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Structure and Regulatory Profile of the Monkeypox Inhibitor of Complement: Comparison to Homologs in Vaccinia and Variola and Evidence for Dimer Formation

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The outbreak of monkeypox in the United States in the summer of 2003 was the first occurrence of this smallpox-like disease outside of Africa. This limited human epidemic resulted from cross-infection of prairie dogs by imported African rodents. Although there were no human fatalities, this outbreak illustrates that monkeypox is an emerging natural infection and a potential biological weapon. We characterized a virulence factor expressed by monkeypox (monkeypox inhibitor of complement enzymes or MOPICE). We also compared its structure and regulatory function to homologous complement regulatory proteins of variola (SPICE) and vaccinia (VCP). In multiple expression systems, 5–30% of MOPICE, SPICE, and VCP consisted of function-enhancing disulfide-linked homodimers. Mammalian cells infected with vaccinia virus also expressed VCP dimers. MOPICE bound human C3b/C4b intermediate to that of SPICE and VCP. Cofactor activity of MOPICE was similar to VCP, but both were ~100-fold less efficient than SPICE. SPICE and VCP, but not MOPICE, possessed decay-accelerating activity for the C3 and C5 convertases of the classical pathway. Additionally, all three regulators possessed heparin-binding capability. These studies demonstrate that MOPICE regulates human complement and suggest that dimerization is a prominent feature of these virulence factors. Thus, our data add novel information relative to the functional repertoire of these poxviral virulence factors. Furthermore, targeting and neutralizing these complement regulatory active sites via mAbs is a therapeutic approach that may enhance protection against smallpox. The Journal of Immunology, 2006, 176: 3725–3734.

Monkeypox virus (MPXV)3 causes a smallpox-like disease in humans as well as in monkeys. The disease was initially characterized as a viral eruption of captive primates in 1958 (reviewed in Refs. 1 and 2). Human infections were first documented in 1970 in the Central African Democratic Republic of Congo (formerly Zaire). The only outbreak of human monkeypox infection outside of Africa occurred in the United States in 2003 (1). MPXV was introduced via infected rodents (from West Africa) that had been imported by an exotic pet trader. Cross-infected prairie dogs housed in the same facility were subsequently distributed to seven states. The illness spread to humans who had close contact with infected animals. The outbreak consisted of 72 confirmed or suspected cases (1). Although the United States epidemic had no cases of human-to-human transmission and no fatalities, in Central Africa this mode of transmission occurs in 4–73% of cases (3) and with fatality rates from 4 to 25% (3, 4).

The fortunate lack of fatalities in the United States outbreak may have been in part secondary to the strain of MPXV (5). MPXV from West Africa and the Congo basin (Central Africa) are genetically distinct (6, 7). The less virulent West African strains lack the monkeypox inhibitor of complement enzymes (MOPICE), whereas the more virulent (i.e., smallpox-like) strains from the Congo Basin express this inhibitor of the complement system (5). The strain causing the infection in the United States was from West Africa and did not possess MOPICE.

MOPICE and other poxviral complement regulators (e.g., smallpox inhibitor of complement enzymes (SPICE) of variola and vaccinia complement control protein (VCP) of vaccinia) are structural and functional homologs of the human regulators of complement activation (RCA) family (8–11). This genetic cluster lies at 1q3.2 and encodes six regulators: two plasma proteins factor H and C4b-binding protein (C4BP); two membrane-anchored proteins, membrane cofactor protein (MCP; CD46) and decay-accelerating factor (DAF; CD55); and complement receptor (CR)-1 (CR1; CD35) and -2 (CR2; CD21) (12). RCA proteins consist largely or entirely of repeating units called complement control protein (CCP) modules (also known as short consensus repeats or sushi domains) that house the sites for C3b-C4b interactions. These consist of ~60 aa, with four invariant cysteines that pair (Cys1 to Cys3 and Cys2 to Cys4) and 10–18 other highly conserved residues (12). Poxviral
complement regulators are >90% homologous to each other (see Fig. 1) and ~35% homologous to their human counterparts.

Native RCA proteins protect host cells from complement-mediated damage using two mechanisms. Cofactor activity (CA) refers to the limited proteolytic degradation of C3b and C4b that requires a cofactor protein plus the plasma serine protease factor I. Decay-accelerating activity (DAA) refers to the irreversible dissociation of the catalytic serine protease domain from complement-activating enzyme complexes or convertases (12). The function of the convertases is to cleave and thereby activate C3 and C5.

Most poxviral inhibitors of complement enzymes (PICES) possess four CCPs as do the human regulators MCP and DAF. In contrast, the Central African strain of MOPICE consists of three CCPs with a truncated fourth that is secondary to the deletion of a single base pair producing a frameshifted stop codon 13 aa into CCP4 (13) (Fig. 1). Our recent study demonstrates that the more virulent Central African strains contain MOPICE, whereas the less virulent strains delete the open reading frame for MOPICE (5). Thus, similar to VCP and SPICE, MOPICE is likely to be a virulence factor in monkeypox infection. Because little is known about MOPICE, our goal was to characterize its complement regulatory activities as compared with SPICE and VCP. In so doing, we have also extended prior structural and functional observations on SPICE and VCP.

We found that, despite its truncation, MOPICE retains C3b/C4b binding and cofactor activities intermediate between those of SPICE and VCP but lacked DAA. Additionally, neither SPICE, VCP, nor MOPICE possessed DAA for the alternative pathway convertases. Unexpectedly, homodimeric forms of MOPICE, VCP, and SPICE were identified that had enhanced functional activity and whose formation was dependent upon an “extra” cysteine in each of the proteins.

Materials and Methods

Molecular engineering, expression, and purification of recombinant poxviral proteins

MOPICE and VCP genes were PCR amplified using genomic DNA from monkeypox central African isolate MPXV-ZAI-V70-I-K23-Zaire (for MOPICE) and vaccinia Western Reserve (for VCP). The 5’ primers were the same (5’-ATGAAAGCTGGAGGGCAGTACGTCGCAATGGTGG-3’). For amplification of the 3’ terminus, MOPICE primer was 5’-TTAGCGTACACATTTTGGAAGTTCCGGC-3’ and VCP was 5’-TTAGCGTACACATTTTGGAAGTTCCGGC-3’. These were cloned into the pCR-Blunt vector per the manufacturer’s directions (Invitrogen Life Technologies). SPICE was constructed from VCP by mutation of the 11 different amino acids (see Fig. 1) using nine primers (Integrated DNA Technologies) and the QuiikChange Multi Site-Directed Mutagenesis Kit (Stratagene). The following summary enumerates the amino acids and respective nucleotides that were changed: Q77H and A288T; H98Y and A698C; and K214T and A763C.

The fidelity of all clones was verified by DNA sequencing. The CDAs were further subcloned into the EcoRI site of plasmid pSG5 (Stratagene). A construct also was prepared in pSG5 that added a cleavable (enterokinase: Asp-Asp-Asp-Asp-Lys) 6× histidine tag using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) with the following two complementary oligonucleotides, each underlining the area of recognition: 5’-CGAAAACTTCTAGGCCGTCGATGATGATGATGCTTATCGTCATCGTCAGCCGCCTAAGTTATCCGTAGGATCATTAGGTATGGATGATTATCTCATCGTCAGCCGCTAAGATTITTCGCG3’. DNA clones in pSG5, with and without the enterokinase/6×histidine tag, were expressed transiently in Chinese hamster ovary (CHO) cells using Fugene-6 (Roche Molecular Biochemicals) as described previously (14). Concentrated supernatants from proteins bearing the 6× histidine tags were purified on ProBond Resin (Invitrogen Life Technologies) per the manufacturer’s directions.

Alanine substitutions were produced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) per the manufacturer’s directions using the parental cDNA in pSG5.

All three poxviral proteins were also expressed in Escherichia coli and Pichia expression systems. The Pichia pastoris system (Invitrogen Life Technologies) was used for expression by cloning into the yeast expression vector pPICZα per the manufacturer’s directions. For protein purification, culture supernatants were purified over a cation exchange column and eluted on a 0 to 1 M NaCl gradient in 25 mM acetate (pH 4.5). Eluates were concentrated and dialyzed vs PBS. The amino-terminal portion of each protein was confirmed by sequencing. All three sequences were preceded by Glu-Ala-Glu-Ala derived from the signal peptide of P. pastoris α-factor.

For E. coli expression, cDNA was cloned into BamH1 and NoI sites in the expression vector pET28a-1. This vector was modified in our laboratory as a derivative of pET28α+ (Novagen) by deleting the thrombin cleavage site and the T7 tag. Clones were transformed into the E. coli strain BL21-CodonPlus-RIL (Stratagene) for expression. Purification of the recombinant proteins was performed following modification of standard procedures. Briefly, frozen pellets were resuspended in 10 ml of 20 mM Tris (pH 8.0), 25% sucrose, 1 mM EDTA, 0.01% sodium azide, and 10 mM DTT. Lysozyme (160 μl of 50 mg/ml containing 250 U benzozase nuclease (Novagen) and 200 μl of 1 M magnesium chloride) was added and mixed with an equal volume of lysis buffer (50 mM Tris (pH 8.0), 1% Triton X-100, 0.1 M NaCl, 0.01% sodium azide, and 10 mM DTT) with room temperature (RT) rotation for 1 h. This was sonicated three times with 15-s pulses.

FIGURE 1. A comparison of the complement regulatory proteins of monkeypox (MOPICE), variola (SPICE), and vaccinia (VCP). Each line represents a single CCP module. The arrows indicate the amino acids that were changed in VCP to create SPICE. Residues or dashes highlighted in blue represent those changed (or deleted) in comparison to SPICE. A single base pair deletion in MOPICE causes a frameshift, leading to an early termination 13 aa into CCP4. The asterisks (*) indicate possible heparin binding sites (the ± shows presence of the site only in VCP). In CCP4, SPICE alters (and MOPICE lacks) a consensus residue for a heparin binding site. Cysteines are highlighted in red. Typically, in human CCP repeats, Cys 1 pairs with Cys 3 and Cys 2 pairs with Cys 4. SPICE and VCP have an unpaired cysteine at the amino terminus, whereas MOPICE has an unpaired cysteine in the CCP4 remnant.
pulses followed by centrifugation at 6000 × g for 30 min. The final pellet, consisting of inclusion bodies, was resuspended in 20 mM Tris (pH 8.0) and 0.01% NaN3 and stored at 4°C. Inclusion bodies were solubilized in 6 M guanidine HCl, 10 mM Tris (pH 8.0), and 20 mM 2-ME, and purified on an AKTA purifier (GE Healthcare) using a 1-ml HisTrap HP column (GE Healthcare) per the manufacturer’s directions. Next, the purified proteins were added dropwise into refolding buffer (100 mM Tris (pH 8.6), 400 mM L-arginine, 2 mM EDTA, 0.02 M ethanolamine, 0.5 mM oxidized glutathione, and 50 mM reduced glutathione) in three increments over a period of 24 h and then stirred overnight at 4°C. Samples were concentrated and dialyzed against PBS. The proteins were assessed by SDS-PAGE, mass spectrometry, and circular dichroism.

Quantification and Western blotting

The quantity of poxviral proteins produced by CHO cells was determined by ELISA. Briefly, the capture Ab 5A10 (15) was coated at 5 µg/ml overnight at 4°C and then blocked for 1 h at 37°C (1% BSA and 0.1% Tween 20 in PBS). Dilutions of concentrated samples and recombinant VCP (rVCP) (as a standard) were incubated for 1 h at 37°C and then washed with PBS containing 0.05% Tween 20. Next, rabbit anti-VCP antiserum (as described above). After washing, HRP-coupled donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories) was added and incubated for 1 h at 37°C. After washing, TMB substrate (Pierce Biotechnology) was added, and absorbance (650 nm) was assessed in an ELISA reader. For Western blots, the supernatants or purified proteins were electrophoresed in nonreduced and reduced conditions (as described above).

Vaccinia virus infection of BSC-1 cells

BSC-1 cells (African green monkey epithelial cells) grown in DMEM with 10% FCS were infected with vaccinia virus at a multiplicity of infection (m.o.i.) of 5 or 20 (or mock infected) for 12 or 24 h. Supernatants were collected and cells were solubilized in 1% Nonidet P-40/PBS followed by analysis on 12% SDS-PAGE and Western blotting using rabbit anti-VCP antiserum.

Cloning and expression of PIECES

The cDNAs for the complement inhibitors of monkeypox and vaccinia were obtained by PCR from genomic DNA. SPICE was produced from VCP by substitution mutagenesis (see Fig. 1). Follow-
that disulfide bonding was responsible for the putative dimer (Fig. 2B). Also, the three proteins migrated closer to the calculated m.w. under reducing conditions (Fig. 2B). The samples in Fig. 2 were derived from mammalian cells, but we have also observed dimers, although to a lesser extent, when the proteins were produced in *P. pastoris* and *E. coli* expression systems (data not shown).

**MOPICE binds human C3b and C4b**

We characterized binding of MOPICE to human C3b and C4b in comparison to SPICE and VCP (Fig. 3 and Table I). MOPICE bound human C3b ~6-fold more efficiently than VCP (Table I). However, SPICE bound C3b 15-fold more efficiently than MOPICE (Table I). In the case of C4b, MOPICE binding activity was more equivalent to VCP, whereas SPICE was 4-fold more efficient than either. These comparative binding data relative to SPICE and VCP are consistent with those noted previously (15, 22). Additionally, rVCP expressed in the Pichia system showed a similar profile (data not shown). From these data we conclude that, despite deletion of most of CCP4, MOPICE retains the ability to bind to human C3b and C4b.

**CA of MOPICE**

CA was performed using biotinylated ligands (human C3b or C4b), and cleavage products were identified via Western blotting (Fig. 4). Human complement regulator, MCP, was used as a positive control (14). As reported previously, SPICE cleaved C3b to iC3b2, whereas VCP cleaved C3b predominantly to iC3b1 (22, 23). Similar to SPICE (Fig. 4, A–D, and F), MOPICE also cleaved C3b

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**Table I. Summary of C3b and C4b binding by MOPICE**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Concentration (nM) for 50% Binding to C3b&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Concentration (nM) for 50% Binding to C4b&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Ratio C3b/C4b</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP</td>
<td>0.009 ± 0.003</td>
<td>0.011 ± 0.003</td>
<td>0.8</td>
</tr>
<tr>
<td>SPICE</td>
<td>0.27 ± 0.03</td>
<td>0.20 ± 0.04</td>
<td>1.3</td>
</tr>
<tr>
<td>MOPICE</td>
<td>4.0 ± 1.4</td>
<td>0.85 ± 0.14</td>
<td>4.7</td>
</tr>
<tr>
<td>VCP</td>
<td>24.0 ± 4.2</td>
<td>0.75 ± 0.13</td>
<td>32.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SEM for 3–5 experiments.

<sup>b</sup> 50% binding point was calculated from A<sub>630</sub>.

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**FIGURE 3.** A comparison of C3b (A) and C4b (B) binding by MOPICE, SPICE, and VCP. For these ELISAs, supernatants from transiently transfected CHO cells containing the poxviral inhibitors (described in Fig. 2) or a solubilized recombinant MCP were incubated on ligand-coated microtiter wells. Representative experiment of three is shown (see Table I).

**FIGURE 4.** A comparison of C3b CA by MOPICE, SPICE, and VCP. Cofactor assays were performed with biotinylated human C3b and human factor I with supernatants from transiently transfected CHO cells containing the recombinant poxviral inhibitors (described in Fig. 2). Western blots of reduced samples were developed with streptavidin-HRP and chemiluminescence substrate. A, Diagram of C3 cleavage showing expected fragments. B–E, Degradation of human C3b by MCP, SPICE, MOPICE, and VCP, respectively. F, An expanded view of the cleavage pattern for the α2 peptide. MOPICE, SPICE, and MCP predominantly produce iC3b₂, whereas VCP primarily produces iC3b₁.
to iC3b2; however, its efficiency was more similar to VCP, requiring ~500-fold higher concentration compared with SPICE (Fig. 4, C–E). Dose-response curves (data not shown) for C3b CA showed 50% cleavage of the α-chain of C3b for SPICE, MOPICE, and VCP at 18.8 ± 0.6, 10,150 ± 353, and 22,515 ± 948 pM, respectively (mean ± SEM for three experiments).

SPICE was also a more efficient cofactor for C4b than MOPICE and VCP (Fig. 5). Dose-response curves (data not shown) for C4b CA demonstrated 50% cleavage of the α-chain for SPICE, VCP, and MOPICE at 19.5 ± 2, 17,400 ± 2,500, and 17,182 ± 1,560 pM, respectively (mean ± SEM for three experiments). Thus, equivalent cleavage of C4b by MOPICE and VCP required ~900-fold higher amounts than SPICE.

These results indicate that CCP4 is not required for CA and that SPICE is the most efficient poxviral cofactor against human complement followed by MOPICE and then VCP.

MOPICE has no DAA against human convertases

DAA was determined using Ab-coated sheep erythrocytes on which membrane-bound complement convertases had been formed (Fig. 6 and Table II). MOPICE had no detectable DAA for either the classical pathway or alternative pathway C3 convertase. In contrast, SPICE and VCP decayed the C3 and C5 convertases of the classical pathway, with SPICE being 5- to 10-fold more efficient than VCP (Table II). Native regulators of classical pathway convertase, sCR1 and C4BP, were more potent. sCR1 was >500-fold more efficient than SPICE relative to the C5 convertase (Table II), probably reflecting the multiple binding sites that cooperate in regulating the classical pathway C5 convertase (17).

We did not detect DAA for the alternative pathway C3 and C5 convertases by the PICES in three separate assay systems, ELISA, surface plasmon resonance or rabbit erythrocytes (data not shown). Two other reports found limited activity by VCP, but only if sheep erythrocytes were used (24, 25). Taken together, these results demonstrate that the PICES primarily target DAA to the classical pathway.

MOPICE, VCP, and SPICE bind similarly to heparin

We next sought to establish the heparin-binding profile of MOPICE in comparison to VCP and SPICE. Previous studies have established that VCP binds heparin (26) and suggested that SPICE binds similarly (8). We performed heparin-affinity chromatography on recombinant PICE proteins produced in E. coli (Fig. 7) and compared that to human factor H. Samples were eluted with an increasing salt gradient from zero to 2000 mM and absorbance at 280 mAU was monitored. MOPICE, VCP, and SPICE eluted at 554 mM, 568 mM, and 592 mM, respectively. Equivalent results were obtained for the same proteins produced in Pichia (data not shown). The VCP data are consistent with that found previously (26). Interestingly, all three bound more tightly than human factor H, which eluted at 412 mM.
Mechanism for formation and function of VCP dimers

As noted above (Fig. 2), 5–30% of expressed recombinant PICES migrated on nonreducing, SDS-PAGE with a mobility consistent with a dimer, especially when expressed in mammalian systems. To address the mechanism of dimer formation, we mutated the extra cysteines; that is, the early termination of MOPICE in CCP4 leaves an unpaired cysteine, whereas both SPICE and VCP contain two cysteines at their amino terminus (Fig. 1). We substituted an alanine in MOPICE in the remnant of CCP4 (i.e., C185A) and separately in the cysteines of VCP and SPICE (i.e., C1A and C2A of CCP1, respectively) (Fig. 8). In all cases, alanine substitution abrogated dimer formation (Fig. 8), indicating that these cysteines are responsible for dimer formation.

To determine whether dimer formation is generated during an in vitro viral infection, we treated mammalian cells with vaccinia virus and performed a Western blot to assess VCP expression in supernatants and solubilized cell pellets (Fig. 9). Cells were infected with either 5 or 20 m.o.i. for 12 or 24 h. In these supernatants and solubilized cell preparations, ~10% of VCP was identified as a dimer (Fig. 9). In cell pellets, several VCP-containing higher m.w. species (>100 kDa) were evident whose signals increased with both m.o.i. and time (Fig. 9B, lanes 3, 4, 6, and 7). This suggests either formation of VCP multimers or interactions with other proteins (26, 27).

To assess functional activity of monomers vs dimers, we chromatographed supernatants of rVCP with a 6× histidine tag expressed by CHO cells over C3b- and C4b-Sepharose columns, eluted bound material with an increasing salt gradient, and then evaluated fractions in Western blot using a rabbit polyclonal Ab to VCP. As demonstrated in Fig. 10, A and B, dimeric VCP eluted maximally under higher salt conditions than monomeric VCP (150 vs ~280 mM) for both C3b and C4b columns, indicating that dimers have a higher affinity than monomers for C3b and C4b.

We next purified larger amounts of monomer and dimer from CHO supernatants of rVCP with a 6× histidine tag using a HisTrap HP column. Dimers eluted at twice the imidazole concentration (325 mM) compared with monomers (162 mM) (data not shown). Pooled monomer and dimer fractions were dialyzed against PBS and shown to be at least 90% pure via Western blot using anti-VCP (Fig. 10C). We next assessed DAA for the classical pathway C3 and C5 convertases using the VCP monomer vs the dimer fractions (Fig. 10, D and E). Dimers inhibited lysis 15- and 19-fold more efficiently than monomers against the C3 and C5 convertases, respectively. We also performed C3b and C4b cofactor assays (data not shown), and dimers had twice the activity of monomers. Thus, dimer formation further enhances complement regulatory activity, especially DAA, and likely virulence of these factors.

Discussion

MPXV produces an illness in humans and other primates similar to smallpox in clinical manifestations and mortality rates (2, 3, 28). The disease is endemic to the rainforest regions of Western and Central Africa, where wild rodents are the likely reservoir. Some consider human monkeypox to be the most important poxviral infection now that smallpox has been eradicated (1, 2, 29). This proposition is supported by the recent epidemic in the United States (2003) that was the first occurrence of monkeypox in the Western Hemisphere. Patients experienced similar signs and symptoms to those afflicted in Africa, namely a prodrome of fever, headache, and sweats, before appearance of skin lesions and lymphadenopathy (1, 2). African cases are associated with hunting, skinning, and ingesting infected rodents and monkeys (3). The United States illness occurred following close contact with infected pets. To date, animal quarantine and prohibition of shipment of exotic pets into the United States has contained the infection. However, because the outbreak spread to seven states before containment, it is possible that it could have been transmitted to the North American rodent population. Monkeypox

Table II. Classical pathway DAA of PICES and human inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>50% Inhibition of C3 Convertase&lt;sup&gt;a&lt;/sup&gt; (fmol)</th>
<th>50% Inhibition of C5 Convertase&lt;sup&gt;a&lt;/sup&gt; (fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPICE</td>
<td>425 ± 48</td>
<td>486 ± 13</td>
</tr>
<tr>
<td>VCP</td>
<td>4125 ± 426</td>
<td>2033 ± 260</td>
</tr>
<tr>
<td>MOPICE&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Nd</td>
<td>Nd</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sCR1</td>
<td>25 ± 5.0</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>C4BP</td>
<td>30 ± 9.0</td>
<td>44 ± 6.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± SEM for three to five experiments.

<sup>b</sup> Nd, Not detectable; up to 30,000 fmol.

Figure 6. SPICE and VCP but not MOPICE possess C3 (A) and C5 (B) convertase DAA for the classical pathway. Poxviral inhibitors (described in Fig. 2) or purified human complement regulatory proteins (sCR1, C4BP) were incubated for 10 min with prepared sheep erythrocytes. Guinea pig serum was then added for 30 min. Percentage of inhibition was calculated from comparisons to conditions without added inhibitor. Representative experiment of three is shown (see Table II).

FIGURE 7. MOPICE, SPICE, and VCP bind similarly to heparin. Poxviral inhibitors produced recombinantly in E. coli were chromatographed over HiTrap heparin columns. Human factor H and BSA (data not shown) served as positive and negative controls, respectively. Samples were eluted with an increasing salt gradient from 0 to 2000 mM, and A<sub>280</sub> (mAU) was monitored. MOPICE, VCP, and SPICE elution patterns overlapped with peaks at 554 mM, 568 mM, and 592 mM, respectively. Although factor H eluted at 412 mM salt, BSA did not bind (data not shown).
reports that rVCP primarily cleaves C3b to iC3b1 and that SPICE in functional studies. Syfrøera et al. (22) expressed rVCP and recombinant SPICE in mammalian cells, they suggested that the initial cleavage to iC3b1 is that is, MOPICE cleaves C3b to iC3b2, whereas VCP cleaves of C3b cleavage by MOPICE is identical with SPICE and MCP; the human regulator, MCP. Interestingly, however, the pattern between that of SPICE and VCP. Both MOPICE and VCP are less DAA. Despite truncation of the fourth CCP, MOPICE retains a solubilized construct of CR1 (sCR1) for the comparisons of CA for C3b and C4b (12). We also used human C4BP as well as comparator because it also contains four CCPs and binds and possesses complement regulatory proteins. Specifically, we examined bind- system and to compare this profile to other human and poxviral functional repertoire of MOPICE against the human complement ICE is a virulence factor in monkeypox infections (5). Because of those studies and the evidence that the United States strain originated from West Africa, we proposed that MOP-ICE is present in the more virulent strains from Central Africa and absent in the less virulent West Africa strains (5). A Central African isolate produced severe mor- bidity and uniform mortality at high dose, whereas the West Afri- can strain caused no deaths and little morbidity. Previously, we found that MOPICE could bind and cleave human C3b and C4b (5). Because of those studies and the evidence that the United States strain originated from West Africa, we proposed that MOP- ICE is a virulence factor in monkeypox infections (5).

Thus, one goal of this report was to further characterize the functional repertoire of MOPICE against the human complement system and to compare this profile to other human and poxviral complement regulatory proteins. Specifically, we examined bind- ing to human C3b and C4b and contrasted that to the human regu- lator, MCP (CD46). MCP was chosen as the primary human com- parator because it also contains four CCPs and binds and possesses CA for C3b and C4b (12). We also used human C4BP as well as a solubilized construct of CR1 (sCR1) for the comparisons of DAA. Despite truncation of the fourth CCP, MOPICE retains ligand binding and CA, which is intermediate in potency between that of SPICE and VCP. Both MOPICE and VCP are less efficient for C3b and C4b cleavage compared with SPICE and the human regulator, MCP. Interestingly, however, the pattern of C3b cleavage by MOPICE is identical with SPICE and MCP; that is, MOPICE cleaves C3b to iC3b2, whereas VCP cleaves C3b primarily to iC3b1.

Sahu et al. (23) expressed rVCP in yeast and performed functional studies. Syfrøera et al. (22) expressed rVCP and recombinant SPICE in E. coli. We have confirmed data from these two reports that rVCP primarily cleaves C3b to iC3b1 and that SPICE cleaves C3b to iC3b2 (22). Because Sahu et al. (23) determined that iC3b2 was unable to form a fluid-phase alternative pathway C3 convertase, they suggested that the initial cleavage to iC3b1 is sufficient to inactivate C3b and that the second cleavage to iC3b2 is not required. MOPICE and SPICE, however, are similar to human regulators in cleaving C3b to iC3b2, suggesting that there may be an advantage for this type of cleavage reaction.

Unexpectedly, we found no DAA for MOPICE, indicating that CCP4 is likely to be important in this interaction. We also detected classical but not alternative pathway DAA for SPICE and VCP, with the former being 5- to 10-fold more efficient. DAA of SPICE has not been reported previously; however, a product produced in baculovirus did not have alternative pathway DAA (D. Hourcade and A. M. Rosengard, unpublished results). That VCP-inhibited formation and accelerated decay of the classical C3 convertase was noted previously (24, 31). It has been suggested that VCP also is a weak decay accelerator of the alternative pathway (24, 25) and that CCPs 2–4 mediate this activity (25). Both studies used sheep erythrocytes for forming C3bBb because rabbit erythrocytes failed to detect any activity. Mullick et al. (25) found that decay of 50% activity required 2400-fold higher concentration (64 vs 0.027 μM) than that required to similarly inhibit the classical pathway C3 convertases. Additionally, factor H was >10,000 times more effective than VCP. Our studies did not detect alternative pathway-mediated DAA using three separate systems: an ELISA; surface plasmon resonance (building of C3bBb on the chip); and a hemo- lytic assay using rabbit erythrocytes. Although the PICES may have very weak DAA for the alternative pathway, our data further establish that PICES primarily target the classical rather than the alternative pathway convertases. This seems somewhat counterintuitive, but may indicate a more important and underappreciated role for the classical pathway (possibly through natural Abs or the lectin pathway) in host defense against these viruses.

We investigated heparin-binding capability of the PICES. This activity may be an important functional asset for the PICES. For example, the ability of VCP to bind heparin may facilitate cellular attachment, production of anti-inflammatory reactions, and interference with receptors for leukocyte chemo- taxis (26). Binding by VCP to heparin or other glycosaminoglycans may play a critical role in the infection cycle by en- abling prolonged tissue retention, modifying host chemokine response, and subverting the host immune response (27, 32). Our studies establish that SPICE, MOPICE, and VCP bind similarly to heparin. Interestingly, all three bind better than human factor H. The retention of this capability among the three PICE proteins suggests that it may be a common mechanism to assist virulence. Four consensus sites for heparin binding on VCP have been suggested, although overall positive charges may

**FIGURE 8.** Dimer formation is abrogated by substitution of an alanine for the extra cysteine in VCP, SPICE, and MOPICE. Substitution mutagenesis was performed in which alanine was substituted individually for the cysteine in both positions of VCP and SPICE (i.e., C1A or C2A) in CCP1 and in the remnant of MOPICE in CCP4 (C185A). The recombinant proteins were expressed transiently in CHO cells, and supernatants were analyzed on 12% nonreducing SDS-PAGE followed by Western blotting (see Fig. 2).

**FIGURE 9.** Mammalian cells infected with vaccinia virus (VV) express a dimer as well as a monomer of VCP. BSC-1 cells were infected with a m.o.i of 5 or 20 for 12 or 24 h. Supernatants (A) and solubilized cell pellets (B) were analyzed on 12% SDS-PAGE (nonreducing) followed by Western blotting (see Fig. 2). rVCP produced in *Pichia* was electrophoresed in the first lane. Arrows identify dimers. The minor band with the fastest r in the supernatants may represent a degradation product of VCP (A, lanes 3, 4, 6, and 7).
contribute to this activity (See Fig. 1) (26, 32). MOPICE and SPICE do not possess site 4 (13). Also, the highly virulent strain of variola (VAR-IND) mutationally alters site 1 (13). Furthermore, a rVCP that included site 3 and CCPs 2 + 3 did not bind heparin (26). Thus, we propose that that the second site may dominate heparin-binding capability, although other positive charges may influence this reaction as well (see Fig. 1). We will investigate this possibility.

An unanticipated finding in our present study was that mammalian expression of MOPICE, SPICE, and VCP produced disulfide-linked dimers. Dimers were also noted to a lesser extent when the proteins were expressed in *Pichia* and *E. coli*. Additionally, we observed dimers in the supernatants and lysates of vaccinia-infected mammalian epithelial cells. PICES are structurally poised to form dimers. MOPICE has an unpaired cysteine in the remnant of CCP4, whereas SPICE and VCP possess an extra cysteine at their amino terminus. Dimer formation was abrogated by substitution of an alanine for the cysteine in the remnant of CCP4 of MOPICE and at the N-terminal cysteines of SPICE and VCP (in either the first or the second position). This provides further evidence that an extra cysteine mediates dimerization.

Previous reports have not noted dimers in VCP or SPICE. rVCP expressed in *P. pastoris*, purified via heparin chromatography and evaluated by gel filtration, produced primarily a monomer (33). In two other reports using the yeast expression system, the protein profile presented was only under reduced conditions (23) or a small amount was visible on nonreducing SDS-PAGE (26). Although VCP-His and SPICE-His produced in baculovirus showed one band on a nonreducing SDS-PAGE (15), the overall signal intensity was light. Thus, expression of the PICES in mammalian cells may more efficiently produce dimers.

Dimer formation has been reported for B5R, an extracellular enveloped viral protein common to multiple orthopoxviruses (34 - 37). B5R produced recombinantly in baculovirus or via vaccinia virus-infected cells produced disulfide-bonded homodimers. Interestingly, the ectodomain of B5R consists primarily of four CCPs. Although the protein does not participate in complement regulation, it is important for induction of actin bundles that assist viral egress (reviewed in Ref. 34). The role of and mechanisms for promoting such dimers have not been investigated.

Our studies provide evidence that dimers enhance functional activity. Dimers show increased affinity for C3b and C4b because they eluted under higher salt conditions than monomers. Additionally, dimers enhanced by 15- and 19-fold DAA for human C3 and C5 convertases of the classical pathway, respectively. We speculate that dimeric forms of poxviral regulators of complement could provide a significant advantage, especially on the surface of infected host cells. In this setting, clusters of C3b and C4b would...
need to be quickly dismantled or degraded to avert destruction by host complement. Also, the ability to bind back to the cell surface via heparin, C3b/C4b, or other mechanisms, would further set the stage for such protection.

Host complement regulators also benefit from dimeric interactions. Human cell surface regulators CD46 and CD55 are synergistic in their complement regulatory activity, suggesting that a physical association between regulators could lead to a functional advantage (38). CR1 (CD35) has multiple sites for C3b/C4b interactions and also clusters on RBCs. Both of these structural adaptations facilitate immune adherence and control of complement activity (39). Also, to efficiently dissociate the C5 convertase, two active sites of CR1 are required (39, 40). In primates, complement regulatory proteins have been shown to dimerize. For example, on human erythrocytes ~5% of DAF is expressed as a dimer (DAF-2) (41). In this case, it is covalently linked but is not monomeric under denaturing conditions. On orangutan RBCs, the majority of DAF is expressed as a nondisulfide covalently linker dimer (42). Taken together, these studies demonstrate that the formation of dimers provides an enhanced ability to regulate human complement.

An intriguing possibility is that dimerization may vary among infected cell types and perhaps may be higher in primary cells. Both of the mammalian cell lines (BSC-1 and CHO) are of epithelial lineage and both produced dimers. We suggest that engineering of this additional unpaired cysteine by poxviruses is a strategy designed to more efficiently subvert the complement system.

In summary, MOPICE possesses C3b and C4b binding and CA but lacks DAA. In the case of SPICE and VCP, our studies detected DAA only for the classical pathway. All three poxviral complement regulators bind similarly to heparin. Also, we provide evidence for dimerization of poxviral complement regulators and demonstrate for VCP that this structural modification increases functional activity. Thus, our studies provide novel information relative to the complement regulatory activities of these virulence factors. Importantly, these results suggest activities to target for neutralization. For example, production of humanized mAbs that specifically target (and abrogate) ligand binding and cofactor activities as well as classical pathway DAA is a therapeutic strategy that could reduce the virulence of poxviral infections. The newly developed animal model for smallpox infection may provide an in vivo system to test such an approach (43).

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Disclosures

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References


