Herpesvirus saimiri (HVS) is a lymphotropic virus that causes T-cell lymphomas in New World primates. It encodes a structural homolog of complement control proteins named complement control protein homolog (CCPH). Previously, CCPH has been shown to inhibit C3d deposition on target cells exposed to complement. Here we have studied the mechanism by which it inactivates complement. We have expressed the soluble form of CCPH in Escherichia coli, purified to homogeneity and compared its activity to vaccinia virus complement control protein (VCP) and human complement regulators factor H and soluble complement receptor 1. The expressed soluble form of CCPH bound to C3b (K_D = 19.2 μM) as well as to C4b (K_D = 0.8 μM) and accelerated the decay of the classical/lectin as well as alternative pathway C3-convertases. In addition, it also served as factor I cofactor and supported factor I-mediated inactivation of both C3b and C4b. Time course analysis indicated that although its rate of inactivation of C4b is comparable with VCP, it is 14-fold more potent than VCP in inactivating C3b. Site-directed mutagenesis revealed that Arg-118, which corresponds to Lys-120 of variola virus complement regulator SPICE (a residue critical for its enhanced C3b cofactor activity), contributes significantly in enhancing this activity. Thus, our data indicate that HVS encodes a potent complement inhibitor that allows HVS to evade the host complement attack.

The complement system is an integral participant in the innate mechanisms of immunity and, thus, has a burden of performing surveillance in the host and protecting it from all the pathogens including viruses (1, 2). Earlier studies have decisively demonstrated that both acute and latent viruses are susceptible to complement-mediated neutralization (3, 4). Thus, complement exerts a strong selective pressure on viruses during infection. These data suggest that for their successful survival, viruses must have developed mechanisms to subvert this system. Consistent with this premise, genome sequencing of poxviruses and herpesviruses have shown that members of these families encode for structural homologs of human regulators of the complement activation (RCA) family (5–9).

The RCA family members are formed by tandemly repeating complement control protein (CCP) domains or short consensus repeats, which fold into a bead-like structure, and multiple CCPs are separated by linkers of 2–7 residues (10–12). These proteins regulate complement by two distinct mechanisms (i) by accelerating the irreversible dissociation of the classical/lectin (C4b,2a) and alternative (C3b,Bb) pathway C3-convertases and (ii) by serving as cofactors in serine protease factor I-mediated inactivation of C3b and C4b (the subunits of C3-convertases) (13, 14). To date detailed characterization of all these activities has been performed for the complement control protein homologs of vaccinia virus (VCP) (15–17), variola virus (SPICE) (18), monkeypox virus (MOPICE) (9), and Kaposi’s sarcoma-associated herpesvirus (Kaposica/KCP) (19, 20).

Sequence comparison of the viral homologs of RCA (vCCPs) show that the sequence similarity between the poxvirus homologs exceeds 91%, whereas that among the herpesvirus homologs varies from 43 to 89%. These data suggest that the herpesvirus homologs are more diverse in structure compared with the poxvirus homologs. Whether this structural diversity in herpesvirus homologs is also reflected in their function is not clear, as among the herpesvirus homologs, detailed functional characterization has been performed only for the Kaposi’s sarcoma-associated herpesvirus homolog (Kaposica/KCP) (19–22).

Herpesvirus saimiri (HVS), the prototype of rhadinoviruses, is regularly found in its natural host, the squirrel monkey. Although it does not cause any disease in its natural host, infection in other New World primates such as tamarins, common marmosets, and owl monkeys causes acute peripheral T cell lymphoma within less than 2 months (23, 24). In addition, the virus is also capable of transforming simian and human T cells in vitro (25, 26). Interestingly, unlike any other viruses, the HVSharbors two homologs of complement regulatory proteins, (i) a

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3 The abbreviations used are: RCA, regulators of complement activation; AP, alternative pathway; CP, classical pathway; HVS, herpesvirus saimiri; CCP, complement control protein; VCP, viral CCP; CCPH, complement control protein homolog; sCCPH, soluble CCPH of herpesvirus saimiri; VCP, vaccinia virus complement control protein; SPICE, smallpox inhibitor of complement enzymes; MOPICE, monkeypox inhibitor of complement enzymes; Kaposica, Kaposi’s sarcoma-associated herpesvirus inhibitor of complement activation; sCR1, soluble complement receptor 1; EA, antibody coated sheep erythrocytes; ORF, open reading frame; RU, response unit.
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homolog of RCA encoded by ORF4 and (ii) a homolog of the terminal complement inhibitor CD59 encoded by ORF15 (27, 28). The ORF4 was predicted to encode a protein containing four CCP modules followed by a transmembrane domain. Analysis of posttranscriptional processing indicated that ORF4 transcript occurs as unspliced as well as single-spliced mRNA. The unspliced mRNA codes for a membrane-bound glycoprotein containing four extracellular CCPs along with a transmembrane region, whereas the spliced mRNA codes for a soluble protein that lacks transmembrane region (29).

Initial characterization of the RCA homolog of HVS (named complement control protein homolog, CCPH) showed that expression of the membrane form of this protein on BALB/3T3 cells inhibited C3d deposition on these cells when they were incubated with whole human serum (30). Although this study demonstrated the complement inhibiting activity of this protein, the mechanism by which it inactivates complement activation was not elucidated. In the present study we describe the mechanism of complement regulation by the RCA homolog of HVS. Our results show that the soluble form of the RCA homolog (sCCPH; CCP1-4 without the transmembrane domain) interacts with complement proteins C3b as well as C4b and accelerates decay of the classical/lectin and alternative pathway C3-convertases. In addition, the protein also has the ability to serve as factor I cofactor and support factor I-mediated inactivation of C3b and C4b. Importantly, we show that its factor I cofactor activity for C3b is 14-fold higher in comparison to VCP, the most completely characterized vCCP, and that Arg-118 plays a critical role in enhancing this activity.

EXPERIMENTAL PROCEDURES

Reagents and Buffers—Antibody-coated sheep erythrocytes (EA) were made by incubating sheep erythrocytes with anti-sheep erythrocyte antibodies procured from ICN Biomedical Inc. (Irvine, CA). Veronal-buffered saline (VBS) contained 5 mM sodium phosphate and 145 mM NaCl. GS, EGTA, and phosphate-buffered saline, pH 7.4, contained 10 mM EDTA, 0.15 mM CaCl2, and 0.5 mM MgCl2.

Complement Proteins and Their Proteolytically Activated Products—The human complement protein C3 was purified as follows. Human plasma was subjected to a stepwise precipitation with 11 and 26% polyethylene glycol. The precipitated fraction was washed with 10 mM EDTA, 100 mM NaCl, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The sample was then centrifuged at 4 °C for 30 min at 11,000 rpm, and the supernatant obtained was loaded onto a Mono S 5/5 column (Amersham Biosciences) in 50 mM sodium phosphate, pH 6.0. Bound proteins were eluted with a linear salt gradient of 0–0.5 M NaCl and analyzed by SDS-PAGE. Homogeneous factor B fractions were pooled and concentrated. The recombinant human soluble form of complement receptor type 1 (sCR1) was a generous gift from Dr. Henry Marsh (AVANT Immunotherapeutics, Inc., Needham, MA.). C3b, the proteolytically activated form of C3, was generated by limited trypsin cleavage of C3 and purified on a Mono Q 5/5 (Amersham Biosciences) column as previously described (16). C4b, the proteolytically activated form of C4, was purchased from Calbiochem. Purity of all the proteins exceeded 95%, as judged by SDS-PAGE analysis.

Cloning, Expression, Purification, and Refolding of the Soluble Form of Herpesvirus Saimiri Complement Control Protein Homolog (sCCPH) and the R118A Mutant—The herpesvirus saimiri CCPH gene (CCP domains 1–4) was PCR-amplified from the CCPH clone pCEX-1 (a kind gift of Drs. John Lambris, Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA and Jens-Christian Albrecht, Institut für Klinische und Molekulare Virologie, Erlangen, Germany) with specific primers 5′-GGAAATTCAGCTGTCCTA- CAGTAAACCAG-3′ (the EcoRI site is underlined) and 5′-CCGTCGACATCATCATTGGAATACGGTGG-3′ (the XhoI site is underlined) and cloned into the bacterial expression vector pET29 at the EcoRI and XhoI sites. The R118A mutant was constructed from this clone by using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). After verifying the fidelity of both the clones by DNA sequencing, they were transformed into Escherichia coli BL21 cells for expression.

Expression of sCCPH and R118A mutant (numbering according to the mature protein sequence (29)) was performed as described below. A single colony of the bacterial clone expressing sCCPH or the mutant protein was inoculated into 5 ml of LB-kanamycin media (LB media containing 30 μg/ml kanamycin) and grown overnight at 37 °C, and 2 ml of this culture was transferred into 100 ml of LB-kanamycin. The culture was grown for 2 h at 37 °C, and thereafter 10 ml of the culture was transferred to 600 ml of LB-kanamycin and grown at 37 °C until the optical density reached 0.6 at A600. Protein expression was induced by the addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside, and the induced culture was further grown for 4 h. The cells were harvested by centrifugation at 8000 rpm at 4 °C.

For purification of the expressed proteins, frozen cell pellets (~16 g) were gently resuspended in 48 ml of 50 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl, and 0.1 mM phenylmethylsulfonyl fluoride. The cell suspension was then treated with lysozyme (0.3 mg/ml), stirred for 20 min, mixed with deoxycholic acid (1.3 mg/g), and stored at 37 °C for 30 min. After incubation, the lysate was sonicated with 15 pulses of 15 s each and centrifuged at 10,000 × g for 20 min at 4 °C. The pellet containing the inclusion bodies was washed twice with 50 mM Tris, pH 8.0, 10 mM EDTA, 100 mM NaCl, and 0.5% Triton X-100 and solubilized in 50 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl, and 8 M urea. The sample was then centrifuged at 4 °C for 30 min at 11,000 rpm, and the supernatant obtained was loaded onto a nickel nitritotriacetic acid-agarose column (Qiagen, Hilden, Germany) pre-equilibrated with 100 mM NaH2PO4, 10 mM Tris, 8 M urea, pH 8.0. The column was washed with the binding
buffer containing 10 mM imidazole, and the bound protein was eluted with 400 mM imidazole.

The purified proteins were refolded by using a rapid dilution method (1:50) described previously (33). In brief, the purified protein was added dropwise with continuous stirring into a refolding buffer containing 0.02 M ethanolamine, 1 mM EDTA, 0.5 M L-arginine, 1 mM reduced glutathione, and 1 mM oxidized glutathione, pH 11.0. The sample was then left static for 36 h. The refolded sample was concentrated, dialyzed against phosphate-buffered saline, and subjected to SDS-PAGE, circular dichroism, and sequencing and mass analysis by mass spectrometry (17).

Measurement of Factor I Cofactor Activity—Analysis of factor I cofactor activities of sCCPH and the mutant was essentially performed as described (34). These assays were performed in physiological ionic strength buffer (phosphate-buffered saline).

Measurement of Decay-accelerating Activity—The classical pathway (CP) decay-accelerating activity of sCCPH and the R118A mutant were determined by forming EAC142 (35), and the alternative pathway (AP) C3-convertase decay-accelerating activity was measured by forming C3b,Bb on sheep (Eo) as well as rabbit (Eo) erythrocytes. The details of these methods have been described previously (17, 36).

Circular Dichroism (CD)—The sCCPH and its mutant R118A were subjected to CD spectra in the far UV region (190 – 360 nm) using a Jasco J18 spectropolarimeter with a cylindrical quartz cell with a path length of 0.01 cm. The resolution was 1 nm, the sensitivity was 20 millidegrees, and the speed was 10 nm/min. Each presented spectrum is the measure of eight measurements. The concentration of both the proteins was 200 µg/ml in 10 mM phosphate containing 145 mM NaCl, pH 7.4. All the data were subtracted against the background data using the spectral analysis software.

Flow Cytometry for Measurement of Inhibition of C3b Deposition—Inhibition of the classical and alternative pathway-mediated C3b deposition on erythrocytes by sCCPH and VCP was measured by flow cytometry (19). For measurement of the classical pathway-mediated C3b deposition, 5 µl of EA (107/ml in GVB2+) was mixed with 2 µl of C8-deficient human serum (Calbiochem) and 2 µM sCCPH or VCP in a total volume of 44 µl and incubated for 30 min at 37 °C. The cells were washed with GVB2+, centrifuged, mixed with 100 µl of 1/500-diluted fluorescein isothiocyanate-conjugated F(ab)2 anti-C3 goat IgG (Cappel Laboratories, Warrington, PA), and further incubated on ice for 1 h. After incubation, the cells were washed twice with 400 µl of GVB, resuspended in 1.0 ml of the same buffer, and analyzed on a FACS Vantage (BD Biosciences). For measurement of alternative pathway-mediated deposition of C3b, 5 µl of rabbit erythrocytes (109/ml in GVB) was mixed with 2 µl of 0.1 M MgEGTA, 3 µl of C8-deficient human serum (Calbiochem), and 30 µl of GVB or GVB containing 2 µM sCCPH or VCP and incubated for 30 min at 37 °C. The cells were washed with GVB, and deposition of C3b was detected as described above. Results are expressed as mean channel fluorescence of 10,000 cells.

Surface Plasmon Resonance Measurements—The kinetics of sCCPH and the R118A mutant binding to C3b and C4b was determined on the surface plasmon resonance-based biosensor BIACORE 2000 (Biacore AB, Uppsala, Sweden). The experiments were performed in phosphate-buffered saline-Tween (10 mM sodium phosphate, 145 mM NaCl, pH 7.4, containing 0.05% Tween 20) at 25 °C. For proper orientation of these proteins, the free SH groups of both C3b and C4b were biontilated and then immobilized on the streptavidin chip (Sensor Chip SA, Biacore AB) (34). FC-2 was immobilized with C3b (1592 RU), FC-3 was immobilized with C4b (1197 RU), and FC-1 (blank flow cell) served as the control flow cell. Because sCCPH showed very little binding to C3b, more C3b molecules were deposited onto FC-2 by forming C3-convertase (37, 38). In brief, ~6000 RUs of C3b were deposited using three cycles of C3b deposition. In each cycle, the C3-convertase was formed by injecting a mixture of factors B and D (5 µg of B and 0.35 µg of D) and then 45 µg of native C3 was injected using the co-inject option. Deposition of C3b onto the chip was performed in veronal-buffered saline containing 1 mM NiCl2. For measurement of binding of sCCPH and the mutant protein to C3b and C4b, various concentrations of these proteins were injected for 120 s at 50 µl/min. Dissociation was measured for 180 s. The sensor chips were regenerated with 30-s pulses of 0.2 M sodium carbonate, pH 9.5. Sensograms obtained for the control flow cell (FC-1) were subtracted from the data for the flow cell immobilized with C3b or C4b, and the surface plasmon resonance data obtained were evaluated by BIAlaevulation software version 4.1 using global fitting.

RESULTS

Expression and Characterization of sCCPH—Because a large quantity of protein was required for conducting multiple
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assays, we chose to express HVS sCCPH in E. coli using the pET expression system. The soluble form of HVS CCPH was amplified from the HVS clone pCEX-1 and cloned into the expression vector pET29. The expressed protein was purified to homogeneity using histidine affinity (Fig. 1), and the identity of the expressed protein was confirmed by sequencing using mass spectrometry. The amino acid sequence of the expressed protein was consistent with the predicted sequence confirming the identity; the sequence coverage obtained was 86%. The expressed protein was refolded according to the method described by R. A. Smith and co-workers (33). This method typically provided a 20% yield. Analysis of the refolded protein by circular dichroism yielded a peak around 230 nm, which is a characteristic of CCP domains (39) (Fig. 1). These data confirmed proper folding of the protein. The expressed protein was >95% pure as judged by SDS-PAGE analysis, and it migrated as a single band of 32,000 Da on the gel. Further mass analysis by mass spectrometry confirmed that its molecular mass was similar to its calculated mass (within error <1%) (Fig. 1).

Previously it has been shown that the membrane form of CCPH inhibits C3d deposition on the target cells (30). To verify if the refolded protein is biologically active, we tested its ability to inhibit C3b deposition on erythrocytes during complement activation. As depicted in Fig. 2, sCCPH inhibited both the classical as well as alternative pathway-mediated deposition of C3b on erythrocytes. Importantly, the data indicated that sCCPH was more active than VCP in inhibiting the alternative pathway-mediated deposition of C3b.

Kinetic Analysis of Interaction of sCCPH with Complement Proteins C3b and C4b—The human complement control proteins inactivate complement by targeting C3b and/or C4b. Because sCCPH inhibited C3b deposition mediated by both the classical and alternative pathways, we sought to analyze its interaction with C3b and C4b by surface plasmon resonance technology. In this assay, C3b and C4b were immobilized in their physiological orientation on a streptavidin chip by labeling their free SH groups with biotin (34), and sCCPH was injected over the chip to measure binding. The sCCPH showed good binding to C4b but very weak binding to C3b (Fig. 3, upper left panel). Binding data obtained by injecting various concentrations of sCCPH fitted well to a 1:1 binding model \( k_\text{a} = 158; k_\text{d} = 5.32 \times 10^{-3} \). More C3b was deposited on the sensor chip to increase the response and reevaluate affinity. More C3b was deposited by forming AP C3-convertase on the chip and flowing native C3

FIGURE 2. Inhibition of C3b deposition on erythrocytes during complement activation by sCCPH and VCP. Top panel, classical pathway-mediated C3b deposition on erythrocytes was measured by incubating EA and C8-deficient human serum with or without 2 \( \mu \)M sCCPH/VCP at 37 °C for 30 min. Deposition of C3b was detected by fluorescence-activated cell sorting using fluorescein isothiocyanate-conjugated \( \text{F(ab'}\text{)}_2\) anti-C3 goat immunoglobulin G. Bottom panel, alternative pathway-mediated C3b deposition on erythrocytes was measured by incubating rabbit erythrocytes and C8-deficient human serum in the presence of MgEGTA with or without 2 \( \mu \)M sCCPH/VCP at 37 °C for 20 min. Deposition of C3b was detected as described above. Control samples contained 10 mM EDTA.

FIGURE 3. Surface plasmon resonance analysis of binding of sCCPH to complement proteins C3b and C4b. Biotinylated C3b and C4b were oriented in their physiological orientation on a streptavidin chip (Sensor Chip SA; Biacore AB), and various concentrations of sCCPH were injected over the chip to measure binding. Top left, binding of sCCPH to C3b and C4b oriented on a streptavidin chip by labeling their free SH groups with biotin (34). Top right, deposition of C3b by forming AP C3-convertase on the chip (37, 38). Factors B and D mix and C3 (in veronal-buffered saline containing 1.0 mM NiCl\(_2\)) were repeatedly injected over the chip using the co-inject option of Biacore 2000. Deposition of C3b was detected as described above. Control samples contained 10 mM EDTA. Top right, sensogram overlay for the interaction between sCCPH and C4b deposited using the AP C3-convertase. The solid lines represent the global fitting of the data to a 1:1 Langmuir binding model \( (A + B \rightarrow AB) \). The concentration of sCCPH injected is indicated at the right of the sensograms. Bottom left, sensogram overlay for the interaction between sCCPH and C4b. The arrow indicates the time point used for evaluating the steady-state affinity data. The concentration of sCCPH injected is indicated at the right of the sensograms.
TABLE 1
Kinetic and affinity data for the interactions of sCCPH, R118A mutant, and VCP with human complement proteins C3b and C4b

| Ligand | Analyte     | $k_d/k_a$ | S.E. ($k_d/k_a$) | $K_D$ | $\chi^2$ |
|--------|-------------|-----------|-----------------|-------|----------|
| C3b    | sCCPH       | 2.63 x 10^{-3}/137 | 2.05 x 10^{-3}/3.39 | 1.92 x 10^{-5} | 1.83 |
| C4b    | sCCPH       | NA        | NA              | 8.04 x 10^{-7} | 2.87 |
| C3b    | R118A       | 4.06 x 10^{-3}/187 | 4.75 x 10^{-3}/0.73 | 2.17 x 10^{-4} | 9.44 |
| C4b    | R118A       | NA        | NA              | 9.64 x 10^{-7} | 1.23 |
| C3b    | VCP         | NA        | NA              | 2.0 x 10^{-6} | 1.47 |
| C4b    | VCP         | NA        | NA              | 3.65 x 10^{-7} | 1.12 |

$^{a}$ Data were calculated by global fitting to a 1:1 Langmuir binding model (BIA evaluation 4.1).
$^{b}$ Data were calculated by steady-state analysis (BIA evaluation 4.1).

**FIGURE 4.** Factor I cofactor activity of sCCPH, VCP, factor H (fH), and sCR1 for complement proteins C3b and C4b. Cofactor activity for C3b cleavage was assayed by mixing 3 μg of C3b, 0.1 μg of factor I, and 1.5 μg of the cofactor (as indicated in the lanes) in 20 μl of physiologic ionic strength buffer and incubating at 37 °C for the indicated time periods. Cofactor activity for C4b cleavage was performed by mixing 3 μg of C4b, 0.1 μg of factor I, and 1 μg of the cofactor (as indicated in the lanes) in 20 μl and incubating at 37 °C for the indicated time periods. Cleavage products were visualized by separating the samples on SDS-PAGE gel (9.5% for C3b and 10% for C4b) under reducing conditions and staining with Coomassie Blue.
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- Cofactor activity of sCCPH and VCP.
- Time course of factor I cofactor activity of sCCPH, VCP, factor H (fH), and sCR1 for complement protein C3b. Cofactor activity was measured by incubating 3 μg of C3b with 0.05 μg of factor I and 0.5 μM concentrations of the cofactors (as indicated in each gel) at 37 °C for the indicated time period. The reactions were stopped by adding the sample buffer containing dithiothreitol, and the cleavage products were visualized by separating the samples on 9.5% SDS-PAGE gel and staining with Coomassie Blue. The intensities of the α′ chain were determined by densitometric analysis and represented graphically (lower panel).

- FIGURE 5. Time course of factor I cofactor activity of sCCPH, VCP, factor H (fH), and sCR1 for complement protein C3b. Cofactor activity was measured by incubating 3 μg of C3b with 0.05 μg of factor I and 0.5 μM concentrations of the cofactors (as indicated in each gel) at 37 °C for the indicated time period. The reactions were stopped by adding the sample buffer containing dithiothreitol, and the cleavage products were visualized by separating the samples on 9.5% SDS-PAGE gel and staining with Coomassie Blue. The intensities of the α′ chain were determined by densitometric analysis and represented graphically (lower panel).

- Functional characterization of HVS CCPH.
- Arginine 118 of sCCPH is crucial for its cofactor activities but not decay-accelerating activities. Because sCCPH showed a robust factor I cofactor activity for C3b cleavage, we sought to identify the determinant that is responsible for its enhanced activity. Recent studies on SPICE have shown that it possesses ~100-fold more factor I cofactor activity for C3b than VCP (18), and residues Lys-108 and -120 are primarily responsible for this enhanced cofactor activity (41). Sequence analysis of sCCPH with SPICE showed that sCCPH has Leu (Leu-106) and Arg (Arg-118) in the corresponding positions. Because earlier studies have shown that ionic interactions play a critical role in C3b-C3b/C4b interactions (34, 42–44), we suspected that Arg-118 might be responsible for the enhanced C3b cofactor activity. To further probe this possibility, we built a three-dimensional model of sCCPH by homology modeling using the crystal structure of VCP (45) as the template. The model structure showed that Arg-118 is exposed to solvent (Fig. 8), which further supported the possibility that this residue could be involved in the cofactor activity.

- To study the involvement of Arg-118 in the functional activities of sCCPH, we mutated the Arg-118 to Ala and expressed the R118A mutant in E. coli using the pET expression system. Purification and refolding procedures used for the mutant were essentially similar to that of sCCPH. Sequencing (sequence coverage obtained was 91%) and circular dichroism analysis confirmed the identity and correct folding of the mutant (Fig. 1), respectively. Functional analysis of the R118A mutant showed that its factor I cofactor activity for C3b was drastically decreased compared with sCCPH; it was 50-fold less active compared with sCCPH (Fig. 9). Interestingly, the mutant also showed a 12-fold decrease in factor I cofactor activity for C4b (Fig. 9).

- Next, we analyzed if this mutation also affects the decay-accelerating activities of sCCPH. The data showed that the mutant was as active as sCCPH in accelerating the decay of the classical as well as alternative pathway C3-convertases (14, 46). To determine whether R118A mutation affected binding, we measured binding of this mutant to C3b and C4b using the surface plasmon resonance assay. The R118A mutant showed about an 11-fold decrease in affinity for C3b and C4b (Fig. 10). Together these data indicated that Arg-118 plays an important role in enhancing the cofactor activities but not the decay-accelerating activities of sCCPH.

- Binding of R118A Mutant to C3b and C4b—Binding of the complement control proteins to C3b and C4b is a prerequisite for imparting factor I cofactor activities and decay-accelerating activities; however, a significant body of literature suggests that binding does not always correlate well with these activities (14, 46). To determine whether R118A mutation affected binding, we measured binding of this mutant to C3b and C4b using the surface plasmon resonance assay. The R118A mutant showed about an 11-fold decrease in affinity for C3b compared with sCCPH (Table 1), which was a result of a 7.3-fold decrease in the on-rate and a 1.5-fold increase in the off-rate. These data are consistent with the substantial decrease in C3b cofactor activity. There was, however, no decrease in the affinity of C4b (Table 1), although the mutant showed a 12-fold decrease in C4b cofactor activity. Furthermore, it is clear from the data provided in Fig. 10 that R118A
mutation had no effect on the decay-accelerating activities; hence, the decrease in $K_D$ value for C3b did not correlate with the AP C3-convertase activity.

**DISCUSSION**

The regulators of human complement belonging to the RCA family contain 4–59 copies of CCP domains. It is believed that the sequence variations imposed on the CCP domain fold and the interdomain dynamics determine the differences in functionality of the complement regulators (8, 11, 47–49). In viruses, homologs of complement regulators have been described in members of Herpesviridae and Poxviridae. The sequence similarity among the poxviral complement regulators exceeds 91%, whereas that among the herpesviral complement regulators ranges between 43 and 89%. Therefore, functional characterization of various herpesviral complement regulators would help in determining whether structural diversity in these regulators has led to any change in their functional diversity. Functional complement regulators in Herpesviridae family have been described in herpesvirus saimiri (30), γ-herpesvirus 68 (50), and Kaposi’s sarcoma-associated herpesvirus (KSHV) (19, 20), but detailed functional analysis for decay-accelerating activities, factor I cofactor activities, and binding to C3b and C4b have been performed only for the KSHV complement regulator (Kaposica/KCP) (19–21). In the present study we have analyzed the functional activities of HVS sCCPH to get insight into the functional diversity of sCCPH against the complement system.

A previous study had shown that the membrane form of HVS CCPH inhibits the classical pathway-mediated deposition of C3d onto the target cells (30). Our data on inhibition of C3b deposition onto the target cells by sCCPH show that it inhibits both the classical as well as alternative pathway-mediated deposition of C3b (Fig. 2). These results are consistent with the previous data on herpesviral (γ-HV68 and Kaposica) and poxviral (VCP and SPICE) complement regulators (16, 18–20, 50), which showed inhibitory activities against both the pathways. Earlier, using hemolytic assays it has been shown that vCCPs are efficient in inactivating the classical pathway ($IC_{50} = 0.1–0.2$ μM) (16, 19); these values are considered significant because the local concentration of these proteins at the site of infection is expected to be very high (2, 20, 51). Measurement of inhibition of the CP-mediated lysis of sheep erythrocytes showed that like other vCCPs, sCCPH is also an effective inhibitor of the classical pathway ($IC_{50} = 0.27$ μM). Further analysis of the CP C3-convertase regulatory activities demonstrated that it contains both effective factor I cofactor activity for C4b (Fig. 6) as well as CP decay-accelerating activity (Fig. 7A). Thus, like other vCCPs, the effective classical pathway inhibitory activity is also conserved in HVS CCPH.

It is clear from the data presented in Fig. 2 that sCCPH is significantly more active than VCP in inhibiting the alternative pathway-mediated deposition of C3b onto erythrocytes. To define the mechanism responsible for this increased activity, we characterized its factor I cofactor activity for C3b and decay-accelerating activity for the AP C3-convertase. The data revealed that sCCPH possesses 14-fold more C3b cofactor activity compared with VCP (Fig. 5). In fact, the cofactor activity was only about 2.5-fold less compared with human complement regulators factor H and sCR1 (Fig. 5). We would like to point out here that the observed difference in the cofactor activ-
ity of sCCPH compared with VCP was not due to the difference in affinity for C3b as sCCPH showed lower affinity for C3b compared with VCP (Table 1). This, however, is not surprising as previously it has been shown that CD46, which has a much lower affinity for C3b than CR1, has a higher cofactor activity than CR1 (52). It is likely that the increased cofactor activity of sCCPH is a result of its better interaction with factor I. Analysis of AP C3-convertase decay-accelerating activity showed that sCCPH is a poor decay accelerator of AP C3-convertase (Fig. 7B). Although its activity was 8-fold better compared with VCP, it was >2000-fold less active compared with factor H and sCR1 (Fig. 7B). Together these data suggest that the increased alternative pathway inhibitory activity of sCCPH was primarily due to its increased factor I cofactor activity for C3b.

Factor I is known to cleave C3b at three different positions depending on the cofactors involved: the first between 1281–1282, which generates iC3b1; the second between 1298–1299, which generates iC3b2; the third between 932–933, which generates C3c and C3dg. Whether vCCPs support the cleavage of C3b to C3c and C3d/C3dg was under debate until recently (18, 20), but it is now clear that viral regulators primarily support the cleavage of C3b to iC3b1 (e.g. VCP (9, 16)) or iC3b2 (e.g. Kaposica (19, 22), SPICE (9, 41), and MOPICE (9)) and not C3c and C3dg. Like most of other viral regulators, the sCCPH also supported the generation of C3b to iC3b2 (Figs. 4 and 5). Because generation of iC3b1 itself is sufficient to inactivate C3b (16), it is not clear whether inactivation of C3b to iC3b2 as opposed to iC3b1 provides any functional advantage to viruses.

Because sCCPH showed about a 14-fold higher cofactor activity for C3b, we sought to examine the basis for this increased activity. Earlier, Rosengard et al. (18) demonstrated that SPICE is about 100-fold more potent than VCP in inactivating C3b. Later, using the site-directed mutagenesis approach, it was established that Lys-108 and -120 residues are principally responsible for better functioning of SPICE (41). When we aligned sCCPH sequence with SPICE to determine whether sCCPH contains positively charged residues at the corresponding positions, we found that sCCPH contains Leu (Leu-106) and Arg (Arg-118) at these positions. Based on these, we predicted that Arg-118 might be responsible for the higher cofactor activity of sCCPH. Modeling of the sCCPH structure based on the crystal structure of VCP demonstrated that the side chain of Arg-118 is exposed to solvent (Fig. 8), which further supported this possibility. Thus, we mutated the Arg-118 to Ala and examined its functional activities. We found that removal of charge at this position drastically affected the factor I cofactor activity for C3b (50-fold decrease) and to some extent C4b (12-fold decrease) but had no effect on the decay-accelerating activities (Figs. 9 and 10). These data along with the previous studies on SPICE clearly point out that the presence of a positive charge at this position enhances the C3b cofactor activity in viral homologs. It is interesting to note that despite
belonging to two different viral families, substitution of positively charged residue at comparable positions has been seen in SPICE as well as sCCPH (Lys-120 in SPICE and Arg-118 in sCCPH). Although a previous study on SPICE (41) examined the role of Lys-120 in enhancing C3b cofactor activity, it did not look at its role either in C4b cofactor activity or in decay-accelerating activities. Based on our data, we suggest that Lys-120 of SPICE might also play a role in enhancing its cofactor activity for C4b.

In summary, our data clearly show that HVS CCPH possesses all the complement regulatory activities present in Kaposica and other viral regulators. Thus, it seems that despite significant sequence differences between herpesviral complement regulators, the functional activities have been conserved. These data along with previous observations, therefore, point out that maintenance of various complement regulatory functions must be important to the pox as well as herpesviruses and inhibition of the lectin/classical pathway is crucial to viral survival than the inhibition of alternative pathway. Whether sequence variations in herpesviral complement proteins have resulted in acquisition of any new functions is not clear at present and requires further studies. Previously, it has been demonstrated that CCP homolog of γHV-68 plays an important role in complement evasion in vivo (53). Given the fact that sCCPH is an efficient complement inactivator, it is likely that sCCPH may also act as an immune evasion molecule in vivo and protect HVS from the host complement during infection.

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