Minireview

Viral Complement Regulators: The Expert Mimicking Swindlers

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The complement system is a principal bastion of innate immunity designed to combat a myriad of existing as well as newly emerging pathogens. Since viruses are obligatory intracellular parasites, they are continuously exposed to host complement assault and, therefore, have imbibed various strategies to subvert it. One of them is molecular mimicry of the host complement regulators. Large DNA viruses such as pox and herpesviruses encode proteins that are structurally and functionally similar to human regulators of complement activation (RCA), a family of proteins that regulate complement. In this review, we have presented the structural and functional aspects of virally encoded RCA homologs (vRCA), in particular two highly studied vRCAs, vaccinia virus complement control protein (VCP) and Kaposi’s sarcoma-associated herpesvirus complement regulator (Kaposica). Importance of these evasion molecules in viral pathogenesis and their role beyond complement regulation are also discussed.

Keywords: Viral complement evasion, Viral molecular mimicry, RCA, Complement, immune evasion, KSHV, HHV-8, Vaccinia virus, Variola virus, Smallpox, HVS, RRV, Kaposica, VCP, HVS-CCPH

1. Introduction

The innate immune system forms the initial barriers of immune defense to foreign invaders and comes into play prior to the adaptive immune assault.

These include anatomical, physiological, inflammatory and phagocytic barriers. The complement system is an integral constituent of the inflammatory barriers of innate immunity and acts as a major host defense against infections. To date, more than 30 complement proteins have been identified that participate in activation and control of three major activation pathways — the classical, alternative and lectin pathways (Fig. 1), which ensure the recognition of existing as well as newly evolving pathogens.

According to a prevailing model, innate immunity utilizes three distinct immune recognition mechanisms to discriminate self from microbial non-self and abnormal self: a) recognition of microbial non-self by pattern-recognition molecules, b) recognition of missing self, and c) recognition of induced or altered self. Consistent with this model, the complement system is known to recognize non-self and altered-self targets using all the three strategies. In classical pathway (CP), antibodies (natural and antigen-induced), C-reactive protein (CRP), specific intracellular adhesion molecule-grabbing nonintegrin receptor 1 (SIGN-R1) and C1q recognize the microbial non-self targets, while in lectin pathway (LP), mannose binding lectin (MBL) and ficolins (L, M and H) participate in the recognition process. Thus, pattern-recognition mole-

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Abbreviations: AP, alternative pathway; CCP, complement control protein; CCPH, complement control protein homolog; mCCPH and sCCPH, transmembrane and soluble forms of CCPH; CP, classical pathway; C3, complement component 3; C3b, proteolytically cleaved form of C3; iC3b2, C3b cleaved by factor I between Arg1281-Ser1282 and Arg1298-Ser1299; C3c, 137 kDa fragment generated after cleavage of C3b by CR1 and factor I; C4b, proteolytically cleaved form of C4; C3dg, 25 kDa fragment generated after cleavage of C3b by CR1 and factor I; DAF, decay-accelerating factor; CFA, cofactor activity; CR1, complement receptor 1; MCP, membrane cofactor protein; DAA, decay-accelerating activity; γ-HIV68, murine γ-herpesvirus 68; γ-HIV68 RCA, murine γ-herpesvirus 68 regulator of complement activation; HVS CCPH, herpesvirus saimiri complement control protein homolog; Kaposica, Kaposi’s sarcoma-associated herpesvirus inhibitor of complement activation; KSHV, Kaposi’s sarcoma-associated herpesvirus; MBL, mannose-binding lectin; MOPICE, monkeypox inhibitor of complement enzymes; RCA, regulator of complement activation; RCP-H and RCP-1, rhesus rhadinovirus complement control protein-H and 1 respectively; SPR, surface plasmon resonance; ORF, open-reading frame; RRV, rhesus rhadinovirus; SPICE, smallpox inhibitor of complement enzymes; VCP, vaccinia virus complement control protein.
Fig. 1—Complement activation pathways [The complement system is activated by three different pathways based on the recognition of target molecules. These pathways lead to the formation of a key enzyme C3 convertase, which cleaves the central C3 molecule into C3a and C3b, the latter being deposited on the target surface. Opsonization of viruses with C3b causes virus neutralization. Further activation of pathways generates another enzyme C5 convertase that cleaves C5 component to C5a and C5b. In some viruses, coating up to C5 is necessary for neutralization. Further activation of pathway results in formation of MAC on surface of viruses, directly lysing the virus. Activation of pathways is tightly regulated at C3 and C5 convertase steps by human RCAs (in black letters) and viral RCAs (in red letters). CRP, C1q and lectins play a crucial role in recognition of pathogens in these pathways.

In alternative pathway (AP), however, recognition molecule is not required for initiation and is initiated in the fluid phase by the enzyme C3 convertase C3(H2O),Bb generated as a result of the spontaneous hydrolysis of thioester bond of C3. This enzyme cleaves C3 into C3b, which then attaches to the target cells. Though attachment of C3b shows preference for certain sugars, it deposits equally well on to the microbial surface and autologous cells. Therefore, further activation of the pathway is principally blocked by a family of proteins termed as regulators of complement activation (RCA) (Fig. 1). A fluid-phase complement regulator factor H that recognizes sialic acid on autologous cells and ubiquitously expressed membrane-bound regulatory proteins, such as membrane cofactor protein (MCP; CD46) and decay-accelerating factor (DAF; CD55) effectively control the activation process. Thus, recognition of missing self triggers the activation of the AP. In analogy with the third strategy (i.e., recognition of induced or altered self), complement system also possesses the ability to recognize the altered-self. For example, C1q and MBL bind to apoptotic cells and cellular debris and help their clearance.

Because the complement system has a potential to recognize microbial non-self with and without the help of any recognition molecule, it possesses the ability to target a vast repertoire of pathogens including viruses. Both acute as well as latent viruses are susceptible to complement-mediated neutralization. However, despite in vitro effectiveness of complement, viral infections are prevalent in humans, suggesting that viruses have evolved specific mechanisms to combat complement. These include harboring of genes that encode structural and/or functional homologs of host complement regulators, piracy of host membrane regulatory proteins, and use of complement receptors for cellular entry.

In this review, the structure, regulatory mechanisms and structure-function analysis of viral mimics of host regulators of complement activation (vRCAs) have been discussed. In addition, a section on how vRCAs assist in pathogenesis has also been included.

2. Structure and Molecular Characterization of vRCAs

In humans, indiscriminate complement activation on host cells is primarily regulated by a family of proteins termed as RCA. The members of two families of DNA-viruses — poxviridae and herpesviridae encode structural and functional mimics of RCA proteins (Fig. 2). It is suggested that these viruses have acquired complement regulatory proteins from ancestrally infected host organisms by horizontal gene transfer. Variations in the gene sequence of different viral homologs of RCA suggest that these proteins have been modified after their acquisition.

The human RCA family comprises of both soluble (factor H and C4b-binding protein) and membrane-anchored forms (DAF, MCP and complement...
Structurally, RCA proteins appear like beaded strings due to the presence of repeating modules referred to as “short consensus repeats” or “complement control protein” (CCP) domains, joined together by short linkers. The RCA proteins vary in size due to difference in the number of CCP domains (Table 1) and length of inter-domain linkers; the CCP domains vary from 4-59 and the linker segment ranges from 3-8 amino acids (aa). Each CCP domain is a compactly folded structure constituted by approximately 60 aa with the characteristic presence of highly conserved four cysteines, a tryptophan and few other conserved hydrophobic residues. These four invariant cysteines link up through disulfide bonds; the first cysteine pairs up with the third and the second with the fourth. The invariant tryptophan is buried inside the small hydrophobic core of the domain. The CCP domain adopts a β-barrel fold consisting of eight or less β-strands and the length and position of β-strands vary between the domains. It is also important to mention here that efficient folding of CCP results in exposure of most of the side chains to the solvent, which provides larger surface area in comparison to its molecular weight.

### 2.1 Poxviral Homologs

In the poxvirus family, members of the subfamily *Chordopoxvirinae*, but not the *Entomopoxvirinae* encode complement regulators. Vaccinia virus, a member of the genus *Orthopoxvirus* that causes severe infections only in immunocompromised individuals is the first virus that has been shown to encode a RCA homolog named vaccinia virus complement control protein (VCP). Infact, VCP is also the most studied and the first viral RCA protein whose complete structure has been determined. It is a secretory viral protein encoded by C21L gene of the vaccinia virus genome and its primary structure comprises of 263 aa including a 19 aa long signal peptide. The protein is organized into four CCP domains separated by linkers of four aa residues (Fig. 2, Table 1). Unlike human RCA proteins and herpesviral complement regulators, it lacks glycosylation and membrane attachment sites.

Initial structural analysis of VCP domains in pairs (CCP2-3 and 3-4) by nuclear magnetic resonance has revealed that they fold into 6-β strand structure. Subsequently, the crystal structure has confirmed its 6-β strand topology and shown that it has an extended structure with no contacts between non-consecutive CCP domains. The data also reveal that CCP1-2 and CCP3-4 are more in contact with one another than CCP2-3, and that terminal modules of the VCP (CCP1 and 4) are inundated with positive charge while central modules are negatively charged. Earlier, several mutagenesis studies have shown that the negatively charged residues on C3b and C4b and positively charged residues on RCA proteins are important for their interactions. Thus, it is likely that the positively charged residues on VCP play a significant role in interacting with C3b and C4b. Mutagenesis data are, however, lacking to confirm the above premise.

Another interesting feature of VCP is the presence of heparin/polyanion binding site. It has been proposed that interaction of VCP with heparin and...
Table 1—Description of various forms and activities of human and viral RCAs

<table>
<thead>
<tr>
<th>RCA</th>
<th>Number of CCPs</th>
<th>RCA forms (CFA)</th>
<th>DAA</th>
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<tr>
<td>Human RCA</td>
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<td>C4BP</td>
<td>59</td>
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<td>Viral RCA</td>
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<td>MOPICE</td>
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<td>Kaposica</td>
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<td>HVS CCPH</td>
<td>4</td>
<td>S/M</td>
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<td>γ-HV68 RCA</td>
<td>4</td>
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<td>RCP-H</td>
<td>4</td>
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<td>RCP-1</td>
<td>8</td>
<td>M**</td>
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CFA: C3b, C4b; DAA: CP, AP; Refs: 75, 12, 87, 88, 89, 52, 53, 36, 38, 38, 44, 42, 48, 49, 49.

S, soluble form; M, membrane bound form; ND, not determined; * - weak activity; ** - soluble form could be generated due to proteolytic cleavage as seen in γ-HV68 48.

glycosaminoglycans might i) prolong its residence in host cells, ii) inhibit cellular cytotoxicity and iii) increase its effectiveness against complement35. Although this interaction is likely to increase the first two functions in vivo, our data do not support its increased effectiveness against complement after interaction with heparin34,35 (see section 3.1 below).

Other poxviruses that encode complement control protein homologs belong to genera Orthopoxvirus (variola, monkeypox, cowpox, camelpox and ectromelia), Leporipoxvirus (myxoma and rabbit fibroma), Suipoxvirus (swinepox) and Yatapoxvirus (yaba monkey tumor). Sequence analyses show that these proteins are highly homologous to each other (sequence similarity exceeds 91%). RCA proteins from variola (the causative agent of smallpox) and monkeypox (the causative agent of smallpox-like disease in monkeys and humans) have been studied in detail for their ability to inhibit complement (Fig. 2, Table 1). Like VCP, both variola RCA homolog (named SPICE) and monkeypox RCA homolog (named MOPICE) are secretory proteins. It is interesting to note that SPICE, which is also a four CCP domain structure like VCP, differs from VCP in only 11 aa residues and yet is 100-1000 fold more potent than VCP36-38. On the other hand, MOPICE contains only three CCP domains and fourth CCP module in this protein is truncated as a result of a stop codon generation due to a frameshift mutation in CCP438,39. To date, it is the smallest RCA protein among all the human and viral complement regulators.

2.2 Herperviral Homologs

*Herpesviridae* is another virus family that encodes the complement regulators. In this family, members of subfamily *Alphaherpesvirinae* and *Gammaherpesvirinae* encode for these evasion molecules. The members of *Alphaherpesvirinae* encode for non-RCA homologs (e.g., glycoprotein C of herpes simplex viruses; for review see40,41), while the members of *γ-herpesvirinae* encode for RCA homologs. In this review, we have limited our discussion only to RCA homologs.

Amongst γ-herpesviruses, members that encode RCA homologs include Kaposis’s sarcoma-associated herpesvirus (KSHV/HHV-8), *Herpesvirus saimiri* (HVS), *Herpesvirus aetelae* (HVA), murine γ-herpesvirus 68 (γ-HV68) and rhesus rhadinovirus (RRV). Their sequence comparison shows that unlike poxvirus homologs, they are more diverse in structure (sequence similarity varies from 43-89%). The herperviral homologs differ from poxviral homologs in two aspects: i) they exist as soluble as well as
membrane-bound forms, and ii) they contain glycosylation sites (Fig. 2).

HVS, a lymphotropic virus that causes T-cell lymphomas in New World primates encodes a four CCP containing complement regulator named complement control protein homolog (CCPH). The protein is encoded by the open reading frame (ORF) 4, which is transcribed into two variants existing as a longer unspliced form of 1.7 kb and a shorter single-spliced form of 1.5 kb, wherein splicing removes a 193 bp intron that encodes for a transmembrane domain. The unspliced mRNA codes for membrane-bound glycoprotein (mCCPH), while the spliced mRNA is translated into a secretory protein (sCCPH) with a different carboxyl terminus. The mCCPH comprises of 360 aa polypeptide in which aa 21-266 form the four CCP domains (Fig. 2, Table 1). The protein contains five putative N-glycosylation sites, four located in CCP1 domain and one in the CCP3 domain. Analysis of proteins from infected cells shows that mCCPH and sCCPH are expressed as 65-75 kDa and 45-52 kDa protein, respectively. Apart from the RCA homolog, HVS also encodes for a homolog of the terminal complement inhibitor CD59.

Kaposi’s sarcoma-associated herpesvirus, the causative agent of Kaposi’s sarcoma and most recently discovered human tumor virus also encodes for a four CCP domain containing complement regulator. The protein is named as kaposica (Kaposi’s sarcoma-associated herpesvirus inhibitor of complement activation) by our group and as KCP (Kaposi’s sarcoma-associated herpesvirus complement control protein) by another group. It is referred to as kaposica henceforth in this review. Kaposica is formed by four extracellular CCP domains, followed by a dicysteine motif, a serine/threonine (S/T) rich region and a transmembrane domain (Fig. 2, Table 1). It contains three potential N-linked glycosylation sites located in CCP1, 2 and 4 and several O-linked glycosylation sites in the S/T region. Analysis of post-transcriptional processing of the gene suggests that in addition to unspliced mRNA, two spliced transcripts are also produced. Both of them contain the transmembrane region, however, they either lack the S/T region or the dicysteine motif and the S/T region. The full-length protein comprises of 550 aa, of which residues 23-268 form the four CCP domains. Characterization of proteins from the ORF4 transfected cells shows that kaposica is expressed as 62, 82 and 175 kDa proteins. Analysis of tissue culture supernatants shows the presence of 175 as well as 82 kDa forms. The protein has also been found on the surface of the virions.

Another member of γ-herpesvirinae in which ORF encoding RCA protein is conserved in position is murine γ-herpesvirus 68 that causes pneumonia or meningoencephalitis in mice. The ORF4 of γ-HV68 is predicted to encode for a 388 aa RCA protein in which 245 amino acids constitute the four CCP domains. It is predicted to have several O-glycosylation sites in the S/T region, while just one N-glycosylation site is located in CCP2 (Fig. 2, Table 1). Transcript analysis reveals that the ORF is transcribed as a 5.2 kb bicistronic late mRNA. Western blot analysis of γ-HV68 infected murine fibroblast cells has shown the presence of three isoforms of this protein — 60-65, 50-55 and 40-45 kDa. The smallest isoform is detected in the supernatants of virally infected cells. It is suggested that the soluble form is generated by proteolysis of the membrane form.

Recently, ORF 4 of rhesus rhadinovirus that encodes a complement control protein has been characterized. Experimental infection of RRV in SIV-infected monkeys causes induction of lymphoproliferative disorder similar to that observed in multi-centric Castleman’s disease. The gene has been characterized from H26-95 and 17577 isolates of RRV. H26-95 encodes a four CCP domain containing protein (named RCP-H), while 17577 encodes an eight CCP domain containing protein (named RCP-1). The RCP-H contains four extracellular CCP domains, followed by a S/T region and a transmembrane domain, while RCP-1 contains a repeat of four CCP domain, followed by a S/T region and a transmembrane domain at the C-terminus (Fig. 2, Table 1).

Analysis of the transcripts reveals that H26-95 generates only a full-length unspliced transcript, but 17577 generates full length as well as two spliced transcripts. The second largest transcript encodes for a protein with truncated CCP1, followed by CCP5-8, a S/T region and a transmembrane domain, while the smallest transcript encodes for a truncated first CCP, followed by C-terminal 17 aa. Protein expression analysis in H26-95 infected rhesus macaque fibroblast cells shows the presence of a 70 kDa protein corresponding to full-length protein. Analysis of 17577 infected cells, on the other hand shows the
3. Functional Characteristics of vRCAs

Because viral complement control proteins are mimics of host RCA proteins, they regulate complement essentially similar to RCA proteins with subtle important differences. Human RCA proteins inhibit complement by targeting C3 and C5 convertases. They employ two different mechanisms to regulate these enzymes: i) decay acceleration of classical/lectin and alternative pathway convertases (termed as decay-accelerating activity; DAA), and ii) inactivation of C3b and C4b, the subunits of convertases by acting as factor I cofactors (termed as cofactor activity; CFA) (Fig. 3). These regulatory activities are divided amongst the human regulators and all activities are present in only one complement regulator CR1, a 30 CCP module containing protein. As discussed above, most viral regulators are formed by four CCP modules, but in spite of their small size they possess both DAA as well as CFA (Table 1). In addition to complement regulatory activities, viral RCAs have also been shown to possess other immunomodulatory functions (see below).

3.1 Complement Regulatory Functions of Poxviral Homologs

VCP is the first viral homolog of RCA identified to be functional and is one of the two major proteins secreted by vaccinia virus infected cells. Initial studies using partially purified VCP secreted by vaccinia infected cells show that like RCA proteins it binds to C3b and C4b and accelerates decay of the CP and AP C3 convertases. VCP expressed using the Pichia system shows that it is a poor inhibitor of the AP, in comparison to CP, but possesses potent factor I cofactor activity against C3b and C4b. Its CFA for C4b is similar to that of CR1, but it differs in the C3b CFA from host RCA proteins in that it primarily supports the factor I-mediated cleavage of C3b at site 1 (between Arg1281-Ser1282). This single cleavage however is shown to be sufficient to render C3b non-functional.

Recently, we have further probed into the DAA of VCP. Our comparative data with human complement regulators show that its CP DAA is only two-fold lower in comparison to CR1, indicating that it is a potent CP complement regulator. VCP, however, does not show any DAA against the AP C3 convertase, when the enzyme is formed on rabbit erythrocytes, but shows a very limited activity against the enzyme formed on sheep erythrocytes (IC_{50} = 62 µM). On a molar basis, VCP is ~30,000-fold less active compared to factor H in decaying AP C3 convertase. In a later study, Liszewski et al. failed to detect any decay activity against the AP C3 convertase in the assay systems they used.

Unlike herpesvirus complement regulators, poxvirus inhibitors, including VCP contain an unpaired cysteine at the N- or C-terminus of the molecule. It has been demonstrated using multiple expression systems that 5-30% of VCP forms disulfide-linked head-to-head homodimers, not detected in earlier study. VCP dimers also display 15 and 19-fold higher activity than monomers against the C3 and C5 convertases, respectively, suggesting that poxviruses have designed an ingenious mechanism to enhance the effectiveness of these immune evasion proteins. Another important feature in VCP and other poxviral complement regulators is
the presence of a heparin-binding site\textsuperscript{33,34}. Earlier, it was proposed that like human complement regulator factor H, the presence of heparin site might function to enhance VCP’s complement regulatory activity\textsuperscript{33}. However, our data have demonstrated that unlike factor H, heparin-binding site in VCP does not play a role in enhancing its AP DAA\textsuperscript{64} or inactivation of non-activator (sheep erythrocyte)-bound C3b\textsuperscript{53}.

Although like host complement regulators, VCP interacts with and regulates both C3b and C4b, the binding mechanisms involved are different\textsuperscript{54}. Our study has shown that i) unlike host regulators, interaction of VCP with C3b and C4b follows a simple 1:1 binding mechanism and these complex formations do not involve conformational changes or multiple-site binding, and ii) VCP possesses very fast on- and off-rates for the target proteins. Since transient binding of VCP is enough to inactivate the target proteins, it is likely that this property allows it to inactivate many target proteins in a relatively short time.

Since VCP is implicated in the pathogenesis of vaccinia virus\textsuperscript{58}, attention is also given to the variola virus complement regulator SPICE. Importantly, sequence analysis shows that the complement regulator is conserved in all the strains of variola, including the Indian strain India-1967\textsuperscript{59}. For performing the functional studies, SPICE has been engineered and analyzed for CFA\textsuperscript{36}. It is found to have 100-fold higher C3b CFA and 6-fold higher C4b CFA than VCP. It is, therefore, believed that this difference, in part, is responsible for the greater virulence of variola over vaccinia. The greater CFA of SPICE over VCP is also confirmed by another study\textsuperscript{37}. Further, SPICE has been shown to be 1000 and 75-fold more efficient than VCP in AP and CP hemolytic assays, respectively\textsuperscript{37}. Though these studies have characterized the CFA of SPICE, none have probed into the decay-accelerating activities. A later study\textsuperscript{58} has shown that apart from cofactor activities, SPICE also possesses DAA for CP C3 and C5 convertases, but not for AP C3/C5 convertases.

Monkeypox virus, another member of the Orthopoxvirus genus expresses a three CCP module containing complement regulatory protein MOPICE\textsuperscript{38}. The protein, however, is encoded only by the more virulent strain of Congo basin, but is absent in the less virulent strain of West Africa\textsuperscript{39}. Like other poxviral complement regulators, it binds to C3b and C4b and displays CFA against them, but has affinity intermediate to that of SPICE and VCP\textsuperscript{38}. It, however, does not show any CP DAA observed in other poxviral complement regulators. This is consistent with VCP data on deletion mutants, wherein deletion of CCP4 results in substantial loss of CP DAA activity\textsuperscript{34}.

3.2 Complement Regulatory Functions of Herpesviral Homologs

Among herpesviruses, the complement control protein homolog (CCPH) of Herpesvirus saimiri is the first complement regulator to be identified and functionally examined\textsuperscript{60}. Initial functional characterization of transmembrane form of CCPH (mCCPH) has shown inhibition of complement-mediated lysis of mCCPH expressing cells and reduction in the C3d deposition on these cells when incubated with whole human serum\textsuperscript{60}. Recently, our laboratory elucidated the mechanism of complement inactivation of CCPH by expressing its soluble form (sCCPH)\textsuperscript{61}. We found that it binds to C3b and C4b, displays CFA against them and possesses DAA against CP and a limited activity against AP C3 convertases. A comparison of its CFA with other regulators shows that it is 14-fold more potent than VCP in inactivating C3b, but only about 2.5-fold less potent than factor H and sCR1\textsuperscript{61}.

Apart from HVS CCPH, efforts have also been made to characterize kaposica. Our group and another group concurrently assigned a complement regulatory function to this protein\textsuperscript{44,62}. Kaposica inhibits C3b deposition on the cell surface and inactivates C3 convertases by supporting factor I-mediated decay of C3b and C4b and decaying the CP C3 convertase\textsuperscript{44,62}. However, its AP activity is very poor, about 1000-fold less than factor H. Although data on DAA of kaposica are consistent between both the groups, there was a conflict in CFA data. Our group showed that kaposica supports the inactivation of C3b to iC3b\textsuperscript{2}, while the other group reported that it supports the inactivation of C3b to iC3b\textsuperscript{2} and further to C3c and C3d. Subsequently, they clarified that the further cleavage of iC3b\textsuperscript{2} to C3c and C3d observed in their assay was due to contaminating proteases\textsuperscript{62}.

Binding of kaposica to C3b and C4b has been analyzed by surface plasmon resonance measurements (SPR)\textsuperscript{62, 63}. Coupling of kaposica on to the sensor chip by amine coupling method and measuring binding by flowing C3b or C4b on to the chip\textsuperscript{62} has shown that its affinity for C4b is about 10-fold higher than C3b. This study, however, does not ascertain the binding mechanism. On the other hand, by orienting C3b and
C4b molecules in their physiological orientation on the sensor chip and measuring binding by flowing kaposica, we have shown that like VCP, kaposica also follows 1:1 binding model for interacting with C3b and C4b. Further, its affinity for C4b is found to be 25-fold higher than for C3b. Our binding data also clearly show that like VCP\(^{33,34}\) kaposica also binds to heparin\(^{46}\). Similar results have been subsequently reported in another study\(^{64}\). They have also shown that kaposica binds to CHO cells, but not to heparan sulfate-deficient CHO cells. It may be mentioned here that though heparin-binding capability is conserved in poxviral complement regulators\(^{34,38}\) and kaposica, it is absent in HVS CCPH (Singh et al., unpublished observation).

In addition to HVS CCPH and kaposica, RCA homologs have also been functionally characterized from murine γ-herpesvirus 68 (γ-HV68) and rhesus rhadinovirus. Expression and functional analysis of γ-HV68 RCA protein shows that it effectively inhibits the murine C3 deposition on activating particles via classical as well as alternative pathways. Although in-depth \(\textit{in vivo}\) study has demonstrated the role of this protein in the viral pathogenesis (see section 5), the mechanism by which γ-HV68 RCA regulates the C3 convertases is not clear. Recently, functional characterization of rhesus rhadinovirus complement control protein (RCP) has also been achieved\(^{40}\). Expression of RCP on the CHO cells has been shown to inhibit C3b deposition on these cells, when incubated with human serum\(^{49}\).

3.3 Non-complement Regulatory Functions of vRCAs

Although the viruses display an array of diverse mechanisms to combat a range of host immune responses, they have a restriction on their genome size. Thus, to be successful pathogens, viruses cleverly assign multiple functions to the molecules they encode. The vRCAs, unlike their name do more than just regulating the complement and thus are multi-faceted small molecules. In addition to the complement inhibitory properties, these molecules display various other properties which either abet infection or assist in defense against various host responses.

The VCP and kaposica also perform functions independent of complement regulation. VCP inhibits the binding of xenoantibody to major histocompatibility complex (MHC) class I molecules on endothelial cells\(^{33}\). In addition, it also inhibits binding of xenoantibody to Gal epitope on pig aortic endothelial cells and NK cell-induced killing of xenoendothelial cells\(^{35,65}\), suggesting that it is capable of inhibiting cellular cytotoxicity. Although the exact mechanism for these inhibitions is not known, it is proposed that binding of VCP to cell surface heparin-like molecules or glycosaminoglycans results in non-specific blocking of cell-cell interactions. Through a similar mechanism, VCP has also been postulated to block MIP-1α activity\(^{35}\).

As kaposica interacts with heparin\(^{44}\) and is present on the envelope of KSHV virions\(^{46}\), it has been tested for its role in virion attachment to the cell surface. Evidences suggest that it plays a significant role in enhancing viral attachment to the permissive cells, as blocking of its interaction with soluble kaposica or mAb that binds to heparin-binding site of kaposica has been found to reduce infection\(^{46}\). Attachment of virions through interaction with cell surface heparan sulfate-like (HS) molecules has been demonstrated for many viruses including KSHV. Other KSHV molecules that interact with HS molecules include glycoprotein B and gpK8.1\(^{66,67}\). The attachment of KSHV virions to HS, is believed to help their entry via endocytosis\(^{68}\) or fusion\(^{69}\).

4. Identification of Functional Domains of vRCAs

It is obvious from the discussions in the above sections that vRCAs harbor decay as well as cofactor activities within the conserved four CCP structure, which is unlike the four CCP containing human regulators like MCP and DAF. It seems that viruses have acquired and modulated important residues within their CCPs to fit these functions to their benefit. Thus, the identification of functional domains/determinants is important for the respective functions, as it would help in elucidating the molecular basis of regulatory mechanisms of these viral regulators, as well as provide new insight into the regulation of C3 convertases.

4.1 Functional Domains in Poxviral Homologs

Initial studies on localization of C3b binding domains in VCP\(^{70,71}\) have reported that all the four domains of VCP are essential for binding and deletion of any one of them results in total loss in activity. These data gave the impression that CCP domains in VCP are indispensable. Our results\(^{34}\) described below clearly indicate that the domains are indeed dispensable, but all the four domains are required for full activities.
We expressed a series of VCP deletion mutants using the *Pichia* expression system to study the contribution of each CCP domain to the cofactor and decay activities and binding to C3b, C4b and heparin\textsuperscript{34}. We observed that CCP1-3 of VCP confers C3b and C4b cofactor activities, however, CCP4 is required to retain the full activity. Earlier studies on human complement regulators (MCP, CR1, C4BP and factor H) have also shown the requirement of three CCPs for the cofactor activities\textsuperscript{72-80}. On analyzing the DAA for the classical/lectin pathway C3 convertase, we observed that the DAA function is retained in CCP1-2 and CCP1-3, indicating that CCP1-2 is the minimum domain required for this activity. There is, however, a dramatic reduction in CCP1-2 and CCP1-3 activity in comparison to the full-length protein as these mutants show 5600- and 2500-fold lower activities, respectively. Therefore, for all practical purposes, the four CCPs are essential for CP DAA. VCP possesses a very limited AP DAA activity, which is primarily imparted by CCP2-4.

A study by Smith *et al*\textsuperscript{33} has shown that VCP binds to heparin and that CCP1 and 4 are the domains responsible for this binding. Heparin binding is mediated by interactions between the negatively charged heparin sulfate groups and positively charged residues on the proteins, hence the above results\textsuperscript{33} also fit well with the VCP crystal structure data which identified CCP1 and 4 to be surrounded by positive field\textsuperscript{25}. Later, it has been shown that heparin binding is confined to CCP4\textsuperscript{81}. In our study on the heparin binding ability of VCP and its deletion mutants, binding has been observed only in mutants that contained CCP1\textsuperscript{34}. Thus, our data is in conflict with the earlier study\textsuperscript{81}, but falls in line with the binding of MOPICE to heparin, which lacks CCP4\textsuperscript{38}.

SPICE is the only other member of poxvirus family that has been studied with respect to identification of domains/residues important for functional activity. Based on the mutagenesis data of MCP and CR1 and crystal structure of VCP, we predicted that Y98 and Y103 are probably important for its increased C4b cofactor activity, while K120 is responsible for its increased C3b CFA\textsuperscript{3}. In another study, using electrostatic modeling approach, K108 and K120 are predicted to be important for increased C3b CFA of SPICE\textsuperscript{37}; the prediction has been confirmed by performing gain-in-function mutations in VCP. Importantly, this study has shown that the overall positive electrostatic potential of VCP correlates well with the binding and activity, suggesting that activities of vRCAs can be predicted using molecular dynamics and electrostatics\textsuperscript{82}.

4.2 Functional Domains in Herpesviral Homologs

Like in VCP, we also made efforts to dissect the functional domains in kaposica using various deletion mutants\textsuperscript{63}. Interestingly, we observed that CCP2-3 confers cofactor activity towards both C3b and C4b\textsuperscript{63}. This property of kaposica is unlike any other known complement regulator as mapping analyses of the human RCA proteins (MCP, C4BP, CR1 and factor H) and VCP suggest the requirement of a minimum of three CCPs for both C3b and C4b CFAs. Characterization of CP DAA of kaposica indicates that the activity resides within CCP1-2, but CCP3 is required for the optimal activity. Interestingly, the activity of CCP1-3 is similar to the full-length protein. This finding on kaposica is different from that of VCP (CCP1-3 of VCP shows negligible activity), but is consistent with human RCA proteins\textsuperscript{83}. Kaposica is a weak regulator of AP C3 convertase and is 1000-fold less active than human factor H and DAF\textsuperscript{62,63}. Further examination of AP DAA of the various mutants demonstrates that all the domains are essential for the activity. The binding of the mutants to C3b and C4b by SPR analysis\textsuperscript{63} shows that a minimum of two N-terminal CCPs are necessary for binding to C4b, while all CCPs are needed for binding to C3b. Heparin binding site has been localized in CCP1\textsuperscript{63}.

A site-directed mutagenesis study based on homology modeling has identified several surface residues important for DAA and CFA in kaposica\textsuperscript{84}. The results suggest that a positively charged and a negatively charged cluster, as well as few other residues located in CCP1-3 are required for C4b binding, CFA and CP DAA. Additional residues in CCP4 also contribute to binding and degradation of C3b. Heparin-binding site is identified as a cluster of positively charged residues located in CCP1 stretching into CCP2. The same group also looked into the importance of individual domains by constructing deletion mutants, as well as domain-shuffling mutants with complement receptor 2\textsuperscript{46}. Their results demonstrate that CCP1-3 displays CP DAA similar to the full length protein, which is essentially similar to our data\textsuperscript{83}. Further, they claim that CCP1-3 and 1-4 are the minimum regions required for the CFA against C4b and C3b, respectively. This is in contrast to our data\textsuperscript{63} which show CCP2-3 as the minimum region required for
these activities. It is likely that the lower sensitivity of the cofactor assays utilized by them is responsible for this discrepancy. From the above studies, it seems that though two CCPs are enough to impart CFA as well as DAA activities, additional residues of kaposica residing in CCP1 and CCP4 also participate in these activities.

In our study, HVS CCPH has been found to be 14-fold more potent than VCP in inactivating C3b. We, therefore, made an attempt to characterize the determinant responsible for this increased activity. Because mutagenesis data established that K108 and K120 are principally responsible for the increased activity of SPICE, we aligned the CCPH sequence with SPICE and looked for the presence of positively charged residues at the corresponding positions. We found that Leu (L106) and Arg (R118) are present at these positions. Further, modeling of CCPH structure depicts that R118 could be responsible for the increased C3b CFA of the protein, since its side chain is exposed to the solvent. Study involving site-directed mutagenesis of this residue confirms our premise. The residue is also found to be important for the C4b CFA, but not for the DAA.

5. Role of vRCAs in Pathogenesis

Although viral RCAs have been extensively studied for their complement regulatory activities and for domains and residues that contribute towards the functional activities, their significance as accomplices of virulence is still not demonstrated in many viruses. To date, only VCP and γ-HV68 RCA have been tested in animal models for examining their role in pathogenesis.

Soon after it was demonstrated that VCP inhibits complement, efforts have been made to establish its role as a virulence determinant. To ascertain its in vivo role, a VCP null mutant has been constructed by deleting a 70-base pair segment from C21L gene of WR strain of vaccinia virus. This deletion has shown no effect on the growth of virus in tissue culture, indicating that it has no effect on replication. However, when the mutant is inoculated intradermally into rabbits and guinea pigs, it produces smaller skin lesions than the wild-type virus, suggesting that VCP plays an important role in virulence. What remains unclear is whether attenuation shown is due to VCP-mediated inactivation of host complement or is independent of complement regulation, as VCP is known to perform roles other than complement regulation (see section 3.3).

Importance of vRCA-complement interaction in viral pathogenesis has been more critically evaluated for γ-HV68 RCA protein. After establishing that deletion of γ-HV68 RCA has no effect on viral replication, it has been tested in two different mouse models of γ-HV68 virulence — acute lethal meningoencephalitis in weanling wild-type mice and mortality during persistent infection of IFNγR knockout mice. The deleted virus shows significant attenuation in both the models, as compared to the wild-type γ-HV68, as well as the marker rescue virus. Significant difference is observed in the acute lethal meningoencephalitis model, where LD₅₀ of γ-HV68 RCA deleted virus is about 100-fold higher, as compared to the wild-type. To examine whether this difference is due to lack of γ-HV68 RCA-mediated inhibition of host complement or other activities, yet to be characterized, infection studies have been performed in complement-knock-out mice (C₃⁻/⁻ or factor B⁻/⁻). The reasoning was, if interaction between γ-HV68 RCA and complement is important for virulence then γ-HV68 RCA-null mutant should be as virulent as the wild-type γ-HV68 in complement deficient animals. Consistent with this, the virulence of deleted virus is restored to the wild-type level in C3-knock-out mice. The virulence, however, is not restored in the factor B deficient mice, suggesting that γ-HV68 RCA primarily targets the classical/lectin pathway.

6. Concluding Remarks

Since the discovery of the first vRCA protein two decades ago, it has been found that such homologs also present in other pox as well as herpesviruses are conserved in position and sequence and are functional. Most importantly, their presence correlates well with the viral virulence. For example, the more virulent strain of monkeypox virus from the Congo Basin encodes vRCA, while the less virulent strain from West Africa lacks this protein. Similarly, deletions of the respective vRCA from vaccinia and γ-HV68 result in decrease in their virulence. These data clearly highlight the importance of vRCA in viral pathogenesis.

There are, however, still many unanswered questions: i) why vRCAs are poor inhibitors of the alternative pathway? Functional analysis of vRCAs shows that they lack AP DAA, which results in poor inhibition of the alternative pathway. These data suggest that classical and lectin pathways play a
predominant role in controlling viral infections. However, this argument does not seem to be entirely true because AP inhibitory activity of the variola RCA (MOPICE) completely lacks DAA and hence the importance of DAA in virulence could be questioned. iii) Why poxviruses encode only for the soluble form of vRCAs, while herpesviruses encode for both soluble and membrane-bound forms? By analogy with the human RCA, the soluble forms would be important for inhibiting the fluid phase complement activation and generation of complement-mediated inflammatory response at the site of infection, while the membrane-bound forms would be important for inhibiting complement activation at the surface of the virions. Therefore, the viruses must encode both these forms. It is likely that the requirement of the membrane-bound regulator in poxviruses is completely taken care of by the host complement regulators acquired by the viruses into the envelope while budding. Given the importance of these proteins in viral virulence, it is conceivable that further understanding of molecular basis of their functioning would shed new light on the pathogenesis of pox and herpesviruses and identification of vital control points in these molecules may provide new targets for contemplating rational drug design.

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