A. K. Singh, J. D. Lambris, and A. Sahu. 2003. Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) open reading frame 4 protein (kaposica) is a functional homolog of complement control proteins. *J. Virol.* 77: 3878–3881.
- Johnson, J. B., K. Grant, and G. D. Parks. 2009. The paramyxoviruses simian virus 5 and mumps virus recruit host cell CD46 to evade complement-mediated neutralization. *J. Virol.* 83: 7602–7611.
- Vanderplasschen, A., E. Mathew, M. Hollinshead, R. B. Sim, and G. L. Smith. 1998. Extracellular enveloped vaccinia virus is resistant to complement because of incorporation of host complement control proteins into its envelope. *Proc. Natl. Acad. Sci. USA* 95: 7544–7549.
- Chung, K. M., M. K. Liszewski, G. Nybakken, A. E. Davis, R. R. Townsend, D. H. Fremont, J. P. Atkinson, and M. S. Diamond. 2006. West Nile virus nonstructural protein NS1 inhibits complement activation by binding the regulatory protein factor H. *Proc. Natl. Acad. Sci. USA* 103: 19111–19116.
- Stoiber, H., M. Pruenster, C. G. Ammann, and M. P. Dierich. 2005. Complement-opsonized HIV: the free rider on its way to infection. *Mol. Immunol.* 42: 153–160.
- Moss, B. 2001. Poxviridae: the viruses and their replication. In *Fields Virology*. D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus, eds. Lippincott Williams and Wilkins, Philadelphia, p. 2849–2883.
- McFadden, G. 2005. Poxvirus tropism. *Nat. Rev. Microbiol.* 3: 201–213.
- Finlay, B. B., and G. McFadden. 2006. Anti-immunology: evasion of the host immune system by bacterial and viral pathogens. *Cell* 124: 767–782.
- Chen, N., G. Li, M. K. Liszewski, J. P. Atkinson, P. B. Jahrling, Z. Feng, J. Schriewer, C. Buck, C. Wang, E. J. Lefkowitz, et al. 2005. Virulence differences between monkeypox virus isolates from West Africa and the Congo basin. *Virology* 340: 46–63.
- Kotwal, G. J., and B. Moss. 1988. Vaccinia virus encodes a secretory polypeptide structurally related to complement control proteins. *Nature* 335: 176–178.
- Henderson, C. E., K. Bromek, N. P. Mullin, B. O. Smith, D. Uhrin, and P. N. Barlow. 2001. Solution structure and dynamics of the central CCP module pair of a poxvirus complement control protein. *J. Mol. Biol.* 307: 323–339.
- Murthy, K. H., S. A. Smith, V. K. Ganesh, K. W. Judge, N. Mullin, P. N. Barlow, C. M. Ogata, and G. J. Kotwal. 2001. Crystal structure of a complement control protein that regulates both pathways of complement activation and binds heparan sulfate proteoglycans. *Cell* 104: 301–311.
- McKenzie, R., G. J. Kotwal, B. Moss, C. H. Hammer, and M. M. Frank. 1992. Regulation of complement activity by vaccinia virus complement-control protein. *J. Infect. Dis.* 166: 1245–1250.
- Sahu, A., S. N. Isaacs, A. M. Soulika, and J. D. Lambris. 1998. Interaction of vaccinia virus complement control protein with human complement proteins: factor I-mediated degradation of C3b to iC3b; inactivates the alternative complement pathway. *J. Immunol.* 160: 5596–5604.
- Liszewski, M. K., P. Bertram, M. K. Leung, R. Hauhart, L. Zhang, and J. P. Atkinson. 2008. Smallpox inhibitor of complement enzymes (SPICE): regulation of complement activation on cells and mechanism of its cellular attachment. *J. Immunol.* 181: 4199–4207.
- Girgis, N. M., B. C. Dehaven, X. Fan, K. M. Viner, M. Shamim, and S. N. Isaacs. 2008. Cell surface expression of the vaccinia virus complement control protein is mediated by interaction with the viral A56 protein and protects infected cells from complement attack. *J. Virol.* 82: 4205–4214.
- Isaacs, S. N., G. J. Kotwal, and B. Moss. 1992. Vaccinia virus complement-control protein prevents antibody-dependent complement-enhanced neutralization of infectivity and contributes to virulence. *Proc. Natl. Acad. Sci. USA* 89: 628–632.
- Rosengard, A. M., L. C. Alonso, L. C. Korb, W. M. Baldwin, III, F. Sanfilippo, L. A. Turka, and J. M. Ahearn. 1999. Functional characterization of soluble and membrane-bound forms of vaccinia virus complement control protein (VCP). *Mol. Immunol.* 36: 685–697.
- Smith, S. A., R. Sreenivasan, G. Krishnasamy, K. W. Judge, K. H. Murthy, S. J. Arjunwadkar, D. R. Pugh, and G. J. Kotwal. 2003. Mapping of regions within the vaccinia virus complement control protein involved in dose-dependent binding to key complement components and heparin using surface plasmon resonance. *Biochim. Biophys. Acta* 1650: 30–39.
- Isaacs, S. N., E. Argyropoulos, G. Sfyroera, S. Mohammad, and J. D. Lambris. 2003. Restoration of complement-enhanced neutralization of vaccinia virus virions by novel monoclonal antibodies raised against the vaccinia virus complement control protein. *J. Virol.* 77: 8256–8262.
- Mullick, J., J. Bernet, Y. Panse, S. Hallihsur, A. K. Singh, and A. Sahu. 2005. Identification of complement regulatory domains in vaccinia virus complement control protein. *J. Virol.* 79: 12382–12393.

35. Nicholson-Weller, A., J. Burge, D. T. Fearon, P. F. Weller, and K. F. Austen. 1982. Isolation of a human erythrocyte membrane glycoprotein with decay-accelerating activity for C3 convertases of the complement system. *J. Immunol.* 129: 184–189.
36. Seya, T., J. R. Turner, and J. P. Atkinson. 1986. Purification and characterization of a membrane protein (gp45-70) that is a cofactor for cleavage of C3b and C4b. *J. Exp. Med.* 163: 837–855.
37. Singh, A. K., J. Mullick, J. Bernet, and A. Sahu. 2006. Functional characterization of the complement control protein homolog of herpesvirus saimiri: ARG-118 is critical for factor I cofactor activities. *J. Biol. Chem.* 281: 23119–23128.
38. Pangburn, M. K. 1987. A fluorimetric assay for native C3: the hemolytically active form of the third component of human complement. *J. Immunol. Methods* 102: 7–14.
39. Lambris, J. D., Z. Lao, T. J. Oglesby, J. P. Atkinson, C. E. Hack, and J. D. Becherer. 1996. Dissection of CR1, factor H, membrane cofactor protein, and factor B binding and functional sites in the third complement component. *J. Immunol.* 156: 4821–4832.
40. Kirkitadze, M. D., M. Krych, D. Uhrin, D. T. Dryden, B. O. Smith, A. Cooper, X. Wang, R. Hauhart, J. P. Atkinson, and P. N. Barlow. 1999. Independently melting modules and highly structured intermolecular junctions within complement receptor type 1. *Biochemistry* 38: 7019–7031.
41. Bernet, J., J. Mullick, Y. Panse, P. B. Parab, and A. Sahu. 2004. Kinetic analysis of the interactions between vaccinia virus complement control protein and human complement proteins C3b and C4b. *J. Virol.* 78: 9446–9457.
42. Pan, Q., R. O. Ebanks, and D. E. Isenman. 2000. Two clusters of acidic amino acids near the NH2 terminus of complement component C4 alpha'-chain are important for C2 binding. *J. Immunol.* 165: 2518–2527.
43. Prydzial, E. L., and D. E. Isenman. 1986. A reexamination of the role of magnesium in the human alternative pathway of complement. *Mol. Immunol.* 23: 87–96.
44. Lukacik, P., P. Roversi, J. White, D. Esser, G. P. Smith, J. Billington, P. A. Williams, P. M. Rudd, M. R. Wormald, D. J. Harvey, et al. 2004. Complement regulation at the molecular level: the structure of decay-accelerating factor. *Proc. Natl. Acad. Sci. USA* 101: 1279–1284.
45. Kuttner-Kondo, L. A., M. P. Dybvig, L. M. Mitchell, N. Muqim, J. P. Atkinson, M. E. Medof, and D. E. Hourcade. 2003. A corresponding tyrosine residue in the C2/factor B type A domain is a hot spot in the decay acceleration of the complement C3 convertases. *J. Biol. Chem.* 278: 52386–52391.
46. Soames, C. J., and R. B. Sim. 1997. Interactions between human complement components factor H, factor I and C3b. *Biochem. J.* 326: 553–561.
47. Harris, C. L., R. J. Abbott, R. A. Smith, B. P. Morgan, and S. M. Lea. 2005. Molecular dissection of interactions between components of the alternative pathway of complement and decay accelerating factor (CD55). *J. Biol. Chem.* 280: 2569–2578.
48. Mullick, J., A. K. Singh, Y. Panse, V. Yadav, J. Bernet, and A. Sahu. 2005. Identification of functional domains in kaposica, the complement control protein homolog of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8). *J. Virol.* 79: 5850–5856.
49. Singh, A. K., V. N. Yadav, K. Pyaram, J. Mullick, and A. Sahu. 2009. Mapping of functional domains in herpesvirus saimiri complement control protein homolog: complement control protein domain 2 is the smallest structural unit displaying cofactor and decay-accelerating activities. *J. Virol.* 83: 10299–10304.
50. Yadav, V. N., K. Pyaram, J. Mullick, and A. Sahu. 2008. Identification of hot spots in the variola virus complement inhibitor (SPICE) for human complement regulation. *J. Virol.* 82: 3283–3294.
51. Wu, J., Y. Q. Wu, D. Ricklin, B. J. Janssen, J. D. Lambris, and P. Gros. 2009. Structure of complement fragment C3b-factor H and implications for host protection by complement regulators. *Nat. Immunol.* 10: 728–733.
52. Fritzing, D. C., B. E. Hew, M. Thorne, M. K. Pangburn, B. J. Janssen, P. Gros, and C. W. Vogel. 2009. Functional characterization of human C3/cobra venom factor hybrid proteins for therapeutic complement depletion. *Dev. Comp. Immunol.* 33: 105–116.
53. Liszewski, M. K., M. K. Leung, R. Hauhart, C. J. Fang, P. Bertram, and J. P. Atkinson. 2009. Smallpox inhibitor of complement enzymes (SPICE): dissecting functional sites and abrogating activity. *J. Immunol.* 183: 3150–3159.
54. Spiller, O. B., L. Mark, C. E. Blue, D. G. Proctor, J. A. Aitken, A. M. Blom, and D. J. Blackbourn. 2006. Dissecting the regions of virion-associated Kaposi's sarcoma-associated herpesvirus complement control protein required for complement regulation and cell binding. *J. Virol.* 80: 4068–4078.
55. Gordon, D. L., R. M. Kaufman, T. K. Blackmore, J. Kwong, and D. M. Lublin. 1995. Identification of complement regulatory domains in human factor H. *J. Immunol.* 155: 348–356.
56. Jokiranta, T. S., J. Hellwage, V. Koistinen, P. F. Zipfel, and S. Meri. 2000. Each of the three binding sites on complement factor H interacts with a distinct site on C3b. *J. Biol. Chem.* 275: 27657–27662.
57. Sharma, A. K., and M. K. Pangburn. 1996. Identification of three physically and functionally distinct binding sites for C3b in human complement factor H by deletion mutagenesis. *Proc. Natl. Acad. Sci. USA* 93: 10996–11001.
58. Blom, A. M., L. Kask, and B. Dahlbäck. 2001. Structural requirements for the complement regulatory activities of C4BP. *J. Biol. Chem.* 276: 27136–27144.
59. Hourcade, D., M. K. Liszewski, M. Krych-Goldberg, and J. P. Atkinson. 2000. Functional domains, structural variations and pathogen interactions of MCP, DAF and CR1. *Immunopharmacology* 49: 103–116.
60. Iwata, K., T. Seya, Y. Yanagi, J. M. Pesando, P. M. Johnson, M. Okabe, S. Ueda, H. Ariga, and S. Nagasawa. 1995. Diversity of sites for measles virus binding and for inactivation of complement C3b and C4b on membrane cofactor protein CD46. *J. Biol. Chem.* 270: 15148–15152.
61. Kalli, K. R., P. H. Hsu, T. J. Bartow, J. M. Ahearn, A. K. Matsumoto, L. B. Klickstein, and D. T. Fearon. 1991. Mapping of the C3b-binding site of CR1 and construction of a (CR1)2-F(ab')₂ chimeric complement inhibitor. *J. Exp. Med.* 174: 1451–1460.
62. Reilly, B. D., S. C. Makrides, P. J. Ford, H. C. Marsh, Jr., and C. Mold. 1994. Quantitative analysis of C4b dimer binding to distinct sites on the C3b/C4b receptor (CR1). *J. Biol. Chem.* 269: 7696–7701.
63. Kuttner-Kondo, L., D. E. Hourcade, V. E. Anderson, N. Muqim, L. Mitchell, D. C. Soares, P. N. Barlow, and M. E. Medof. 2007. Structure-based mapping of DAF active site residues that accelerate the decay of C3 convertases. *J. Biol. Chem.* 282: 18552–18562.
64. Harris, C. L., D. M. Pettigrew, S. M. Lea, and B. P. Morgan. 2007. Decay-accelerating factor must bind both components of the complement alternative pathway C3 convertase to mediate efficient decay. *J. Immunol.* 178: 352–359.