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Influence of Electrostatics on the Complement Regulatory Functions of Kaposica, the Complement Inhibitor of Kaposi’s Sarcoma-Associated Herpesvirus

Kalyani Pyaram,* Chris A. Kieslich,† Viveka Nand Yadav,* Dimitrios Morikis,† and Arvind Sahu*

Kaposica, the complement regulator of Kaposi’s sarcoma-associated herpesvirus, inhibits complement by supporting factor I-mediated inactivation of the proteolytically activated form of C3 (C3b) and C4 (C4b) (cofactor activity [CFA]) and by accelerating the decay of classical and alternative pathway C3-convertases (decay-accelerating activity [DAA]). Previous data suggested that electrostatic interactions play a critical role in the binding of viral complement regulators to their targets, C3b and C4b. We therefore investigated how electrostatic potential on Kaposica influences its activities. We built a homology structure of Kaposica and calculated the electrostatic potential of the molecule, using the Poisson–Boltzmann equation. Mutants were then designed to alter the overall positive potential of the molecule or of each of its domains and linkers by mutating Lys/Arg to Glu/Gln, and the functional activities of the expressed mutants were analyzed. Our data indicate that 1) positive potential at specific sites and not the overall positive potential on the molecule guides the CFAs and classical pathway DAA; 2) positive potential around the linkers between complement control protein domains (CCPs) 1–2 and 2–3 is more important for DAAs than for CFAs; 3) positive potential in CCP1 is crucial for binding to C3b and C4b, and thereby its functional activities; 4) conversion to negative or enhancement of negative potential for CCPS 2–4 has a marked effect on C3b-linked activities as opposed to C4b-linked activities; and 5) reversal of the electrostatic potential of CCP4 to negative has a differential effect on classical and alternative pathway DAAs. Together, our data provide functional relevance to conservation of positive potential in CCPs 1 and 4 and the linkers of viral complement regulators. The Journal of Immunology, 2010, 184: 000–000.

The complement system is an ancient yet highly effective immune surveillance system against a wide array of pathogens including viruses (1, 2). It is known to directly assail viruses by means of opsonization, leading to aggregation, phagocytosis, and lysis (3, 4). In addition, it also promotes virus-specific B and T cell responses (5, 6). Owing to the intense threat that pathogens, including viruses (1, 2), pose, it is not surprising that many viruses have learned to escape from the complement surveillance to become successful human pathogens (1, 7–9). The formidable complement evasion mechanisms developed by viruses include encoding of structural and/or functional homologues of host complement regulators (e.g., herpesviruses and poxviruses) (10–14), capturing of host complement regulatory proteins (e.g., poxviruses, herpesviruses, paramyxoviruses, and retroviruses) (3, 15, 16), and use of host complement receptors for cellular entry (e.g., herpesviruses, paramyxovirus, and picornaviruses) (4, 17).

Kaposi’s sarcoma-associated herpesvirus (also known as human herpesvirus 8) is a γ-herpesvirus belonging to genus Rhadinovirus (18). It is etiologically associated with Kaposi’s sarcoma and also with lymphoproliferative disorders like primary effusion lymphoma and multicentric Castleman’s disease (19, 20). The virus encodes >90 open reading frames (ORFs), of which ORF4 encodes a protein that shares prominent homology with human regulators of complement activation (RCA) (19, 20). The ORF4 protein is composed of four complement control protein domains (CCPs) connected by four residue linkers, followed by a dicysteine motif, a serine/threonine (S/T)-rich region and a transmembrane domain for membrane attachment. Studies in primary lymphoma effusion–derived cell line indicated the presence of at least two alternatively spliced transcripts in addition to the full-length mRNA. The spliced transcripts retained the CCP and transmembrane domains, but lacked either the S/T region or the dicysteine motif and the S/T region (21). Functional characterization of the protein by us and others revealed that both the soluble (CCPs 1–4) (22) and the membrane-associated forms (21) possess the ability to inhibit the proteolytically activated form of C3 (C3b) deposition on the cell surface. The protein was named Kaposica (Kaposi’s sarcoma-associated herpesvirus inhibitor of complement activation) by us (22) and KCP by others (21). The protein was also found to be expressed on Kaposi’s sarcoma-associated herpesvirus–infected cells as well as on the virion envelope (23), supporting the premise that the virus indeed has the capability to evade complement.

Studies on the mechanism of complement inhibition by Kaposica showed that it regulates C3-convertases by supporting serine...
protease factor I-mediated inactivation of complement proteins C3b and the proteolytically activated form of C4 (C4b), the subunits of C3-convertases (termed cofactor activity [CFA]), and by accelerating irreversible dissociation of the classical/lectin (C4b,2a) pathway and, to a limited extent, of the alternative pathway (AP) (C3b,Bb) C3-convertases (termed decay-accelerating activity [DAA]) (22, 24). The protein has also been mapped for its functional domains (25). Data revealed that CCPs 1–2 are the minimum essential domains required for classical/lectin pathway C3-convertase DAA, but CCP3 contributes to optimal activity. The AP DAA required all the CCPs. Interestingly, unlike human complement regulators, CCPs 2–3 (only two CCPs) displayed CFA for C3b and C4b; CCPs 1 and 4, however, contributed to optimal C3b CFA. In a subsequent domain mapping study utilizing the full-length protein, data obtained were largely similar, with the exception that CCPs 1–3 and 1–4 were shown to be required for C4b and C3b CFAs, respectively, as opposed to CCPs 2–3 (23). It is likely that the lower sensitivity of the assays used by the later study was responsible for these discrepancies.

The mechanism of molecular recognition between Kaposica and its target proteins C3b and C4b indicated that it involves ionic contacts (26), which was also observed in other viral and human complement regulators (27–31). Although it is clear that ionic interactions form an essential component of binding interface between human as well as viral complement regulators, and their target proteins C3b and C4b, what largely remained unanswered is this: Does overall electrostatic potential of a complement regulator or of its individual CCP domains affect the complement regulatory activities? And what is the relative contribution of each of the individual CCP domains? In recent immunophysical studies on vaccinia virus complement control protein (VCP) and variola smallpox inhibitor of complement enzyme (SPICE), it was observed that the overall positive electrostatic potential of VCP and SPICE correlates well with C3b binding and CFA (32, 33). In addition, localization of the positive electrostatic potential was shown to play a role in VCP and SPICE (32); for example, increase in the positive electrostatic potential of CCPs 1 and 4 due to decrease in the negative electrostatic potential at the interface area of CCPs 2 and 3 resulted in greater C3b binding, inhibition of the AP, and CFA. On the basis of these data, it was proposed that long-range electrostatic interactions drive the docking of complement regulators onto C3b/ C4b and such correlations can be used to predict binding and functional activities of complement regulators (33). These data therefore suggested that the effectiveness of viral complement regulators as immune evasion proteins is dictated by the degree of positive electrostatic field surrounding them. To date, however, no systematic study has been performed to answer how electrostatic potential of a complement regulator affects complement regulatory activities. Intriguingly, the positive electrostatic potential is conserved in CCPs 1 and 4 as well as the links between CCPs 1–2 and 2–3 in various herpes and pox viral complement regulators. In the current study, we therefore used Kaposica as a model protein to understand how spatial distribution of charge on the CCP domain(s) influences the function of viral regulators. We analyzed the physicochemical properties of Kaposica and conducted a systematic mutagenesis study to decipher whether overall positive electrostatic potential of the molecule or of its individual domains influences its various complement regulatory activities and whether it has a differential effect on various functional activities of Kaposica.

Materials and Methods

Proteins, reagents, and buffers

The human complement proteins C1, C2, C4, factor D, C4b, and factor I were purchased from Calbiochem (La Jolla, CA). Human C3 and factor B were purified as previously described (34, 35). Native C3 was separated from C3 (H2O) by running the sample on Mono S column (36). C3b, the proteolytically activated form of C3, was generated by limited tryptic cleavage of human C3 and purified using Mono Q 5/5 column (Amersham Pharmacia Biotech, Uppsala, Sweden), as described (37). All the proteins were >95% pure, as judged by SDS-PAGE analysis. Ab-coated sheep Es were prepared by incubating sheep E Abs obtained from ICN Biomedical (Irvine, CA). Veronal-buffered saline contained 5 mM barbital, 145 mM NaCl, and 0.02% sodium azide, pH 7.4. Dextrse gelatin veronal-buffered saline (DGSBV) contained 0.02% gelatin, 2.5 mM NaCl, 0.1% gelatin, 2.5% dextrse, and 0.02% sodium azide, pH 7.4. DGVB2+ was DGVB with 0.5 mM MgCl2 and 0.15 mM CaCl2, PBS contained 10 mM sodium phosphate and 145 mM NaCl, pH 7.4.

Molecular modeling

Our atomic-level calculations are possible when three-dimensional structures are known at high resolution. However, the structure of Kaposica is yet to be solved; therefore, our initial step was the generation of a Kaposica homology model. The three-dimensional structure of a known homologue, has been experimentally determined [Protein Data Bank Code: 1G40 (38)] and was used for the homology modeling of Kaposica. To generate the homology model for Kaposica, we first obtained the amino acid sequence alignment for the comparison of VCP, our template, and Kaposica, our target, using ClustalW (39). The generated amino acid sequence alignment was manually optimized to enhance the level of sequence identity. The optimized sequence alignment and template structure, 1G40, were imported into a DeepView, Swiss Institute of Bioinformatics (http://spdbv.vital-it.ch) project and submitted to the Project Mode of the SWISS-MODEL online server (40). The generated Kaposica model was inspected for van der Waals clashes, secondary structure quality, and disulfide bond correctness. The Kaposica model was created with all four CFP modules intact. Models for each CCP module were extracted from the whole Kaposica model. In this study, the CCP module is defined as the sequence between and including the first and fourth conserved cysteines. The theoretical mutations were generated by importing coordinates, corresponding to either the entire Kaposica molecule or individual CCP modules, into Deep View and selecting the mutation site and type. Because multiple rotamers can be generated for each mutation, the rotamer that showed the most favored contacts, primarily hydrogen bonds, while minimizing the number of van der Waals clashes, was selected.

Electrostatic potential calculations

Electrostatic potential calculations were made with the Adaptive Poisson–Boltzmann Solver (41), which uses a grid-based method to solve the linearized Poisson–Boltzmann equation. Prior to electrostatic potential calculations, partial charges and van der Waals radii were assigned using the software PDB2PQR (42) and the PARSE force field (43). A dielectric coefficient of 2 was used for the protein, whereas a dielectric coefficient of 78.5 was used for the solvent. The calculations were carried out at an ionic strength corresponding to a 0 mM concentration, assuming +1/−1 charges for the counterions. The dielectric surface was defined by selecting the contact surface, using a sphere with a probe radius of 1.4 Å. The ion accessibility surface was defined using a sphere with a probe radius of 2.0 Å. Because calculations were performed on the intact Kaposica macromolecule, as well as individual CCP modules, the physical dimensions of the box used for each set of calculations were set to different values to ensure that the largest magnitude values were captured, while still providing maximum resolution. The grid size was 129 × 129 × 129 points. A box size of 130 Å × 120 Å × 190 Å was used for calculations of whole Kaposica, whereas a box size of 100 Å × 100 Å × 140 Å was used for calculations of individual CCP modules. The spatial distributions of electrostatic potential were visualized and analyzed using isopotential contours using the molecular graphics program Visual Molecular Dynamics [44]; Theoretical and Computational Biophysics Group, Urbana, IL; www.ks.uiuc.edu/Research/vmd].

Cloning of the soluble form of Kaposica and construction of its mutants by site-directed mutagenesis

The soluble form of Kaposica (CCPs 1–4) was PCR amplified from the Kaposica clone in pPICZαA (22), with the specific primers 5′-CATgC- CATgggCAAgTgTTCCCAAAAAACCTTAATTgg-3′ (the NcoI site is underlined) and 5′-CCCAAAGCtCtCCAAAGCtCtAACTtAAAATTgg-3′ (the NcoI site is underlined), using Pfu DNA Polymerase (Qagen, Hilden, Germany) and cloned into the pGEM-T Easy Vector System (Promega, Madison, WI). This clone then served as a template to generate mutants, using site-directed mutagenesis. The amplified Kaposica was also cloned into the bacterial expression vector pET28a at the NcoI and HindIII

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s. The fidelity of both the clones was verified by DNA sequencing, using an automated 3730 DNA analyzer (Applied Biosystems, Foster City, CA).

The mutants of Kaposica with altered positive charge were generated by substituting the positively charged lysine (K) or arginine (R) with the negatively charged glutamate (E) or the neutral glutamine (Q). The template (pGEMT-Kaposica clone) was mutated using the commercially available QuickChange II or QuickChange Multisite-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), according to the manufacturer’s instructions. Two sets of mutants were generated. In the first set of mutants, the positive charge in CCP1 and/or the links between CCPs 1–2 and 2–3 was negated. These mutants were K5E/K9E (mutant 1, M1), K5E/K9E/K17E (M2), K6E/K65E (M3), K131E/K133E (M4), K64Q/K65Q (M5), K131Q/K133Q (M6), and K5E/K9E/K17E/K131E/K133E (M7). Here, the number, the letter represents the location of the amino acid in the mature protein, and the letters before and after the number denote the amino acid in Kaposica and the amino acid to which it was mutated, respectively. In the second set of mutants, the charge on each of the domains was completely changed to negative, or the negative character was further enhanced. These were K9E/R15E/R20E/R43E (domain mutant, D1), K77E/K88E/R106E (D2), R136E/K138E (D3), and K194E/K212E/K214E/R228E (D4). The mutants were then cloned into PET28 expression vector, and the authenticity of all the above mutants was validated by automated DNA sequencing. For expression, the PET clones of Kaposica and the mutants were transformed into Escherichia coli BL21 cells.

Expression, purification, and refolding of Kaposica and its mutants

Expression and purification of Kaposica and its mutants was performed using the PET expression system as described before (35, 45). The purified proteins were then refolded by the rapid dilution method (35, 45, 46) and further purified on a Superose-12 gel filtration column (Pharmacia) to obtain the monodispersed population of proteins. The proteins thus obtained were concentrated and subjected to SDS-PAGE and circular dichroism analyses (35).

Measurement of factor I CFA for C3b and C4b

Analysis of factor I CFAs of Kaposica and its mutants was performed in PBS, pH 7.4, as previously described (27).

Measurement of C3-convertase DAA

The classical pathway (CP) and AP DAAs of Kaposica and its mutants was measured by forming CP C3-convertase (C4b2a) and AP C3-convertase (C5b,6b) on sheep and rabbit Es respectively as described (34, 47).

Surface plasmon resonance measurements

Binding of Kaposica and its mutants with human C3b and C4b was assessed using a surface plasmon resonance (SPR)-based biosensor, Biacore 2000 (Biacore AB, Upplands, Sweden). Binding analysis of all the proteins with C4b was performed in HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 50 μM EDTA, 0.05% surfactant Tween 20, pH 7.4) at 25°C as described (45). In brief, a nitrotriacetic acid chip coated with nickel using 500 μM NiSO₄ was activated with 500 μM NISO₄ and immobilized with Kaposica or its mutants (~500 response units [RU]) through their C-terminal His® tag on the test flow cells (FC-2, FC-3, and FC-4), whereas the control flow cell (FC-1) was immobilized with equivalent RU of an unrelated protein from Mycobacterium tuberculosis (hypothetical protein, TlyA). Binding of C4b to the immobilized ligand was measured by flowing 125 nM human C4b at 10 μl/min for 180 s, following dissociation for 180 s. The sensor chip was regenerated with 30-s pulses of 0.2 M sodium carbonate, pH 9.5, and regeneration buffer (10 mM HEPES, 150 mM NaCl, 0.35M EDTA, 0.005% surfactant P20, pH 7.4). The specific binding response to C4b for each ligand protein was obtained by subtracting the response generated by the control flow cell (FC-1) from the data obtained for the flow cell immobilized with the test ligand.

Because the binding response of Kaposica to C3b was much lower than that to C4b, the same ligand-analyte orientation did not provide adequate binding response when ligands were immobilized at a 500-RU level. Further increase in the immobilization level resulted in a significant drift in the baseline. We therefore reversed the setup for measuring binding of Kaposica/mutants to C3b. C3b was immobilized on the chip (27), and Kaposica/mutants were flown. In brief, ~1000 RU of biotinylated C3b was immobilized onto a test flow cell (FC-2) of streptavidin chip (Sensor Chip SA; Biacore AB). The flow cell was blocked by flowing BSA-biotin, and then more molecules of C3b (6000 RU) were deposited by forming the AP C3-convertase on the test flow cell (M1–M7). The control flow cell (FC-1) had C3b immobilized by biotinylating BSA-biotin. Binding of Kaposica and its mutants to C3b was measured by flowing various concentrations of these proteins for 120 s at 50 μl/min. Subsequently, dissociation was followed for 180 s. The sensor chips were regenerated with 30-s pulses of 0.2 M sodium carbonate, pH 9.5. The specific binding response of each protein to C3b was obtained by subtracting the response generated by the control flow cell from the data obtained for the flow cell immobilized with C3b.

Results

Electrostatic calculations, theoretical design, and expression of Kaposica mutants

For this study, 11 Kaposica mutants were designed to delineate the role of the electrostatic character of each individual CCP module and linker. The designed mutants were generated using soluble Kaposica (CCPs 1–4) as a template and substituting positively charged Lys/Arg residues with negatively charged Gln or neutral Gln. During mutant design, numerous theoretical mutants were created and Poisson–Boltzmann electrostatic calculations were made to analyze the effect of these mutations on the spatial distribution of electrostatic potential. The Poisson–Boltzmann method was used in this study owing to its ability to account for variability in the dielectric medium and the presence of counterions, which is essential when modeling the complex environment of proteins in aqueous solutions. The theoretical mutants that provided the largest effect on electrostatic potential with the fewest number of mutations were selected for experimental analysis. The two sets of theoretical mutants were analyzed; The first set involved mutants with varying electrostatic properties generated by muta- tions within the CCP1 module and/or linkers (M1–M7, Fig. 1A), whereas the second set of mutants were designed with the goal of reversing the charge of the individual CCP modules (D1–D4, Fig. 5A). The D1–D4 mutants were designed by performing electrostatic calculations on each individual CCP module. However, the mutations were incorporated in the full-length Kaposica (CCPs 1–4). All the mutants, including Kaposica, were then expressed in E. coli and purified to homogeneity (Figs. 1B, 5B). The proteins yielded a peak at around 230 nm upon circular dichroism analysis, emblematic of CCP domain-containing proteins, validating the proper conformation of the refolded proteins.

Does overall positive electrostatic potential guide the CFAs of Kaposica?

Kaposica supports inactivation of C3b and C4b by the serine protease factor I, by acting as a cofactor. To elucidate the importance of overall positive electrostatic potential of Kaposica in performing the function of a cofactor, we expressed a series of seven mutants (M1–M7) with decreased overall positive electrostatic potential and analyzed their C3b and C4b CFAs. If the overall positive electrostatic potential guides CFAs, then the overall positive potential on the regulator should correlate well with the CFAs and the mutant with the most negative potential should show the least activity. Consistent with this premise, the mutant with the most overall negative electrostatic potential (M7) was largely inactive for C3b and C4b CFAs (Figs. 2, 3, Table I). Further, mutants M3–M6, in which overall positive electrostatic potential was reduced by replacement of positive residues at the linkers between CCPs 1–2 and 2–3, also showed reduction in CFAs; the effect, however, was more pronounced on C3b CFA than C4b CFA. Conversely, this trend was not followed by other mutants. For example, mutants M1 and M2, in which overall positive potential was reduced owing to reduction in the positive potential around the CCP1 (Fig. 1), showed no difference in C3b and C4b CFAs in comparison with the wild-type protein (Figs. 2, 3, Table I). These data thus suggest that the decrease in CFAs observed by imparting negative potential in the molecule depended more on the site of negation (interface between CCPs 1–2 and 2–3) than on the overall decrease in positive potential in the molecule.
Does overall positive electrostatic potential direct the DAAs?

In addition to supporting the protease-mediated inactivation of subunits of C3-convertases, Kaposica also regulates CP and AP C3-convertases by accelerating their decay. We therefore next compared the DAAs of Kaposica and its mutants to evaluate whether overall positive potential influences these activities. To measure the decay activities, CP(C4b,2a) and AP(C3b,Bb) C3-convertases were formed on sheep and rabbit Es, respectively, using purified complement proteins. These enzymes were then allowed to decay in the presence of increasing concentrations of Kaposica or each of the mutants, and the remaining C3-convertase activity was estimated by measuring hemolysis following addition of EDTA-sera (a source of C3–C9).

The data presented in Fig. 4 show that, as with CFAs, the M7 mutant, which possessed maximum negative potential, displayed only residual CP DAA. Among other mutants, N-terminal positive potential reduction mutants (M1 and M2) did not show any effect on the CP DAA, but mutants in which overall positive potential was reduced owing to negation of charge at the linkers (M3–M6) showed considerable reduction in CP DAA (Fig. 4, Table I). It is therefore apparent that positive potential around the linker regions, and not the overall positive potential, is important for driving CP DAA.

Earlier it had been shown that Kaposica possesses a very weak AP C3-convertase DAA (25, 49). We thus also sought to analyze the dependence of AP DAA on overall positive electrostatic potential. Interestingly, unlike the CP DAA and CFAs, all the mutants except M1 showed considerable reduction in AP DAA (Fig. 4, Table I), suggesting that in contrast to these activities AP DAA is more susceptible to reduction in positive potential on CCP1.

Influence of electrostatic potential of individual domains of Kaposica on its CFAs

The data obtained from the first set of mutants indicated that decrease in functional activities (except AP DAA) of Kaposica depended more on the site of reduction of positive electrostatic potential (e.g., interface between CCP 1–2 and 2–3) than on decrease in overall positive potential in the molecule. Hence, we next sought to determine the influence of alteration of electrostatic potential of each of the CCP domains on the CFAs. For this purpose, a set of four mutants were designed and expressed wherein electrostatic potential of the individual domains with positive (CCP1 and CCP4) or neutral (CCP2) potential was changed to negative, and the domain with negative potential (CCP3) was mutated to increase its negativity (Fig. 5A).

The data presented in Fig. 6 clearly show that the effect of negative potential on various domains of Kaposica was greater on C3b CFA than on C4b CFA. The mutants D1–D4 displayed ∼7–83-fold decrease in C3b CFA, compared with Kaposica. In contrast, except D1, none of the mutants displayed >2-fold decrease in C4b CFA (Table I). Thus, increase in negative potential around any of the domains in the molecule is not tolerated for C3b CFA, suggesting that maintenance of intrinsic potential on all the domains is critical for C3b CFA. Such, however, is not the case for C4b CFA, as, except for CCP1, negative potential is well tolerated around domains 2–4.

Influence of electrostatic potential of individual domains of Kaposica on its DAAs

Having dissected out the importance of intrinsic electrostatic potential of individual CCP domains in the CFAs, we next examined the role of the same in decay acceleration of CP and AP C3-convertases. The CP DAAs of the four domain mutants varied interestingly (Fig. 7, Table I). D1 showed a complete elimination of CP DAA, indicating that positive potential on CCP1 of Kaposica is indispensable for this activity. Imparting negative electrostatic potential on CCP2 (D2) and CCP3 (D3) did not lead to any significant change in CP DAA. A fascinating finding was that the D4 mutant that carried negative potential instead of positive potential at CCP4 of Kaposica displayed a remarkable 4.5-fold increase in CP DAA (Fig. 7, Table I).

The assessment of AP DAA of the CCP domain mutants, as depicted in Fig. 7, revealed that imparting negative electrostatic potential on any of the four CCP domains led to either considerable decrease or abolishment of activity. Thus, as with C3b CFA, maintenance of intrinsic electrostatic potential on all four CCP domains of Kaposica is also critical for AP DAA.
Influence of electrostatic potential on binding of Kaposica to C4b and C3b

Our electrostatic modeling predicts direct binary interactions between Kaposica and its target proteins C3b and C4b; therefore, we next measured binding of Kaposica and its mutants (M1–M7 and D1–D4) to C3b and C4b, to assess how electrostatic potential around various domains influences binding. In addition, we also examined whether binding correlates with functional activities. It should, however, be pointed out that ligand binding does not always correlate with CFA and DAA (50, 51). CFA involves binding of viral complement regulator to C4b or C3b, followed by binding of factor I to C4b or C3b and the complement regulator (52). Decay acceleration, in contrast, entails interaction of viral complement regulator with C4b or C3b as well as with C2a or Bb (53, 54).

We used an SPR-based binding assay described in our previous studies (27, 45) for measurement of binding responses. There was a remarkable reduction in the binding of M7 mutant to C4b, which carries the most negative electrostatic potential (Fig. 8). Significant decrease in binding to C4b was also observed in mutants with altered electrostatic potential in the linker region (M3–M7) and CCP1 (M2); M1, with two-residue replacement in CCP1, did not show any effect on C4b binding. Among domain electrostatic potential modulation mutants, D1–D3 showed significantly reduced binding, whereas D4 showed marginally reduced binding to C4b. These data together point out that positive potential around CCP1 and the linkers between CCPs 1–2 and 2–3 of Kaposica are vital for binding to C4b. When we looked for dependence of functional activities on binding, we observed a significant correlation between C4b binding response and C4b CFA ($r = 0.719$, $p = 0.008$) and between C4b binding response and CP DAA ($r = 0.618$, $p = 0.032$).

In the case of binding to C3b, flowing of 4 μM Kaposica over a C3b-deposited sensor chip showed good binding response to C3b (Fig. 9). Flowing of similar amounts of mutants showed that binding of M7 to C3b, like that to C4b, was also substantially reduced (Fig. 9). Binding of M3–M6 showed moderate to substantial reduction, but no reduction was observed in binding of M1 and M2, suggesting that C3b binding is less susceptible than C4b binding to partial negation of positive potential on CCP1. Among the domain electrostatic negation mutants, binding was highly attenuated in D1, D2, and D4 (Fig. 9). These results suggest that, as with C4b binding, positive potential around CCP1 and the linkers between CCP1–2 and 2–3 is important for C3b binding. Unlike for C4b binding, however, positive potential on CCP4 is also equally important for C3b binding. A comparison of C3b binding with functional activities showed a significant correlation between C3b binding and C3b CFA ($r = 0.881$, $p = 0.0002$) and between C3b binding and AP DAA ($r = 0.779$, $p = 0.003$).

Discussion

Viral complement regulators have been shown to play an essential role in viral pathogenesis (55–57), and thus it is imperative to understand the molecular mechanisms underlying the regulation of host complement by these molecules. Among these viral
FIGURE 3. Time course analysis of factor I CFA of Kaposica and its charge reversal mutants for complement protein C4b. For assessing the CFA, 1 μg of Kaposica or each of the mutants (M1–M7) was incubated with 3 μg of C4b in the presence or absence of 100 ng of factor I in PBS, pH 7.4, at 37°C. The reactions were stopped at the indicated time periods by the addition of sample buffer containing DTT, and cleavage products were visualized by subjecting the reactions to 11% SDS-PAGE under reducing conditions, followed by staining with Coomassie blue. Cleavage of αc-chain of C4b by factor I results in the generation of N-terminal 27-kDa, C-terminal 16-kDa (not visualized in the gel), and central C4d fragments indicating inactivation of C4b. The amount of αc-chain remaining after various intervals was quantitated by densitometric analysis and is represented graphically against time.

There is a long-standing view in the field of complement biology that ionic interactions play a vital role in complement regulation. This premise is primarily based on salt-dependent binding of complement regulators to C3b/C4b and mutagenesis data demonstrating that acidic residues on C3b/C4b, and basic residues on complement regulators, are important for the interaction between these proteins (28–31). More recently, on the basis of mutagenesis and electrostatic modeling studies of VCP and SPICE, it has been proposed that overall positive potential on these viral regulators directs the initial recognition of C3b (termed step I). Recognition is then followed by binding, which involves electrostatic interactions (hydrogen bonds, salt bridges, and medium/weak coulombic interactions), hydrophobic and van der Waals interactions, complement regulators, the structural homologues of host RCA proteins encoded by herpes and pox viruses showed ionic strength-dependent binding to their target proteins (26, 27), which suggested that electrostatic potential in the form of long-range interactions and/or surface charge in the form of short-range interactions play a prominent role in their binding and thereby function. In the current study, we investigated the influence of electrostatic potential on the functional activities of Kaposica. Like most viral mimics of RCA, Kaposica is formed by four CCP domains and inactivates C3-convertases by accelerating the decay of C3-convertases as well as by supporting factor I-mediated cleavage of subunits of C3-convertases (C3b and C4b); thus it provides a good example for studying how electrostatics modulate functioning of viral RCA proteins.

Table I. Summary of complement regulatory activities of Kaposica and its mutants

<table>
<thead>
<tr>
<th>Wild-Type/ Mutant</th>
<th>Total Charge</th>
<th>Time for 50% Cleavage of Human C3b αc-Chain (min)</th>
<th>Relative Percentage of C3b CFAa</th>
<th>Time for 50% Cleavage of C4b αc-Chain (min)</th>
<th>Relative Percentage of C4b CFAa</th>
<th>CP DAA IC50 (μM)</th>
<th>Relative Percentage of CP DAAa</th>
<th>AP DAA IC50 (μM)</th>
<th>Relative Percentage of AP DAAa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaposica</td>
<td></td>
<td>3.3 ± 0.3</td>
<td>100</td>
<td>6.5 ± 1.8</td>
<td>100</td>
<td>0.22 ± 0.05</td>
<td>100</td>
<td>6.0 ± 2.1</td>
<td>100</td>
</tr>
<tr>
<td>M1</td>
<td>−2</td>
<td>3.1 ± 0.3</td>
<td>106.4</td>
<td>6.3 ± 2.3</td>
<td>103.2</td>
<td>0.19 ± 0.02</td>
<td>115.8</td>
<td>11.45 ± 3.2</td>
<td>52.4</td>
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<tr>
<td>M2</td>
<td>−4</td>
<td>3.8 ± 0.2</td>
<td>86.8</td>
<td>6.7 ± 1.1</td>
<td>97.0</td>
<td>0.3 ± 0.03</td>
<td>73.3</td>
<td>22.2 ± 6.1</td>
<td>27.0</td>
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<tr>
<td>M3</td>
<td>−2</td>
<td>17 ± 3.6</td>
<td>19.4</td>
<td>11.7 ± 2.4</td>
<td>55.5</td>
<td>1.2 ± 0.2</td>
<td>18.3</td>
<td>57.7 ± 13</td>
<td>10.4</td>
</tr>
<tr>
<td>M4</td>
<td>−2</td>
<td>9.2 ± 1.3</td>
<td>35.9</td>
<td>8.2 ± 1.6</td>
<td>79.3</td>
<td>0.96 ± 0.5</td>
<td>22.9</td>
<td>37.7 ± 4.6</td>
<td>15.9</td>
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<tr>
<td>M5</td>
<td>0</td>
<td>9.2 ± 0.7</td>
<td>35.9</td>
<td>15 ± 2.6</td>
<td>43.3</td>
<td>2.8 ± 0.2</td>
<td>7.9</td>
<td>29.5 ± 5.0</td>
<td>20.3</td>
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<tr>
<td>M6</td>
<td>0</td>
<td>19.7 ± 2.5</td>
<td>16.7</td>
<td>16.2 ± 4.9</td>
<td>40.1</td>
<td>1.5 ± 0.5</td>
<td>14.7</td>
<td>64.7 ± 26.8</td>
<td>9.3</td>
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<tr>
<td>M7</td>
<td>−8</td>
<td>&gt;480b</td>
<td>&lt;0.7</td>
<td>&gt;480b</td>
<td>&lt;0.7</td>
<td>5.7 ± 1.2</td>
<td>3.9</td>
<td>&gt;50 c</td>
<td>&lt;12</td>
</tr>
<tr>
<td>D1</td>
<td>−6</td>
<td>38.8 ± 6.7</td>
<td>8.5</td>
<td>16 ± 1.0</td>
<td>40.6</td>
<td>&gt;10c</td>
<td>&lt;2.2</td>
<td>&gt;50 c</td>
<td>&lt;12</td>
</tr>
<tr>
<td>D2</td>
<td>−4</td>
<td>278 ± 19.3</td>
<td>1.2</td>
<td>11.3 ± 0.3</td>
<td>57.5</td>
<td>0.32 ± 0.04</td>
<td>68.7</td>
<td>&gt;50 c</td>
<td>&lt;12</td>
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<tr>
<td>D3</td>
<td>−2</td>
<td>34.5 ± 4.5</td>
<td>9.6</td>
<td>11 ± 1.0</td>
<td>59.1</td>
<td>0.17 ± 0.03</td>
<td>129.4</td>
<td>60.7 ± 5.0</td>
<td>9.9</td>
</tr>
<tr>
<td>D4</td>
<td>−6</td>
<td>22 ± 5.2</td>
<td>15</td>
<td>10.2 ± 3.3</td>
<td>63.7</td>
<td>0.048 ± 0.007</td>
<td>458.3</td>
<td>200 ± 130</td>
<td>3.0</td>
</tr>
</tbody>
</table>

aRelative activity compared with Kaposica: >50%, no effect; 30–50%, limited reduction; 5–30%, considerable reduction; <5%, abolished activity; >200%, significant increase.

bOnly minimal cleavage was observed even after 8 h incubation.

cHighest concentration of mutant that could be tested in the assay. Data are reported as mean ± SD.
and entropic effects such as solvent exclusion and local structural rearrangements at the binding sites (termed step II) (32, 33). According to this association model, step I influences predominantly the \( k_{on} \) rate and the formation of a weak, nonspecific encounter complex, whereas step II influences predominantly the \( k_{off} \) rate and the formation of a strong, specific bound complex (33, 58). In the earlier studies of VCP/SPICE, correlation was found between the reduction of negative character of the CCP2/3 interface and C3b binding, inhibition of AP, and CFA (32). Indeed, because of spatial cancellation of opposite electrostatic potentials, which is modulated by the dynamic character of VCP/SPICE, an increase of positive potential in one module may contribute to the decrease of negative potential in a nearby module, depending on potential magnitude (33).

We therefore asked whether the overall or localized electrostatic potential guides the complement regulatory activities of Kaposica. To answer this, we applied structural perturbations by mutating positively charged residues. We designed two sets of mutants; the first (Fig. 1) was designed to reduce or eliminate positive potential in the whole molecule, and the second (Fig. 5) was designed to delineate the role of electrostatic potential of the individual CCP modules. The electrostatic potential of Kaposica and the mutants was then calculated, and activities of the expressed proteins were compared to determine the dependence of activities on positive potential in the molecule. The electrostatic calculations depict binary interactions between Kaposica and C3b/C4b, which can be more directly validated by SPR data (Figs. 2–4, 6, 7). When other molecules are involved (factor I or convertase components), additional complexity is introduced, which cannot be incorporated into our modeling at this time because of lack of three-dimensional structures of the multicomponent assemblies.

The SPR data showed that the constructs containing mutations in the CCP1–CCP2 and CCP2–CCP3 linkers, M3–M7, nearly eliminated C4b binding (Fig. 8). The construct with only two CCP1 mutations, M1, had no effect on C4b binding, whereas that with three CCP1 mutations, M2, had significant effect in reducing C4b binding (Fig. 8). This is because the negative electrostatic potential of M2 is distributed more uniformly in the surrounding space of CCP1 (Fig. 1). All M2–M7 mutants had significant effect in reducing the overall positive electrostatic potential and enhancing the overall negative electrostatic potential, with M7 showing the most profound effect (Fig. 1). The effect of mutations appears to be cooperative and with variable contributions to C4b binding (Fig. 8). Although reduction of the positive electrostatic potential of CCP1 contributes to reduction in C4b binding, it is actually the increase in the negative electrostatic potential of the linkers CCP1–CCP2 and CCP2–CCP3 (Fig. 1) that has the most profound effect on C4b binding (Fig. 8). From the electrostatic negation mutants, D1–D3 nearly eliminated C4b binding, whereas D4 slightly reduced C4b binding (Fig. 8). On the whole, these data suggest that an overall positive electrostatic potential in CCP1 and the CCP1–CCP2 and CCP2–CCP3 linkers is essential for Kaposica’s C4b binding ability. There is more variability in the C3b binding data (Fig. 9). The C3b binding strength of the M1–M7 mutants follows the pattern M1 > Kaposica > M2 > M5 > M4 > M3 > M6 > M7. The C3b binding strength of the electrostatic negation data follows the pattern Kaposica > D3 > D4 > D2 > D1. Overall, the theoretical electrostatic potential data correlate well with the experimental binding data (Figs. 8, 9). Differences in the C4b/C3b binding data suggest possible differences in the interaction sites between Kaposica and C4b/C3b. In both cases, the M7 mutant, which has the largest overall negative electrostatic potential (Fig. 1), has the most profound effect in binding. This suggests that a cooperative effect of the electrostatic potentials of CCP1 and the CCP2–CCP3 area contributes to the C3b/C4b binding ability of Kaposica (or lack of binding ability for the studied Kaposica mutants). This observation is in agreement with...
FIGURE 6. Time course analysis of factor I CFAs of Kaposica and its domain charge modification mutants for complement proteins C3b and C4b. For assessing the CFA, 1 μg of Kaposica or each of the mutants (D1–D4) was incubated with 3 μg of C3b or C4b in the presence or absence of 100 ng of factor I in PBS, pH 7.4, at 37°C. The reactions were stopped at the indicated time periods by the addition of sample buffer containing DTT, and cleavage products were visualized by subjecting the reactions to SDS-PAGE under reducing conditions followed by staining with Coomassie blue. The amount of α'-chain of C3b/C4b remaining after various intervals was calculated by measuring the intensity by densitometric analysis. Bottom panels are graphical representations of percentage α'-chain of C3b (left) and C4b (right) versus time.

the mode of binding previously proposed for VCP/SPICE (32, 33). Subtleties are noted in Kaposica’s interactions with C4b and C3b. For example, CCP4 may not be very important for C4b binding (Fig. 8), a finding similar to that observed in our earlier study, in which we observed that affinity of the Kaposica deletion mutant CCP1–3 for C4b, but not for C3b, is similar to that of Kaposica (CCPs 1–4) (25). It is somehow surprising that further electrostatic negation of CCP3 (mutant D3) abolished C4b binding ability but not that of C3b (Figs. 8, 9). As noted previously in the case of VCP/SPICE mutants (33), correlated motions of CCP modules are expected to influence (and be influenced by) the electrostatic potentials of the individual modules and linkers. Alteration of the electrostatic potential in a CCP module or linker affects the electrostatic potential of neighboring modules. In addition, the spatial distributions of electrostatic potentials are fluctuating. As shown in the case of VCP/SPICE mutants (33), correlated motions of CCP modules are expected to influence (and be influenced by) the electrostatic potentials of the individual modules and linkers. Alteration of the electrostatic potential in a CCP module or linker affects the electrostatic potential of neighboring modules. In addition, the spatial distributions of electrostatic potentials are fluctuating. As shown in the case of VCP/SPICE mutants (33), the intermodular dynamics affects spatial distributions of electrostatic potentials and the degree of spatial cancellation of opposite-sign electrostatic potentials or spatial enhancement of same-sign electrostatic potentials; however, a local density of electrostatic potential with positive or negative sign will always be present in each module or linker. According to our model, any of the mutations that affect C3b/C4b binding may affect the intermodular orientation and dynamics. Previous studies (59) have suggested that open arrangement between two successive CCPs is important for decay, and tilt is important for CFA. We believe that the effect of mutations on the complexity of Kaposica’s dynamics cannot be assessed without extensive molecular dynamics simulations and analysis of intermodular correlated motions. Even with knowledge of dynamics, we expect a large variety of interconverting conformers, as was the case with VCP/SPICE mutants (33), and anticipate that conformational selection will be responsible for binding of a single conformer that will result in the lowest free energy complex. Although binding is a requirement for DAA and CFA, we cannot directly assess the effect of mutations on DAA or CFA in the absence of structures of Kaposica with C3b/C4b or structures of the multicomponent assemblies involving factor I and convertases.

At this point we should mention that the qualitative agreement between the computational predictions and the experimental SPR data are not depicted when comparing charges alone. For example, the correlation coefficient for C4b binding is 0.262 (Fig. 8), with that for C3b having a somewhat higher value of 0.537 (Fig. 9). This is because electrostatic potential is a more refined quantity than is charge. Electrostatic potential is, of course, generated by charge but, because of spatial cancellations or enhancements, provides more detailed information. Electrostatic potential is spatially distributed in the volume surrounding the protein, whereas charge, as calculated in Figs. 8 and 9, is localized at the protein surface at ionizable amino acid side chains and N- and C-terminal backbones. In addition, the electrostatic potential calculations take into account not only unit charges of ionizable chemical groups but also partial charges of dipole moments of all polar groups. As mentioned above, the electrostatic potential is the
driving force of the recognition step, through long-range inter-
actions (60), whereas surface charge is effective at the binding 
step through hydrogen bond and salt bridges. Analysis of the data 
of Figs. 8 and 9 reveals significant correlations between the 
binding response to C4b/C3b and C4b/C3b CFA/DAA (in the 
ranges of 0.6–0.9, see figure legends), and thus an indirect cor-
relation of the spatial distributions of electrostatic potentials to 
CFA/DAA. Correlation analysis of C4b/C3b CFA/DAA with 
charge alone yielded no significant correlations. This is not un-
expected because, as mentioned above, surface charge alone does 
not represent the details of the spatial distributions of electrostatic 
potentials or their contributions to protein–protein recognition.

The activity data obtained in the current study revealed that the 
constructs with altered positive potential in the linkers (M3–M6) 
display considerable reduction in CP and AP DAAs (4.4–12.6-fold 
reduction; Fig. 4). The CFA of the M3–M6 mutants, however, was 
less affected: The mutants exhibited considerable limited re-
duction in C3b CFA (2.8–6.0-fold reduction) and limited to no 
effect on C4b CFA (1.3- to 2.5-fold reduction; Figs. 2, 3). The M3– 
M6 mutants also showed reduction in binding to both C4b and C3b 
(Figs. 8, 9). The most likely explanation is that either these residues 
in the linkers are involved in direct interaction with the binding 
partners or reduction in activity is due to change in the preferred 
relative orientation of successive domains. The latter view is similar 
to the idea that interdomain angles are important in dictating the 
decay activity and CFAs (59). Like other CCP-containing RCAs 
that have been studied by molecular dynamics simulations, VCP/ 
SPICE (33) and CR2 (58), Kaposica is expected to participate in 
complex correlated rigid body modular motions about their flexible 
intermodular loops. Intermodular linker dynamics may mediate, 
via electrostatic coupling, allosteric effects upon binding, as has 
been suggested before for CR2/C3d binding (58).

Analysis of functional activities of electrostatic negation mutants 
showed that the mutant D1, in which the positive potential in CCP1 
was changed to negative, showed no detectable DAA and 60–90% 
loss in CFAs (Table I). Given that binding of complement regu-
lator to C3b and C4b is a common step in decay acceleration and 
factor I-mediated C3b/C4b inactivation, it is likely that positive 
potential in CCP1 is important for C3b/C4b binding. This hy-
pothesis is further supported by the fact that mutant D1 displayed 
negligible binding to C3b and C4b (Figs. 8, 9). If positive potential 
is critical for C3b/C4b binding and complement regulation by 
viral complement regulators, then the positive potential in CCP1 
must be conserved in all the viral regulators. Consistent with this 
premise, CCP1 of both herpes (Kaposica and Herpesvirus saimiri 
complement regulator, rhesus rhadinovirus complement control 
protein, murine γ-herpesvirus 68 RCA) and pox (VCP and SPICE, 
monkeypox inhibitor of complement enzymes) viral complement 
regulators carry positive field around them. On the basis of these 
data, we now propose that the positive potential of CCP1, but not 
the overall potential of the complement regulator, directs the 
initial recognition of C3b/C4b (i.e., step I of the two-step asso-
ciation model).

FIGURE 7. Decay acceleration of CP and AP C3-convertases by Ka-
posica and its domain charge modification mutants. Top panel, CP DAA of 
Kaposica and its mutants (D1–D4). Bottom panel, AP (AP) DAA of Ka-
posica and its mutants (D1–D4).

FIGURE 8. Binding of Kaposica and its 
muts to human complement protein C4b. 
Left panels, Sensogram overlays for the 
interaction of Kaposica and its mutants with 
C4b. Kaposica and the mutants were ori-
ented onto an NTA sensor chip by the his-
tidine tag (45), and C4b (125 nM) was 
injected over the chip to measure binding. 
Right panels show correlation graphs. Right 
top panel, Correlation between binding re-
response to C4b and C4b CFA (r = 0.719, p = 
0.008). Right middle panel, Correlation be-
tween binding response to C4b and CP DAA 
(r = 0.618, p = 0.032). Right lower panel, 
Correlation between total charge on mutant 
and C4b binding response (r = 0.262, p = 
0.41). Correlation coefficient (r) was calcu-
lated using Pearson product moment corre-
lation. Binding response was normalized by 
considering 100% binding response equal to 
binding response shown by Kaposica.
Our data on CCPs 2–4 charge negation mutants showed that overall negative charge on these domains have a pronounced inhibitory effect on C3b-linked activities as opposed to C4b-linked activities (Fig. 6, Table I). In particular, C3b CFA was reduced by 85–99% and AP DAA was reduced by 90–100%, but no inhibitory effect was observed on C4b CFA or AP DAA. If anything, there was an enhancement in CP DAA of the D4 mutant (Fig. 7, Table I). These data clearly suggest that intrinsic charge on CCPs 2–4 is indispensable for C3b-related activities. It is likely that negatively charged residues on C3b and AP C3-convertase form binding site(s) for Kaposica; therefore, loss in activity is a result of unfavorable electrostatic interactions. These data also point out that the functional surface for C3b-related activities is larger in comparison with C4b-related activities and is scattered over CCPs 1–4. This view is also supported by our earlier observation made by utilizing deletion mutants in which all four CCP domains of Kaposica are required for optimal C3b CFA, AP DAA, and C3b binding (25). The recently solved crystal structure of C3b in complex with factor H (CCPs 1–4) indicated that all four CCP domains interact with C3b (61). It is thus likely that the interaction of Kaposica with C3b is similar to the factor H–C3b interaction.

The mechanism of decay acceleration of C3-convertases is not yet clearly understood at the molecular level. For the classical C3-convertase, it is suggested that binding of RCA proteins to C4b (53, 59) or to both C4b and C2a (54, 59) leads to dissociation of C2a from the convertase. Our previous SPR binding and activity data on Kaposica indicated that CCPs 1–2 and 1–3 display C4b binding and CP DAA; optimal activity was observed in CCPs 1–3 (25). In the case of human decay-accelerating factor and C4BP, CCPs 2–3 (similar to Kaposica 1–2) and CCPs 1–3 (similar to Kaposica 1–3), respectively, are required for efficient decay of CP C3-convertase (52, 63). These data suggested that like human regulators, two to three domains of Kaposica display CP DAA and efficient decay is provided by the three N-terminal CCPs. The question, then, is whether modulation of electrostatic potential of the fourth CCP domain affects CP DAA. In this study, we show that mutant D4, which possesses intrinsic potential on CCPs 1–3 and negative potential on CCP4, possesses 4.5-fold enhanced CP DAA in comparison with soluble Kaposica. It is clear that negative potential in CCP4 enhances the decay activity, but the mechanism behind this remains unclear. The previously solved crystal structure of decay-accelerating factor has shown the presence of a large negative surface potential on CCPs 3 and 4, and the suggestion has been made that it probably interacts with a positive patch on the C3-convertase (53). It should be noted that CCP3 of Kaposica carries a negative charge; thus it is likely that in the mutant D4, negative potential on CCPs 3 and 4 leads to favorable electrostatic interactions with CP C3-convertase, which in turn result in effective decay of CP C3-convertase. Alternatively, it is also likely that increased decay activity in the D4 mutant is due to electrostatic repulsion between negative potential on CCPs 3–4 and positively charged hotspots on C2a.

In summary, our data suggest that the positive electrostatic potential of CCP1, and not the overall positive electrostatic potential of Kaposica, plays a vital role in its complement regulation. In addition, the positive potential at the linkers between CCPs 1–2 and 2–3 plays a prominent role in CP and AP DAAAs. These findings could categorically explain why positive potential in CCP1 and the linkers is conserved in herpers as well as poxviral complement regulators. Further, the data also indicate that negative potential in CCP4 differentially affects C3b- and C4b-related activities: Charge reversal on CCP4 notably enhanced CP DAA but substantially reduced C3b CFA and AP DAA. These data indicate that negative potential on CCP4 of Kaposica favors inhibition of the classical/lectin pathway, whereas positive potential favors inhibition of the AP. The fact that positive electrostatic potential in CCP4 is conserved in the herpers as well as pox virus regulators supports the view that in addition to inhibition of the CP and lectin pathway, inhibition of the AP must be crucial to the survival of these viruses.
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Disclosures

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References


