Kaposi's sarcoma-associated herpesvirus (KSHV) is closely associated with Kaposi's sarcoma and certain B-cell lymphomas. The fourth open reading frame of the KSHV genome encodes a protein (KSHV complement control protein (KCP, previously termed ORF4)) predicted to have complement-regulating activity. Here, we show that soluble KCP strongly enhanced the decay of classical C3-convertase but not the alternative pathway C3-convertase, when compared with the host complement regulators: factor H, C4b-binding protein, and decay-accelerating factor. The equilibrium affinity constant ($K_{d}$) of KCP for $C3b$ and $C4b$ was determined by surface plasmon resonance analysis to range between 0.47–10 μM and 0.025–6.1 μM, respectively, depending on NaCl concentration and cation presence. Soluble and cell-associated KCP acted as a cofactor for factor I (FI)-mediated cleavage of both $C4b$ and $C3b$ and induced the cleavage products $C4d$ and $iC3b$, respectively. In the presence of KCP, FI further cleaved $iC3b$ to $C3d$, which has never been described before as a complement receptor 1 only mediates the production of $C3dg$ by FI. KCP would enhance virus pathogenesis through evading complement attack, opsonization, and anaphylaxis but may also aid in targeting KSHV to one of its host reservoirs since $C3d$ is a ligand for complement receptor 2 on B-cells.

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Kaposi's sarcoma-associated herpesvirus (KSHV) is the likely etiologic agent of Kaposi's sarcoma and the most recently identified member of the human Herpesviridae family (1, 2). KSHV is also associated with the B-cell tumors body cavity-based primary effusion lymphomas and the plasma cell variant of multicentric Castleman's Disease (for reviews, see Refs. 3 and 4). KSHV belongs to the rhadinovirus genus of the Gam- maherpesvirinae subfamily, the prototype of which is Herpes- virus saimiri (HVS). The long unique region of the KSHV genome comprises 140.5 kb and contains over 80 open reading frames (ORFs) (5). Several of the ORFs encode host cell homologues (e.g. viral cyclin D, viral interleukin-8 G protein-coupled receptor, and a bcl-2 homologue) with the potential to regulate the cell cycle and the immune response thereby contributing to the virulence and the pathogenesis of KSHV (5, 6).

The fourth open reading frame, ORF4, was initially speculated to have complement regulatory abilities based on its homology to human complement regulators decay-accelerating factor (DAF) and membrane cofactor and to previously described virus-encoded complement inhibitors (5). The KSHV ORF4 gene is predicted to encode a KCP protein of 550 amino acids (data base reference SPTREMBL:O40912), and the first 280 amino acids are predicted to encode four complement control protein (CCP) domains. CCP domains are defined by a consensus sequence of 60 amino acids containing four invariant cysteine residues that form disulfide links, which results in the CCP forming a globular domain with a hydrophobic core enclosed by β-strands (7, 8). While the CCP domain is not exclusive to complement control proteins, all but one of the C3-convertase-regulating proteins identified to date, either mammalian or viral, are composed of CCPs. Analyses of KCP mRNA expressed in B-cells isolated from primary effusion lymphoma patients revealed three major transcript variants (one unspliced full-length form and two alternatively spliced variants), all containing the N-terminal four CCP domains but with different amounts of internal coding sequence removed by alternative splicing (9). All three transcripts also encode the transmembrane region indicating that all forms are membrane-associated. The HVS-encoded complement regulator is also alternatively spliced, but the transmembrane region is lost in the single alternatively spliced form due to a frameshift (10). The complement regulator homologue encoded by murine gammaherpesvirus 68 (MHV68) has also been reported to be expressed as cell-bound and soluble forms (11); however, no alternate splicing of the MHV68 mRNA that could yield the latter form was observed by the authors, and how the soluble protein arises has yet to be resolved.

During our initial characterization of the KCP mRNA iso- lated from primary effusion lymphoma cell lines, we found that expression of the KCP cDNA in CHO cells significantly reduced C3b deposition on antibody-sensitized CHO cells following incubation with whole human serum; however, the mechanism of complement regulation was not elucidated (9). Here we de- scribe the mechanism of KCP complement regulation. We ex-
amined the ability of KCP to enhance the decay of C3-conver-
tases of both the alternative and classical pathways of complement. These activities were compared with those of the host complement regulators, factor H (FH), C4-binding protein (C4BP), and DAF. The ability of KCP to act as a factor I (FI) cofactor for the cleavage and inactivation of both C3b and C4b was also examined in the soluble phase and on the surface of cells. Importantly, we show that KCP has FI cofactor activity previously undescribed as it promotes FI production of the C3d fragment.

MATERIALS AND METHODS

Proteins—Human C4BP (12), C1 (13), C2 (14), C4 (15), FH (16), and FI (17) were purified from plasma as described previously. C3, C1b, C3b, factors B, D, and properdin were purchased from Advanced Re-
search Technologies. C1 and C2 were functionally pure (i.e. they were devoid of complement factors), while C4BP, FH, FI, C4, C3, C4b, and C3b were at least 95% pure as determined by Coomassie staining of proteins separated by SDS-PAGE. All proteins were stored in aliquots at -80 °C. Protein concentrations were determined by measuring absorbance at 280 nm. C3b and C4b were labeled with 125I using the chloramine T method (18), and the specific activity was determined to be 0.4–0.5 MBq/µg of protein. Human erythrocyte ghosts were used as a source of C1r and were made through repeated washing (until all red color was removed) in ice-cold phosphate buffer (7.5 mM phosphate, 1 mM EGTA, 0.2 M phenylmethylsulfonyl fluoride) by resuspension and centrifugation at maximum speed in a benchtop microfuge (4 °C) fol-
lowed by discarding the supernatant.

Expression and Purification of KCP—Soluble recombinant Fc fusion proteins were constructed based on the naturally occurring isoforms of KCP (described in Ref. 9) using the same strategy and vectors as previously described to construct DAF-Fc (19). Cloning and character-
ization of full-length KCP cDNA are described elsewhere (9). Briefly, PCR primers were designed to truncate the recombinant KCP isoforms immediately following the CCP domains (short: 5′-AGTCGTCTGAT-
GGTGTCTTCA-3′), prior to the Ser/Thr-rich region (medium; 5′-TG-
GCTGGGATGTAGTTTTCTCAT-3′), or to the transmembrane re-
region (long: 5′-AATGGGAGGGATGTTGGTTCT-3′). All of these primers were designed with a Naf restriction site to allow the in-frame addition of the human IgG1-Fc C terminus when used for PCR in combination with the common forward primer (F: 5′-GGCGCTTCTAGA-
GCTAGCAGTGCCCTTTTTAAGAAACAA-3′), followed by cloning into the previously described expression vector pDR21PE1a (19). The re-
resultant plasmids were transfected into CHO cells (European culture collection) using lipofectamine (Invitrogen). Cells were propagated in RPMI 1640 cell medium containing 10% fetal calf serum, and stable transfectants were created by propagating cells in medium containing 400 µg/ml of hygromycin B (Invitrogen) for 2 weeks. Cell supernatants were collected, and the Fc fusion proteins isolated and purified as previously described using protein A-Sepharose (Amersham Bio-
sciences) (19). The resulting three KCP-Fc fusion proteins of different sizes resemble IgG molecules with the Fab portion replaced with KCP

Inhibition of Classical Pathway C3-convertase—Sheep erythrocytes were washed twice with DGBV+ (2.5 mM veronal buffer, pH 7.3, 72 mM NaCl, 140 mM glucose, 0.1% gelatin, 1 mM MgCl2, and 0.15 mM CaCl2), suspended at a concentration of 10 6 cells/ml, and incubated for 20 min at 37 °C with an equal volume of amboceptor (Roche Molecular Bio-
chemicals) diluted 1:3000 in DGBV2+ to terms of KCP with 0.1% SDS were made by washing the EA cells twice with ice-cold DGBV2+ and resuspending in 10 6 cells/ml; then C1 was added to 10 10 cells drop-wise to a final concentration of 5 µg/ml, and the mixture incubated with agitation for 20 min at 30 °C. EA1 cells were washed twice with ice-cold buffer and incubated with agitation for 20 min at 30 °C with 1 mM EGTA, 0.2 M phenylmethylsulfonyl fluoride, and 7.5 µg/ml of KCP. The samples were then placed on ice for 1 min, centrifuged, and resus-
pended in prewarmed DGBV2+. An equal volume of these EA1C42 cells was added to a range of preidiluted inhibitors and allowed to incubate at 30 °C with constant shaking for 5 min. 100-µl aliquots of each sample were removed and added to 100 µl of guinea-pig serum-diluted 1:50 in 50 mM EDTA-GVB, and the resultant lysis was determined following incubation at 37 °C for 60 min. The amount of released hemoglobin was directly proportional to the residual C3-convertase activity remaining on the EA142 cells and was measured at 405 nm in the supernatant after pelleting the unlysed cells by centrifugation. Soluble recombinant DAF and purified C4BP were used as positive controls in these experi-
ments, and all inhibitors were compared with the amount of lysis observed in the absence of added inhibitors.

Inhibition of the Alternative Pathway C3-convertase—Ten nl of EAIC14 (1.5 × 10 7/ml), prepared as described above, were incubated with 0.25 µg/ml C2 and 50 µg/ml C3 for 30 min at 30 °C with agitation. The cells were washed once in 10 ml EDTA-GVB, resuspended in the same buffer, and incubated for 2 h at 37 °C to allow dissociation of C1 and C2 from the cells. The resultant EAC43 were washed twice in EDTA-GVB, then twice in Mg 2+-EGTA buffer (2.5 mM veronal buffer, pH 7.3, 70 mM NaCl, 140 mM glucose, 0.1% gelatin, 7 mM MgCl2, and 10 mM EGTA), and then resuspended at 2 × 10 6 cells/ml. EAC43 were then incubated with properdin in C1 (1 µg/ml), FD (31 µg/ml), FD (10 µg/ml), and tested inhibitor or Mg 2+-EGTA (final volume 150 µl). The mixtures were kept shaking at 30 °C and 100-µl aliquots removed after 30 min and added to 100 µl of guinea pig serum diluted 1:30 in GVB-EDTA. After 1 h at 37 °C, the samples were centrifuged, and the amount of erythrocyte lysis was determined spectrophotometrically as above. Solu-
ble recombinant DAF-Fc and FH served as positive controls in these experiments, and all inhibitors were compared with the amount of lysis observed in the absence of added inhibitors.

N-terminal Protein Sequencing—C3b was treated with FI in the presence of the short form of KCP as described in the degradation assay above. The proteins containing in total 7.5 µg of C3 were separated by SDS-PAGE on 10–15% gradient gels and transferred by semi-dry elec-
troblotting onto polyvinylidene difluoride membrane. The membrane was stained with Coomassie and the appropriate bands were excised and subjected to N-terminal sequencing in an automated sequenator (494 Protein Sequencer ProCelC; Applied Biosystems).

Surface Plasmon Resonance (Biacore)—The interaction between C3b/ Fc and KCP was analyzed using surface plasmon resonance (Biacore 2000; Biacore). Each of four flow cells on a CM5 sensor chip was activated with 20 µl of 0.1 M ethanola-
mine, pH 8.5. A negative control was prepared by activating and sub-
sequently blocking the surface of flow cell 1.

The association kinetics were studied for a range of C3b and C4b concentrations (methylamine activation of C3 and C4 was used to convert native C3 and C4 to C3b and C4b conversion, respectively) (20) using the standard flow buffer (10 mM Hepes-KOH, pH 7.4 sup-
plemented with 150 mM NaCl, 0.005% Tween 20). In some experiments, 75 mM NaCl was used, and occasionally standard flow buffer was supplemented with physiological concentrations of calcium and zinc (2.5 mM CaCl2, 20 µM ZnCl2, 50 µM EDTA) to achieve saturation during the association phase at a constant flow rate of 30 µl/min. The sample was injected first over the negative control surface and then over immobilized KCP flow cells and analyzed for a dissociation phase of 200 s at the same flow rate. Signals were

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normalized by subtracting the nonspecific signal measured by control flow cell 1. Between each different concentration of C4b or C3b tested these flow cell surfaces were regenerated with a 30-μl injection of 2 M NaCl to remove bound ligands. All sensograms were analyzed using the Biacore Evaluation 3.0 software (Biacore) to calculate equilibrium affinity constants.

Flow Cytometry—Deposition of C3b and various fragments thereof was measured by flow cytometry using polyclonal antibodies. C3b was deposited in the absence of sensitizing antibody, on the surface of sheep erythrocytes (EC3) after three rounds of incubation with C3, FB, FD, and nickel ions as previously described (16). The cells were then incubated overnight at 37 °C with FI alone or in combination with FH, the short form of KCP, or a source of CR1 (human erythrocyte ghosts) in DGVB2. Aliquots of each of these treated EC3 cells were then incubated with a panel of rabbit polyclonal antibodies directed against C3d (Dakopatts), C3c (two different antibodies from Dakopatts), or whole C3 molecule (generated in house against whole purified C3). EC3 cells were washed once in flow cytometry medium (FCM, phosphate-buffered saline, 15 mM EDTA, 30 mM NaH2PO4, 1% bovine serum albumin) and resuspended to a concentration of 10^6 cells/ml. Primary antibodies (purified IgG fractions) were diluted 1/100 in FCM and allowed to bind for 30 min at 4 °C. Unbound antibody was removed by three washes in FCM prior to incubation with secondary fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin antibody (Dakopatts). After three final washes in FCM the cells were analyzed on a flow cytometer (FACSCalibur, BD Biosciences). All treated EC3 cells were compared with EC3 cells that were incubated overnight in DGVB2 as a negative control. All analyses were performed in triplicate, and the experiment was performed twice.

RESULTS
Expression and Purification of Three Splice Variants of KCP—Three soluble recombinant Fc fusion forms of KCP were constructed based on the naturally occurring transcript variants observed in KSHV-infected cells (9). The shortest construct is composed of only the N-terminal CCP domains fused with the hinge and C terminus of human IgG1 Fc region. The medium and longest recombinant fusion proteins were truncated prior to the Ser/Thr-rich region or prior to the transmembrane regions, respectively. Fig. 1 shows the molecular mass of all three purified KCP-Fc forms compared with human DAF-Fc separated by SDS-PAGE under reducing conditions. However, it should be noted that Fc fusion proteins appear as IgG molecules with the Fab portion replaced by KCP, and purified KCP-Fc proteins are therefore dimeric. Additionally, because the longest KCP form retained both of the mRNA splice donor sites and the single splice acceptor site, the resultant fusion protein existed as a mixture of full-length, medium, and short Fc fusion proteins.

KCP Accelerates Decay of the Classical Pathway C3-convertase—Decay of classical pathway C3-convertase was measured in the presence of soluble KCP (Fig. 2). Putative or known inhibitory proteins were added after the formation of the classical C3-convertase on the surface of sheep erythrocytes, and decay acceleration was measured after 5 min. All three forms of KCP-Fc accelerated the decay of C3-convertase equivalently and were ~10-fold less active than the positive control DAF-Fc (Fig. 2A). Monomeric KCP forms were released from KCP-Fc with papain and found to be about 5-fold less active than C4BP at enhancing the decay (Fig. 2B). Dimeric KCP-Fc was found to be equally as effective as monomeric KCP, indicating that the Fc fusion proteins did not cause steric hindrance of the active site, nor did the close proximity of two active sites increase the decay-accelerating activity. No decay of classical pathway C3-convertase was observed when dimeric CAR-Fc (Coxackie adenovirus receptor) or monomeric CAR were tested, confirming that the decay-accelerating activity measured was specific to KCP (Fig. 2).

KCP Shows Limited Inhibition of the Alternative Pathway C3-convertase—The decay of the alternative pathway C3-convertase was also assessed by hemolytic assay. The alternative C3-convertase was assembled on the surface of sheep erythrocytes coincident with the addition of putative and known inhibitors, and decay acceleration was measured after 30 min. All three forms of KCP-Fc accelerated the decay equivalently, but they were 1000-fold less efficient compared with positive control DAF-Fc (Fig. 3A). Monomeric KCP proteins were also tested after being released by papain cleavage, but the decay-accelerating activity was not increased compared with dimeric Fc fusion proteins, and activity was found to be 1000-fold lower than the positive control FH (Fig. 3B). While KCP had poor alternative pathway decay-accelerating activity, it was still much greater than the negative control CAR-Fc and monomeric CAR, which had no significant decay-accelerating activity (Fig. 3).

KCP Acts as FI Cofactor in Cleavage of C4b—Cofactor activity of KCP was assessed in a C4b-degradation assay in the presence of FI (Fig. 4, left panel). FI degradation of radiolabeled C4b was assessed in the presence or absence of putative or control cofactors under physiological saline conditions. All three forms of KCP-Fc were found to catalyze FI cleavage of the C4b α-chain from 85 kDa to the major fragment of 46 kDa, consistent with the cleavage of C4b to C4d (Fig. 5). Cleavage of C4b was not seen if C4b was incubated with KCP alone or FI alone, confirming co-operative activity. Further, no C4b cleavage was observed following incubation with DAF-Fc and FI (not shown), indicating the specificity for the KCP portion of the KCP-Fc molecule. All three forms were roughly equivalent in
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FIG. 3. Acceleration of decay of alternative C3-convertase for Fc fusion proteins (A) or released monomers (B). Increasing concentrations of inhibitors were added to EC435 together with properdin, FD, and FB. The remaining active C3-convertases were measured by measuring released hemoglobin after the addition of guinea pig serum diluted in EDTA-containing buffer. Data are given as mean ± S.D. of quadruplicates.

FIG. 4. Diagrammatic depiction of the FI cleavage sites for C3b and C4b in the presence of various cofactors.

their ability to act as FI-cofactors, and 50% of the total C4b was converted to C4d by FI when KCP was present at 1.85 μM, 2.7 μM, and 1.55 μM, respectively, for short, medium, and long KCP-Fc forms compared with C4BP, which had a 50% total cofactor activity at 0.2 μM (not shown). However, this quantitative difference does not take into account that C4BP has seven active sites compared with the two active sites of KCP—short, medium, or long isoforms. This level of activity was calculated relative to FI, which catalyzed 50% FI cleavage of total C3b to the 64-kDa C3dg degradation product produced by FI in the presence of CR1 (Fig. 6A). N-terminal sequencing of the 30-kDa species yielded the sequence HLVTPS/GCGEQNMIGMTPT. This sequence confirmed that the first 47 amino acid residues of C3dg were missing, which is consistent with the molecular mass and sequence of C3d. Fifty percent of the C3b was degraded by FI to smaller fragments when KCP-Fc was present at concentrations of 1.9 μM, 2.15 μM, and 1.25 μM, respectively, for short, medium, and long isoforms. This level of activity was calculated relative to FH, which catalyzed 50% FI cleavage of cell-bound C3b to the 64- and 40–38-kDa cleavage products (as FH did not induce production of C3dg) when present at a concentration of 50 nM. No cleavage was observed in the absence of FI (Fig. 6B), and incubation of C3b with KCP alone did not result in any cleavage (not shown). All the cell-associated forms of KCP, including the naturally occurring form, were also found to be as effective as soluble KCP for the cleavage of C3b to iC3b and C3dg, while control CHO cells did not act as FI cofactors (Fig. 6C).

The ability of KCP to degrade cell-bound C3b was also measured by flow cytometry analysis. Sheep erythrocytes were coated with C3b in the absence of sensitizing antibody (EC3 cells) through three consecutive rounds of incubation with C3, FB, FD, and properdin under alternative pathway activating conditions. EC3 cells were then incubated overnight with FI alone or in the presence of various cofactor proteins, and the degree of C3b cleavage attained was measured by flow cytometry using a panel of C3d- and C3c-specific antibodies (Table I). The same amount of cell-surface C3d was detected irrespective of enzyme or cofactor added. However, C3c epitopes were almost entirely removed from cells incubated with FI in the presence of CR1 and KCP but not FH. The amount of C3c remained the same at overnight treatment with either FI alone or with buffer alone, indicating that no significant non-specific cleavage of C3b occurred (Table I). The same trend in specific decreased fluorescence for cells incubated with KCP or CR1 was observed with two separate polyclonal antibodies against C3c as well as a polyclonal antibody against intact C3.

Binding of C3b and C4b to KCP Proteins Assessed by Surface Plasmon Resonance (Biacore)—The affinity of binding between the KCP proteins and C3b/C4b was studied with a Biacore—Plasmon Resonance (Biacore) system. The affinity of binding between the KCP proteins and C3b/C4b was studied with a Biacore system. The affinity of binding between the KCP proteins and C3b/C4b was studied with a Biacore system.
chip using amino coupling. The three different constructs occupied different flow cells of the chip. C3b and C4b were then injected for sufficient time to reach saturation (Fig. 7A), and then $K_D$ was calculated from a binding curve showing response at equilibrium ($Req$) plotted against concentration (steady state affinity model; Fig. 7B). Binding of C3b/C4b to all three forms of KCP was evaluated both in dimeric form as well as papain-released monomeric form (Table II). Buffers used contained of KCP was evaluated both in dimeric form as well as papain-released monomeric form (Table II). Human erythrocyte ghosts were used as a source of CR1 used as a positive control (A) or FH (B), whereas untransfected CHO cells were included as a negative control (C). Cofactors were incubated with $^{125}$I-labeled C3b and FI for 1.5 h at 37 °C. Radioactive C3b fragments were visualized by autoradiography after separation by 10–15% gradient SDS/PAGE.

**Table I**

|                        | Anti-C3d | Anti-C3c (1)* | Anti-C3c (2) | Anti-C3 |
|------------------------|----------|---------------|--------------|---------|
| EC3                    | 71.2 ± 4.9 | 200 ± 2.7     | 274.8 ± 7    | 88.8 ± 2.3 |
| EC3 + FI               | 107.9 ± 4.5 | 264.6 ± 12.9 | 295.4 ± 14.4 | 131.3 ± 11.5 |
| EC3 + FH + FI          | 82.4 ± 0.7  | 166.6 ± 3.9   | 176.3 ± 17.1 | 71.04 ± 0.5 |
| EC3 + CR1 + FI         | 67.4 ± 3.8  | 21.3 ± 4.7    | 25.16 ± 3.8  | 34.64 ± 1.1 |
| EC3 + KCP + FI         | 80.8 ± 2.8  | 22.1 ± 0.3    | 21.7 ± 2.2   | 45.0 ± 7.2  |

* Two separate anti-C3c polyclonal antibodies, designated 1 and 2, were used.

### DISCUSSION

Virus-encoded proteins with homologies to host complement regulators have been previously described in two families: *Herpesviridae* and *Poxviridae*. Vaccinia complement control protein (VCP) was the first described and is the most completely characterized (21–26). Other poxviruses that retain homologues to VCP in their genome include variola virus (smallpox), cowpox, and monkeypox, the latter missing the fourth CCP domain and perhaps not functional (27–30). With the exception of Herpes Simplex virus type 1, which encodes glycoprotein C, a complement regulator that does not have homology to any known inhibitor, all herpesviruses that encode complement regulators belong to the rhadinovirus genus. Such members include HVS, M-HV68, and KSHV. The genomic organization has been conserved between the different rhadinoviruses so that the gene encoding the complement regulator is designated as ORF4 (5, 10, 31–33).

Although the functional complement-regulating ability of all the above listed proteins has been studied, there are no reports testing decay-accelerating activity separately from FI cofactor activity as we have detailed here for KSHV. We used a FI-deficient purified protein system, and we showed that KCP has true decay-accelerating activity for the classical pathway C3-
convertase but is poor at enhancing the decay of alternative pathway C3-convertase. The ability of KCP to decay classical C3-convertase is 10-fold lower than for DAF-Fc but only 5-fold lower than that observed for C4BP. However, it is important to note that C4BP is a heptamer with several equivalent binding sites and therefore activity of the single active site in KCP is comparable to that of C4BP. Moreover, the local concentration of KCP in vivo produced from KSHV-infected cells undergoing lytic replication would be high at the site of complement activation (i.e. the virally infected cell) and therefore would be far more effective than soluble host complement regulators anyway. Since KCP seems to be fairly specific for the regulation of the classical pathway as its ability to decay alternative C3-convertase was not very strong (1000-fold lower than DAF-Fc and FH), it suggests that the alternative pathway may not play a significant role in clearance of the virus in vivo. This is in contrast to the homologous viral complement regulator encoded by M-HV68, which has been reported to inhibit both alternative and classical pathways of whole mouse and human serum (11).

We determined that KCP has FI cofactor activity for the cleavage of C4b and C3b. FI cofactor activity has not been tested for any of the other rhadinoviruses, but VCP and the variola virus-encoded complement regulator SPICE have both been shown to act as FI cofactors in the cleavage of C4b and C3b (23, 24, 29). Interestingly, SPICE but not VCP has been suggested to mediate FI cleavage to the C3dg fragment, previously only described for CR1 (29, 34). However, SPICE and KCP cofactor activities are distinctly different in that the final FI degradation product for C3b in the presence of KCP was C3d not C3dg as was reported for SPICE (Fig. 6 and Ref. 29), which we confirmed through N-terminal sequence of the isolated degradation product. FI has never been shown to cleave at this site before, even in the presence of CR1, and the C3d fragment has only been reported as a cleavage product of trypsin, plasmin, or elastase (35). The most common FI cleavage sites in C3b are the two Arg-Ser sequences that result in the release of C3f; however, in the presence of CR1, FI cleaves iC3b at an Arg-Glu sequence and under non-physiological conditions has been reported to cleave iC3b at a Lys-Glu sequence (36). It is therefore surprising that under physiological saline conditions we observed a FI cleavage of C3b at a Glu-His sequence in the presence of KCP. The mechanism by which this occurs is under further investigation. It is important to point out that KCP is the only viral protein that has been shown to degrade cell-associated C3b to C3d, proving that this activity is not an artifact of soluble phase experiments (Table I).

We measured the binding affinity of KCP to C4b and C3b (Table II) using Biacore. These data indicate that all three KCP species are capable of binding C3b and C4b but that the affinity of KCP for C4b is at least 10-fold higher than for C3b. The addition of physiological levels of zinc increased the binding of KCP to C4b 10-fold under all conditions. The higher binding affinity of KCP for C4b, relative to C3b binding, also supports our findings that KCP is better at accelerating the decay of the classical, compared with the alternative, C3-convertase. In comparison to host-encoded complement regulators, we found the that the affinity of KCP for C4b was 7-fold less than that measured for C4BP (K_D = 0.5 µM in 150 mM NaCl and 5 mM EDTA), and for C3b was 20-fold less than FH (K_D = 0.4 µM in 150 mM NaCl and 5 mM EDTA) under the same conditions (not shown).

The ability of KCP to catalyze the cleavage of C3b to C3d by FI is unique among the virus and host complement regulators. It will be important to study the FI cofactor activity for the other rhadinovirus members HVS and M-HV68, as these have not yet been investigated. There may be an evolutionary reason for KSHV or KSHV-infected cells to promote the production of C3d: the receptor for C3d is CR2, expressed predominantly by B-cells, which has been identified as one of the KSHV viral reservoirs (37, 38). Thus KSHV may have evolved a mechanism that not only allows evasion of clearance of virions and virus-infected cells but may be using the complement system to target the virus to primary sites of pathogenesis during natural infection as B-cell infection by KSHV is a central aspect of primary effusion lymphoma and Castleman’s disease (reviewed in Ref. 3 and 4). However, KCP is only expressed in infected cells during lytic replication in B cells (9). While this expression profile may facilitate KSHV transmission from infected cells to uninfected B-cells, whether KCP facilitates infection by extracellular virions through expression on the envelope is currently under investigation.

The importance of complement evasion by rhadinoviruses has recently been underscored in a thorough pathological examination of M-HV68 infection compared with a recombinant virus lacking the complement regulating homologue (39). Deletion of ORF4 from M-HV68 resulted in a dramatic decrease in virulence during acute central nervous system infection of immunocompetent mice and in mortality in persistently infected immunocompromised mice, and this attenuation was not seen in C3-deficient mice. Furthermore, C3 played a role in regulating latency of the virus, but the M-HV68 ORF4 could not counteract this effect (39). This also emphasizes the importance of KCP inhibition of the classical pathway C3-convertase and degradation of cell-bound C3b and C4b that we have detailed here. Extended to in vivo infections, these complement-regulating activities would facilitate KSHV replication and enhance virus pathogenesis through a combination of blocking virion and/or infected cell opsonization, blocking complement-mediated virolysis, and inhibiting anaphylaxis.

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O. Brad Spiller, David J. Blackbourn, Linda Mark, David G. Proctor and Anna M. Blom

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