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J Immunol 2012; 189:1431-1439; Prepublished online 25 June 2012;
doi: 10.4049/jimmunol.1200946
http://www.jimmunol.org/content/189/3/1431

Supplementary Material
http://www.jimmunol.org/content/suppl/2012/06/25/jimmunol.1200946.DC1

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Variola and vaccinia viruses, the two most important members of the family Poxviridae, are known to encode homologs of the human complement regulators named smallpox inhibitor of complement enzymes (SPICE) and vaccinia virus complement control protein (VCP), respectively, to subvert the host complement system. Intriguingly, consistent with the host tropism of these viruses, SPICE has been shown to be more human complement-specific than VCP, and in this study we show that VCP is more bovine complement-specific than SPICE. Based on mutagenesis and mechanistic studies, we suggest that the major determinant for the switch in species selectivity of SPICE and VCP is the presence of oppositely charged residues in the central complement control modules, which help enhance their interaction with factor I and C3b, the proteolytically cleaved form of C3. Thus, our results provide a molecular basis for the species selectivity in poxviral complement regulators. The Journal of Immunology, 2012, 189:1431–1439.

Smallpox, an abysmal disease caused by variola virus, claimed millions of human lives for centuries until its successful eradication in 1977 as a result of extensive mass vaccinations using vaccinia virus as the inoculating agent (1). Despite this success, there are looming concerns about its re-emergence owing to the possible usage of variola virus as a bioterrorism agent, because a large percentage of the current human population is susceptible to smallpox (2). Thus, there is a need to further define the factors that influence variola virus virulence, pathogenesis, and control (3, 4).

Variola virus exhibits strict human tropism; however, molecular factors that govern this specificity are still not defined (5, 6). Overall, host tropism is determined by the immune as well as nonimmune factors (6). Because complement is one of the major innate immune mechanisms that can target cell-free virus and virus-infected cells (7–9), as well as boost antiviral adaptive immune responses (10, 11), Rosengard et al. (12) tested the hypothesis that the strict human tropism of variola virus is due, in part, to its ability to evade neutralization by human complement. Their study revealed that the variola-encoded complement regulator called smallpox inhibitor of complement enzymes (SPICE) is ~100- and 6-fold more potent in inactivating the key human complement proteins C3b (the proteolytically cleaved form of C3) and C4b (the proteolytically cleaved form of C4), respectively, than is vaccinia virus complement control protein (VCP), the complement regulator of vaccinia virus that does not cause disease in immunocompetent humans. These results suggested that SPICE may possibly be a virulence factor of variola virus. This notion gets further support from the fact that SPICE is conserved in all the strains of variola, including the highly virulent strains India-1967 and Bangladesh-1975 (13).

Because SPICE and VCP vary only by 11 aa, it was apparent that the functional differences between these two proteins ought to be due to one or more of these variant residues. Initial efforts to identify the residues responsible for the functional advantage of SPICE over VCP used the electrostatic modeling approach and showed that substitution of two SPICE residues (E108K/E120K) in VCP appreciably enhances its C3b inactivation ability (14). Later, we examined the contribution of each of the variant amino acids of SPICE and demonstrated that substitution of four SPICE residues (H98Y/S103Y/E108K/E120K) in VCP is enough to formulate VCP as potent as SPICE in inactivating human C3b as well as C4b (15). The functional advantage provided by K120 in SPICE was also demonstrated by Liszewski et al. (16).

Unlike variola virus, which infects only humans, vaccinia virus is known to infect a range of domestic animals (observed during smallpox vaccinations) and its outbreaks are frequently reported in dairy cattle in Brazil (17). However, whether VCP exhibits preference in inhibiting bovine complement remained unclear. Given that poxviral complement regulators help evade host complement-mediated neutralization (18–20), modulate humoral and T cell-mediated responses against the virus (21), and serve as virulence determinants (18, 22), it is likely that they function in a species-specific manner. The phenomenon of species-selective complement regulation or “homologous restriction” is well recognized in mammalian regulators of complement activation (RCA) proteins (23, 24), but the molecular basis for it remained elusive (25). In this study, we show that VCP exhibits preference in inhibiting the bovine alternative complement pathway. Furthermore, our biochemical data

Received for publication March 30, 2012. Accepted for publication May 21, 2012.

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Received for publication March 30, 2012. Accepted for publication May 21, 2012.

This work was supported by a project grant from the Department of Biotechnology, India (to A.S.). The authors also acknowledge the financial assistance by the Council of Scientific and Industrial Research, New Delhi, India (to V.N.Y. and K.P.), and by the University Grants Commission, New Delhi, India (to M.A.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: AP, alternative pathway; C3b, proteolytically cleaved form of C3; C4b, proteolytically cleaved form of C4; RCA, regulator of complement activation; SPICE, smallpox inhibitor of complement enzymes; SPR, surface plasmon resonance; VCP, vaccinia virus complement control protein.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1200946

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obtained utilizing various substitution mutants of SPICE and VCP, as well as purified bovine and human complement components, provide a mechanistic insight into the ingenious mechanism of species specificity in poxviral complement regulators.

Materials and Methods

Purification of complement proteins

Human and bovine C3 were purified (Supplemental Fig. 1A) from the respective plasma as described (26) and their native forms were separated from C3(H2O) by loading the sample onto a Mono S column (27). Human C3b was generated by limited tryptic cleavage of C3 (28), and bovine C3b was generated by cleavage of C3 with CVF-Bb (29). Both of these were then purified using a Mono Q column. Human factor B was purified according to the earlier established procedure (30), whereas bovine factors B (31) and I (32) were purified as described with few modifications. For bovine factor B purification, one part of inhibitor solution (1 M KH2PO4, 0.2 M Na2EDTA, 0.2 M benzamidine, and 1 mM PMSF) was mixed with 19 parts bovine plasma and sequentially precipitated with 12 and 26% polyethylene glycol at 0°C. The 26% pellet was then dissolved in 50 mM sodium phosphate (pH 6.0) and further precipitated using 20% (w/v) anhydrous sodium sulfate. The supernatant obtained was subjected to dialysis and then fractionation by ion-exchange chromatography as below. First, the sample was loaded onto a Mono S 10/10 column (Amersham Pharmacia Biotech, Uppsala, Sweden) in 50 mM sodium phosphate (pH 6.0), and bound proteins were eluted with a linear gradient of 0–0.5 M NaCl. Factor B-containing fractions, as identified by SDS-PAGE and Western blot analysis, were pooled and loaded onto a Mono Q 10/10 column (Amersham Pharmacia Biotech) after buffer exchange in 10 mM sodium phosphate (pH 7.4) (Supplemental Fig. 1B). The bound proteins were eluted with a similar salt gradient as above and the fractions containing homogeneous factor B were pooled, concentrated, and dialyzed against PBS (pH 7.4) (Supplemental Fig. 1B).

To purified bovine factor I, bovine sera was dialyzed against 0.02 M sodium phosphate (pH 8.0) containing 0.01 M EDTA and 0.12 M NaCl and loaded onto a QAE-50 Sephadex column (Sigma-Aldrich) equilibrated with the same buffer. The bound protein fraction was eluted with a linear gradient of 0–0.5 M NaCl. Factor B-containing fractions, as identified by SDS-PAGE and Western blot analysis, were pooled and loaded onto a Mono Q 10/10 column (Amersham Pharmacia Biotech) after buffer exchange in 10 mM sodium phosphate (pH 8.5). The bound proteins were eluted with a similar salt gradient as above and the fractions containing homogeneous factor B were pooled, concentrated, and dialyzed against PBS (pH 7.4) (Supplemental Fig. 1B).

Site-directed mutagenesis, expression, and purification of SPICE and VCP mutants

The single and multiple amino acid mutants of SPICE as well as the double amino acid mutant of VCP were generated by using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), and the fidelity of all the cloned products was verified by automated DNA sequencing. These mutants were then expressed in Escherichia coli (BL21 cells), purified, and refolded as previously described (15, 33). The monodispersed population of the refolded mutants was obtained by passing them through a Superpose-12 gel filtration column (Amersham Pharmacia Biotech).

Alternative pathway inhibition assay

Inhibition of alternative pathway (AP) complement activity of various animal sera by SPICE, VCP, and the various mutants was studied by measuring their ability to inhibit the lysis of rabbit erythrocytes (34).

C3-convertase decay acceleration and factor I cofactor assays

The AP C3-convertase C3b,Bb was formed on rabbit erythrocytes by incubating the cells with human/bovine C3, human/bovine factor B, and human factor D in the presence of NiCl2 (35). These preformed C3-convertases were then allowed to decay in the presence of SPICE, VCP, or the mutants, and the remaining enzyme activity was quantitated by incubating the enzyme-coated cells with EDTA sera and measuring lysis (34). The ability of SPICE, VCP, and the mutants to act as a cofactor for the factor I-mediated cleavage of human/bovine C3b was measured by performing the fluid phase cofactor assay (26).

Surface plasmon resonance measurements

The binding abilities of SPICE, VCP, and the mutants to bovine C3b were determined using surface plasmon resonance (SPR)-based biosensor Biacore 2000 (Biacore, Uppsala, Sweden) (36). Bovine C3b (∼5000 response units, RU) labeled through its free thiol group with biotin (Supplemental Fig. 2A, 2B) was oriented on a streptavidin chip (Sensor Chip SA; Biacore, Uppsala, Sweden) and binding of SPICE, VCP, and the mutants to C3b was measured by allowing the purified proteins to flow over the flow cell immobilized with bovine C3b. The binding studies for all interactions were performed at 25°C in PBS containing 0.05% Tween 20 at a flow rate of 50 μl/min to avoid mass transport effect. Association was measured by injecting the mutants for 120 s, and dissociation of the complex was measured for 180 s by replacing the 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 bovine C3b to obtain the specific binding responses.

Results

VCP exhibits preference in inhibiting bovine alternative complement pathway

Because variola and vaccinia show differential host tropism, and vaccinia is known to infect a range of domestic animals, we asked in this study whether VCP displays preference in inhibiting complement of domestic animals compared with SPICE. This premise also derives support from earlier studies wherein VCP was demonstrated to be a better inhibitor of the AP of dog complement compared with SPICE (12, 15). A comparison of the relative activities of VCP and SPICE against the alternative complement pathway of various animals showed that VCP preferentially inhibited complement of nonprimate species, whereas SPICE preferentially inhibited complement of primates. The order of inhibition by VCP was calf ≈ buffalo > sheep > dog > pig > cat > monkey > human (Fig. 1). The maximum inverse inhibitory activity of VCP and SPICE was observed in calf and human complement: VCP was ∼34-fold better in inhibiting calf complement compared with SPICE, whereas SPICE was 23-fold better in inhibiting human complement compared with VCP. Intriguingly, these findings are in line with the species tropism exhibited by vaccinia and variola viruses.

Four variant residues in VCP direct its higher functional activity against bovine alternative complement pathway

VCP and SPICE differ by 11 aa (Fig. 2A), and therefore next we sought to determine which of these variant residues in VCP are responsible for its better inhibitory activity toward bovine complement. Being a homolog of the human RCA proteins, VCP possesses the ability to inactivate AP by targeting the AP C3-convertase by supporting the cleavage of C3b by serine protease factor I (termed cofactor activity) and by accelerating the dissociation of C3-convertase (termed decay-accelerating activity) (26, 37). We thus generated 11 single amino acid mutants by substituting each of the 11 variant VCP residues onto SPICE template (Fig. 2B) and measured their cofactor and decay-accelerating activities using purified bovine complement components.

Examination of cofactor activity of VCP compared with SPICE showed that VCP is 12-fold more efficient than SPICE in inactivating bovine C3b (Fig. 3, Table I). Among the 11 mutants of SPICE, only Y103S, K108E, K120E, and N144E exhibited notably higher (3- to 6-fold) cofactor activity compared with SPICE, suggesting that primarily negatively charged residues of VCP are responsible for shaping the higher cofactor activity of the mole-
cule against bovine C3b (Fig. 3, Table I). Because VCP is 34-fold more efficient than SPICE in inactivating bovine complement, its 12-fold higher cofactor activity did not entirely explain its better activity toward bovine complement. We therefore next measured whether the increased activity is also due, in part, to its enhanced decay-accelerating activity. Interestingly, VCP displayed 36-fold more potent decay activity than did SPICE, and among the single amino acid substitution mutants, K108E depicted a substantial (~16-fold) increase (Fig. 4A, Table I), indicating that the negatively charged Glu at 108 position of VCP is largely responsible for its increased AP decay-accelerating activity.

For bringing about the inactivation of AP C3-convertases by means of either cofactor or decay-accelerating activity, the viral regulator is engaged in trimolecular complexes wherein it interacts

**FIGURE 1.** Species selective complement inhibition by VCP. Relative inhibition of alternative complement pathway of various species by VCP (○) and SPICE(●) was measured by employing a hemolytic assay using rabbit erythrocytes (ER) as complement activator. IC50 values for VCP: calf, 0.017 μM; buffalo, 0.016 μM; sheep, 0.024 μM; dog, 0.029 μM; pig, 0.076 μM; cat, 0.25 μM; monkey, 0.79 μM; human, 6.4 μM. IC50 values for SPICE: calf, 0.57 μM; buffalo, 0.18 μM; sheep, 0.058 μM; dog, 0.39 μM; pig, 0.2 μM; cat, 4.6 μM; monkey, 0.095 μM; human, 0.28 μM.

**FIGURE 2.** Single and multiple amino acid substitution mutants of SPICE. (A) Amino acid sequence alignment of SPICE and VCP. The arrows indicate the 11 variant amino acids in SPICE that were changed with the corresponding amino acid of VCP to generate the single and multiple amino acid substitution mutants of SPICE. The numbers denote their corresponding position in the mature protein. (B) SDS-PAGE analysis of purified VCP, SPICE, and the substitution mutants of SPICE on a 12% SDS-PAGE. The proteins were run under reducing conditions and stained with Coomassie blue. SPICE-double, K108E/K120E; SPICE-triple, K108E/K120E/N144E; SPICE-tetra, Y103S/K108E/K120E/N144E.
with C3b, a component of C3-convertase (38, 39). To assess whether the increased functional activities of VCP toward bovine complement is a result of its increased binding to bovine C3b, we measured the relative binding abilities of VCP, SPICE, and the mutants to bovine C3b using an SPR-based assay (Fig. 4B). VCP exhibited substantially increased binding to bovine C3b compared with SPICE. With respect to the four mutants that demonstrated increased functional activities, only K120E and N144E showed enhanced binding to bovine C3b, whereas the other two mutants showed binding similar to SPICE.

Table I. Summary of the functional activities of VCP, SPICE, and the single and multiple amino acid mutants of SPICE against bovine C3b and AP C3-convertase

<table>
<thead>
<tr>
<th>Wild Type/Mutant</th>
<th>Time (min) for 50% Cleavage of Bovine C3b α'-Chain</th>
<th>Relative C3b Cofactor Activitya</th>
<th>AP-DAA IC50 (μM)</th>
<th>Relative AP-DAAa</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPICE</td>
<td>24</td>
<td>1.0</td>
<td>3.6</td>
<td>1</td>
</tr>
<tr>
<td>H77Q</td>
<td>28</td>
<td>0.85</td>
<td>2.2</td>
<td>1.63</td>
</tr>
<tr>
<td>L131S</td>
<td>24</td>
<td>1.0</td>
<td>2.6</td>
<td>1.38</td>
</tr>
<tr>
<td>N178D</td>
<td>19</td>
<td>1.3</td>
<td>3.2</td>
<td>1.12</td>
</tr>
<tr>
<td>Y98H</td>
<td>17</td>
<td>1.4</td>
<td>4.9</td>
<td>0.73</td>
</tr>
<tr>
<td>L193S</td>
<td>14</td>
<td>1.7</td>
<td>3.05</td>
<td>1.18</td>
</tr>
<tr>
<td>T214K</td>
<td>12.5</td>
<td>1.9</td>
<td>2.3</td>
<td>1.56</td>
</tr>
<tr>
<td>Q236K</td>
<td>11</td>
<td>2.2</td>
<td>1.9</td>
<td>1.89</td>
</tr>
<tr>
<td>Y103S</td>
<td>8</td>
<td>3.0b</td>
<td>3.8</td>
<td>0.94</td>
</tr>
<tr>
<td>N144E</td>
<td>7.8</td>
<td>3.1b</td>
<td>2.0</td>
<td>1.8</td>
</tr>
<tr>
<td>K108E</td>
<td>5.0</td>
<td>4.8b</td>
<td>0.23</td>
<td>15.6b</td>
</tr>
<tr>
<td>K120E</td>
<td>4.0</td>
<td>6.0b</td>
<td>1.5</td>
<td>2.4</td>
</tr>
<tr>
<td>SPICE-double mutant</td>
<td>2.2</td>
<td>10.9b</td>
<td>0.23</td>
<td>15.6b</td>
</tr>
<tr>
<td>(K108E/K120E)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPICE-triple mutant (K108E/K120E/N144E)</td>
<td>2.3</td>
<td>10.4b</td>
<td>0.17</td>
<td>21.0b</td>
</tr>
<tr>
<td>SPICE-tetra mutant (Y103S/K108E/K120E/N144E)</td>
<td>2.1</td>
<td>11.4b</td>
<td>0.14</td>
<td>25.7b</td>
</tr>
<tr>
<td>VCP</td>
<td>2.0</td>
<td>12b</td>
<td>0.1</td>
<td>36b</td>
</tr>
</tbody>
</table>

aRelative activity compared with SPICE.
bA >3-fold difference in activity was considered meaningful.
AP-DAA, Alternative pathway decay-accelerating activity.
Glutamates at positions 108, 120, and 144 are the key functional determinants of VCP for its specificity toward bovine alternative complement pathway.

The above results obtained utilizing single amino acid substitution mutants suggested that 4 (S103, E108, E120 and E144) of the 11 variant residues of VCP are vital for imparting specificity toward bovine complement. We thus asked how many of the four residues are needed to alter the specificity of SPICE toward bovine complement. To answer this, we generated double (K108E/K120E), triple (K108E/K120E/N144E), and tetra (Y103S/K108E/K120E/...
N144E residue mutants of SPICE (Fig. 2B) and measured their inhibitory activity toward bovine C3b and AP C3-convertase. Functionally, all three multiresidue mutants of SPICE exhibited equally good ability to inactivate bovine C3b (Fig. 5A, 5B, Table I), but only triple and tetra mutants were more similar to VCP in their ability to decay the bovine AP C3-convertase (Fig. 5C, Table I). Binding analysis revealed that binding of triple and tetra mutants, but not the double mutant, was comparable to VCP (Fig. 5D). Taken together, these data indicate that primarily the glutamates at 108, 120, and 144 direct the specificity of VCP toward bovine complement.

Switch in host complement specificity of VCP and SPICE is primarily determined by the charge reversal

It is notable that Glu at positions 108 and 120 in VCP is substituted by Lys in SPICE (Fig. 2A). Additionally, VCP also contains Glu at 144, which is substituted by Asn in SPICE. Thus, it was tempting to speculate that the major determinant of switch in host specificity of VCP and SPICE is the opposite charge at these positions. To validate this premise, we measured the inhibitory activities of double (K108E/K120E), triple (K108E/K120E/N144E), and tetra (Y103S/K108E/K120E/N144E) mutants of SPICE (Fig. 2B) as well as double (E108K/E120K) (Supplemental Fig. 1D) and tetra (S103Y/E108K/E120K/E144N) (15) mutants of VCP toward bovine and human complement. As speculated, the triple mutant of SPICE, wherein Glu was substituted in SPICE at three positions, displayed an essentially similar inhibitory activity against bovine complement to that of VCP (Fig. 6A), and there was a loss in activity of this mutant toward human complement (Fig. 6B). In contrast, the double mutant of VCP wherein Lys was substituted in VCP at two positions exhibited appreciable gain in activity toward human complement and loss in activity toward bovine complement (Fig. 6C, 6D); admittedly, complete gain in activity toward human complement was observed only in VCP tetra mutant. In short, largely the reversal in charge switched the host complement specificities of VCP and SPICE.

Species selective cofactor activity of VCP and SPICE is primarily dictated by their interaction with factor I

Given that cofactor activity is a result of interaction between three proteins (C3b, viral regulator, and the protease factor I), we sought to determine whether the enhanced cofactor activity of VCP and
Wherein VCP and multiresidue mutants of SPICE showed better activity when compared with SPICE. This notion is also supported by our direct binding data (SPR data) that VCP and the multiresidue mutants of SPICE displayed moderate cofactor activity (Fig. 7A).

When bovine C3b in the above assay was replaced with human C3b (Fig. 7B), VCP was still the most efficient cofactor for inactivation of human C3b, and double and tetra mutants of SPICE depicted almost similar activity. Similarly, SPICE and the VCP double and tetra mutants displayed relatively poor cofactor activity for human C3b. These data therefore indicate that it is the interaction of VCP with bovine factor I that makes VCP a superior inactivator of C3b, and the two residues of VCP, E108 and E120, are the key mediators of this interaction.

Next, to further support the above contention, we performed cofactor assays using bovine C3b and human factor I in place of bovine factor I (Fig. 7C). In this case, VCP was the weakest cofactor for inactivation of bovine C3b, and two multiresidue mutants of SPICE showed moderate activity; however, SPICE and the multimeric mutants of VCP were most efficient. As expected, when both bovine C3b and bovine factor I were replaced with human C3b and human factor I (Fig. 7D), SPICE and double and tetra mutants of VCP were most efficient cofactors, whereas VCP and double and tetra mutants of SPICE were least effective. Thus, the species-specific cofactor activity of SPICE is also dictated by its interaction with human factor I.

Species selective C3-convertase decay activity of VCP is primarily dictated by its interaction with C3b

VCP displays efficient decay-accelerating activity toward bovine AP C3-convertase compared with human AP C3-convertase, whereas SPICE is a poor inhibitor of both the C3-convertases (Fig. 8A, 8B). We therefore next attempted to determine the basis of the selectivity of VCP against the bovine convertase.

The process of dissociation of the catalytic subunit Bb from the C3-convertase C3b,Bb is preceded by the binding of regulator to both the subunits of C3-convertase, C3b and Bb (40, 41). We therefore asked whether the superior decay-acceleration activity of VCP against bovine C3-convertase is determined by its interaction with C3b or with Bb. Consequently, the C3-convertase C3b,Bb was formed by using different combinations of bovine and human complement proteins C3b and factor B, and the decay activity of VCP, SPICE, and their multiresidue mutants was assessed. The data revealed that the C3-convertase composed of bovine C3b and bovine Bb was very effectively dissociated by VCP as compared with SPICE, and the double, triple, and tetra mutants of SPICE showed activity comparable to VCP (Fig. 8A). However, when the C3-convertase was formed using human complement components, VCP and the multiresidue mutants of SPICE displayed a very poor decay activity (Fig. 8B).

We then generated C3-convertase constituted of bovine C3b and human Bb and measured the abilities of VCP, SPICE, and the mutants to dissociate the enzyme. It was observed that VCP and the multiresidue mutants of SPICE were much more potent compared with SPICE and the double and tetra mutants of VCP (Fig. 8C). It is therefore clear that the specificity of VCP toward bovine C3-convertase is primarily due to its better interaction with C3b. This notion is also supported by our direct binding data (SPR data) wherein VCP and multiresidue mutants of SPICE showed better binding to bovine C3b compared with SPICE (Fig. 5D). To further substantiate this view, we also tried to assess the decay activities of VCP, SPICE, and the mutants toward C3-convertase composed of human C3b and bovine Bb. We however failed to generate this convertase on the cell surface. This was verified by forming fluid phase C3-convertase; incubation of human C3b with bovine factor B and factor D did not result in the cleavage of bovine factor B into Ba and Bb fragments (Supplemental Fig. 2C).

Discussion

Variola and vaccinia viruses, which hold a special place in the history of humankind, differ markedly in their host tropism despite exhibiting a remarkable genome homology (6). It is therefore likely that their immune evasion proteins are more suited to subvert the immune response of the species in which they cause...
Glu108, Glu120, and Glu144 are fully exposed to the solvent (42). This conclusion is consistent with the cocrystal structure of human C3b and factor I showed that interaction of these residues in VCP enhances its negative electrostatic potential of the molecule: the presence of glutamates (108, 120, and 144) in VCP enhances its negative electrostatic potential favoring its binding to bovine C3b, whereas the presence of lysines (and lack of glutamate at 144) in SPICE enhances its positive electrostatic potential favoring its binding to human C3b. Notably, this contention is also supported by the charge distribution on human and bovine C3b. Overall, bovine C3b is mostly electronegative, whereas human C3b is mostly electronegative (45, 46). Besides cofactor activity, we also examined the molecular basis for improved decay-accelerating activity of VCP against bovine AP C3-convertase. Our data suggest that apart from enhancing the cofactor activity of VCP, Glu108, Glu120, and Glu144 are also critical in improving the AP decay-accelerating activity of VCP (Fig. 5C, Table I). To dissect whether interaction of these residues with C3b or Bb is responsible for the enhancement in the activity, we performed decay-acceleration assays using a combination of bovine/human C3-convertase components. It was observed that VCP and the SPICE substitution mutants containing the above-mentioned amino acids are potent against the convertases only when they are formed using bovine C3b (Fig. 8). Taken together, these data suggested that the interaction of VCP with C3b and not Bb dictates its potent activity against the bovine C3-convertase, and that the three glutamates are responsible for this enhancement.

In summary, our results indicate that oppositely charged residues in the central domains of poxviral complement regulators serve as a “molecular switch” that dictates species selectivity in these molecules. Intriguingly, the positively charged residues (at positions 108 and 120) were found to be conserved in all the strains of variola virus, whereas the negatively charged amino acids (at positions 108, 120, and 144) were found to be conserved in different strains of vaccinia, cowpox, ectromelia, rabbotpox, camelpox, and horsepox (Supplemental Fig. 3). Although it would be premature to describe the viral complement regulators as mediators of poxviral tropism, their role as one of the same cannot be ruled out.

Acknowledgments

We thank Dr. Jayati Mullick (National Institute of Virology, Pune, India) for comments and critical reading of the manuscript, and we express our appreciation to Yogesh Panse and Sandeep Walujkar for excellent technical assistance. We also thank Dr. Kapole Srikant and Snigdha Dhal (Proteomic Facility, National Centre for Cell Science, Pune, India) for sequencing bovine complement components.

Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Fig. 1. SDS-PAGE analyses of purified bovine complement proteins and the double-mutant of VCP. (a) Purified bovine C3 and human C3. (b) Purified bovine factor B (in duplicate) and human factor B. (c) Purified bovine factor I and human factor I. All the bovine proteins were subjected to electrophoresis on a 9% SDS-PAGE under reducing conditions and stained with Coomassie blue. (d) The purified double-mutant of VCP (E108K/E120K; marked by an arrow) was run on a 12% SDS-PAGE gel under reducing conditions and stained with Coomassie blue.
Supplementary Fig. 2. Site-specific biotinylation of bovine C3b and analysis of formation of fluid phase alternative pathway C3-convertase using human C3b and bovine factor B. (a, b) The free -SH group of C3b was labelled with PEO-maleimide biotin. The labelled C3b was cleaved into C3c and C3dg by soluble complement receptor 1 and factor I and then analyzed by SDS-PAGE (a) and Western blotting using avidin-HRP (b). Specific labelling of the free –SH group is indicated by the presence of signals in C3b α'-chain (lane 1) and its the cleavage product C3dg (lane 2). (c) The formation of C3-convertase was determined by incubating human C3b with bovine factor B (fB) and human factor D (fD) in presence of Mg²⁺ for 1 h 37°C. The reaction was stopped by adding the sample buffer and formation of C3-convertase was assessed by the presence of Ba and Bb on SDS-PAGE gel. Control was formed by incubating human C3b with human fB and fD under the same conditions.
Supplementary Fig. 3. Sequence alignment of CCP2 and CCP3 domains of different poxviral complement regulators. The protein sequences of the CCP domains 2 and 3 of the RCA homologs from different orthopoxviruses were aligned using CLUSTAL X (version 2.0) program. The arrows in red indicate the 108, 120 and 144 positions in the mature VCP/SPICE proteins. Abbreviations: VAR-G, Variola virus-Garcia-1966; VAR-C, Variola virus-Congo; VAR-S, Variola virus-Somalia; VAR-B, Variola virus-Bangladesh-1975; VAR-I67, Variola virus-India-1967; VAR-I71, Variola virus-India-1971; VACV-WR, Vaccinia virus-WR; VACV-LC, Vaccinia virus-LC16m8; VACV-GLV, Vaccinia virus-GLV; CPV-GRI, Cowpox virus-GRI-90; CPV-BR, Cowpox virus-Brighton Red; RPV, Rabbit pox virus; CMPV, Camelpox virus-M-96; ECT-MP5, Ectromelia virus-Mousepox; ECT-N, Ectromelia virus-Naval; ECT-M, Ectromelia virus-Moscow; HSPV, Horsepox; MPV-Z, Monkeypox virus-Zaïre-96-I-16; BPXV, Buffaloopox virus; VACV-TT, Vaccinia virus -Tian Tan.