

Interaction of Vaccinia Virus Complement Control Protein with Human Complement Proteins: Factor I-Mediated Degradation of C3b to iC3b₁ Inactivates the Alternative Complement Pathway¹

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Vaccinia virus complement control protein (VCP) is a virulence determinant of vaccinia virus that helps protect the virus from the complement attack of the host. To characterize the interaction of VCP with C3 and C4 and understand the mechanism by which VCP inactivates complement, we have expressed VCP in a yeast expression system and compared the biologic activity of the purified protein to that of human factor H and complement receptor 1 (CR1). Recombinant VCP bound to C3 and the proteolytically cleaved form of C3 (C3b), but not to the 135,300-m.w. fragment of C3 generated using elastase (C3c) and the 35,000-m.w. fragment of C3 generated using elastase (C3d) and inhibited both the classical and alternative pathways of complement activation. Although rVCP was less effective at inhibiting the alternative pathway than factor H or CR1, it was more effective than factor H at inhibiting the classical pathway. Unlike factor H, rVCP was unable discriminate between alternative pathway-mediated lysis of rabbit and sheep E. A comparison of the cofactor activity in factor I-mediated cleavage of C3b suggested that in contrast to factor H and CR1, which displayed cofactor activity for the three sites, rVCP displayed cofactor activity primarily for the first site, leading to generation of C3b cleaved by factor I between Arg¹²⁸¹-Ser¹²⁸² (iC3b₁). Its cofactor activity for C4b cleavages was similar to that of soluble complement receptor type 1. Purification and functional analysis of iC3b₁ showed that it was unable to interact with factor B to form the alternative pathway C3 convertase, C3b,Bb. These results suggest that the interaction of VCP with C3 is different from that of factor H and CR1 and that VCP-supported first cleavage of C3b by factor I is sufficient to render C3b nonfunctional. *The Journal of Immunology*, 1998, 160: 5596–5604.

The complement system is a major immune defense against foreign microorganisms, including viruses (1, 2). Activation of the complement system by invading viruses, either in the presence or absence of Abs, can lead to virus neutralization, phagocytosis of the proteolytically cleaved form of C3 (C3b)³-coated viral particles, lysis of infected cells, and generation of inflammatory and specific immune responses (3, 4). To combat these host responses and succeed as pathogens, certain viruses have developed/acquired defenses against complement (5, 6), a process that is well documented for large DNA viruses (7, 8). Sequence analysis of DNA viruses has revealed that some mem-

bers of this group possess a gene (or genes) with striking homology to complement control proteins containing short consensus repeats (SCRs) (9). These compact domains, composed of approximately 60 to 70 amino acids, are characterized by a conserved motif that includes four disulfide-bonded cysteines, proline, tryptophan, and many hydrophobic residues.

In vivo, regulation of complement activation is mediated by a family of complement control proteins containing SCRs known as regulators of complement activation. These include the serum proteins factor H and C4-binding protein, and the membrane proteins complement receptor 1 (CR1), decay-accelerating factor, and membrane cofactor protein. These proteins act at the level of C3 and C4 and function by dissociating the subunits of C3 and C5 convertases and/or by acting as cofactors for the factor I-dependent cleavage of C3b and/or C4b.

The first known example of a viral homologue of these complement control proteins is the vaccinia virus complement control protein (VCP) (10). VCP, which is encoded by the C3L open reading frame (ORF) of the vaccinia genome, is a 35-kDa secretory protein of vaccinia virus that contains four SCRs (11). The protein shows sequence homology to C4-binding protein and to many other members of the complement control protein group. VCP is thought to protect the infected cells and released virions from the complement attack of the host. Support for this theory has come from experiments in which VCP was shown to abrogate complement-mediated, Ab-dependent neutralization of vaccinia virions (12). In addition, studies with recombinant vaccinia viruses that do not express VCP have clearly shown that these viruses are attenuated in vivo (12).

VCP has been shown to regulate complement activation in vitro. Both partially purified VCP and culture medium containing

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³ Abbreviations used in this paper: C3b, the proteolytically cleaved form of C3; SCR, short consensus repeats; CR1, complement receptor type 1; VCP, vaccinia virus complement control protein; ORF, open reading frame; iC3b₁, C3b cleaved by factor I between Arg¹²⁸¹-Ser¹²⁸²; iC3b₂, C3b cleaved by factor I between Arg¹²⁸¹-Ser¹²⁸² and Arg¹²⁹⁸-Ser¹²⁹⁹; C3c, the 135,300-m.w. fragment of C3 generated using elastase; C3d, the 35,000-m.w. fragment of C3 generated using elastase; E, erythrocytes; E_R, rabbit erythrocytes; E_S, sheep erythrocytes; sCR1, soluble complement receptor type 1.

secreted VCP have been shown to inhibit complement-mediated lysis of sheep E (E_S), to bind to C3b and C4b, and to accelerate the decay of the classical as well as alternative pathway C3 convertases (11, 13). VCP shows considerable sequence similarity to other factor I cofactor proteins. Thus, it is important to study its cofactor activity. A previous study has suggested that VCP had cofactor activity in the factor I-mediated cleavage of C3b and C4b (13). However, in that study, the C3b and C4b cleavage products were not visualized, and conclusions about the nature of cofactor activity could not be obtained.

In the present study, we have characterized the interaction of rVCP with C3 and C4 and compared its interaction to that of other known regulators, factor H and CR1. Our results show that the factor I cofactor activity of rVCP for C4b is similar to soluble complement receptor type 1 (sCR1). However, in contrast to factor H and CR1, which function as a cofactor for inactivation of C3b by factor I for sites 1 (Arg¹²⁸¹-Ser¹²⁸²), 2 (Arg¹²⁹⁸-Ser¹²⁹⁹), and 3 (Arg⁹³²-Glu⁹³³), VCP primarily functions as a cofactor for the first site. We also present data suggesting that C3b, when cleaved at site 1 (C3b cleaved by factor I between Arg¹²⁸¹-Ser¹²⁸² (iC3b₁)), is unable to form the alternative pathway C3 convertase C3b,Bb. These results suggest a mechanism for complement evasion by vaccinia virus and demonstrate that the interaction of VCP with C3 is different from all the other factor I cofactors characterized to date.

Materials and Methods

Reagents and buffers

Yeast nitrogen base without amino acids was purchased from Difco (Detroit, MI). BSA and D-biotin were obtained from Sigma (St. Louis, MO). Rabbit and sheep blood were purchased from Cocalico Biologicals (Reamstown, PA), and E_S coated with Abs were prepared by incubating the E_S with anti- E_S Abs (Cordis, Miami, FL). Antisera recognizing rVCP and human C3b were raised in rabbits. Factor H-depleted serum was prepared by passing human serum containing 20 mM EDTA through an anti-factor H Sepharose column pre-equilibrated in veronal-buffered saline (VBS) containing 20 mM EDTA. VBS, pH 7.4, contained 5 mM barbital and 145 mM NaCl. Gelatin veronal-buffered saline (GVB) was VBS containing 0.1% gelatin, GVB²⁺ was GVB containing 0.5 mM MgCl₂ and 0.15 mM CaCl₂, and GVBE was GVB with 10 mM EDTA. MgEGTA contained 0.1 M MgCl₂ and 0.1 M EGTA. PBS, pH 7.4, contained 10 mM sodium phosphate and 145 mM NaCl. Blocking buffer was PBS containing 0.5% milk and 1% BSA.

Complement proteins and C3 degradation products

The human complement proteins C3 (14), factor B (15), factor H (16), and factor I (17) were purified as previously described. The purified C3 used in this study contained 61% native C3 and 39% C3(H₂O), as determined by analyzing the protein sample on a Mono S column (Pharmacia, Piscataway, NJ) (18). Factor D was kindly provided by Dr. J. Volanakis (Department of Medicine, University of Alabama at Birmingham, AL), and the recombinant human CR1 (soluble complement receptor type 1; sCR1) was a generous gift of Dr. S. Makrides (T Cell Sciences, Needham, MA). C3b was generated by limited trypsin cleavage (19). iC3b was produced by incubating 1 mg of trypsin-generated C3b with 175 μ g factor H and 3.5 μ g factor I in 1.2 ml PBS, pH 7.4, at 37°C for 2 h. Both C3b and iC3b were purified on a Mono Q column. The 135,300-m.w. fragment of C3 (C3c) and the 35,000-m.w. fragment of C3 (C3d) were generated by elastase treatment of C3 and further purified on a Mono Q column (19). C4b generated during C4 purification from human plasma was purified as described (20). Purity of all the proteins exceeded 95%, as determined by SDS-PAGE and immunodiffusion analysis.

Cloning, expression, and purification of rVCP

rVCP was expressed using a yeast expression system. As detailed below, a segment of the VCP ORF was amplified by the PCR and ligated into the *Pichia pastoris* expression vector, pHIL-S1 (Invitrogen, Carlsbad, CA). VCP was cloned into the vector pHIL-S1 behind an inducible strong yeast promoter and in-frame with a yeast signal peptide. Such a construct should result in high-level expression of a secreted recombinant protein. To allow isolation of a recombinant yeast, the yeast expression plasmid also con-

tained a selectable marker and flanking sequences that permitted homologous recombination into the yeast genome. Oligonucleotide primers 5'-GCACGCGTCCGACTATGCTGTACTATTCCGTCACGACCC-3' (the *SalI* site is underlined) and 5'-CGCGGATCCTTAGCGTACACATTTT GGAAGTTCCG-3' (the *BamHI* site is underlined and the stop codon is in boldface), were used to amplify a 735-bp segment of the C3L ORF from a plasmid containing the *HindIII*-*BamHI* segment of the *HindIII* C fragment of vaccinia virus (strain WR) DNA. The PCR conditions were: 20 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min 20 s. After amplification, the PCR product was gel purified and digested with *SalI* and *BamHI*. The yeast expression vector, pHIL-S1, was digested with *XhoI* and *BamHI*. The digested PCR product and plasmid were gel purified and ligated together with T4 DNA ligase. The cloned insert and the ligated ends were sequenced to confirm the DNA structure. A recombinant *Pichia pastoris* was isolated following the manufacturer's instructions as described in the *Pichia* Expression Kit (Version 1.8; Invitrogen).

The recombinant yeast cells were grown in 10 ml of sterile BMGY medium (100 mM potassium phosphate, pH 6.0, 10 g/L yeast extract, 20 g/L peptone, 13.4 g/L yeast nitrogen base, 0.4 mg/L biotin, and 1% glycerol) on a shaker at 30°C for 18 h. The liquid culture obtained was used as an inoculum for 1 liter of BMGY medium, which was incubated at 30°C with vigorous shaking until the culture reached an OD₆₀₀ \geq 6 (usually at 72 h). The cells were centrifuged at 1500 \times g for 5 min, resuspended in 200 ml of BMMY medium (BMGY containing 0.5% methanol, but without 1% glycerol) and incubated with vigorous shaking at 30°C. After 24 h, the liquid culture was centrifuged and the supernatant containing the rVCP was collected.

Expressed rVCP was purified from the culture supernatant as follows. In a typical purification procedure, culture supernatant (250 ml) was first precipitated with 20% ammonium sulfate and the supernatant was then precipitated with 60% ammonium sulfate at 0°C. The resulting pellet containing rVCP was dissolved, dialyzed into PBS, and loaded onto a DEAE-Sephacel column (1.5 cm \times 10.5 cm) pre-equilibrated with PBS, and the flow-through containing the unbound proteins was collected. The flow-through obtained was diluted in 10 mM phosphate, pH 7.9, and applied to a Mono Q HR 5/5 column (Pharmacia). The bound proteins were eluted with a linear salt gradient from 0 to 0.5 M NaCl. Fractions containing rVCP were identified by SDS-PAGE and Western blotting, pooled, and dialyzed against PBS.

Mass spectrometry and N-terminal sequencing

The molecular mass of the rVCP was determined by mass spectrometry. Matrix-assisted laser desorption-ionization mass spectrometry was performed using a VG ToFSpec time-of-flight mass spectrometer (Fisons Instruments, Beverly, MA). The sample was dissolved in 50 μ l of 50% acetonitrile containing 0.1% trifluoroacetic acid and diluted before analysis using 2-(4-hydroxyphenylazo)benzoic acid (Aldrich, Milwaukee, WI) as the matrix. Data were analyzed by OPUS software (Fisons Instruments) (21). To determine the N-terminal sequence of rVCP, the sample was applied to a conditioned glass-fiber filter coated with BioBrene Plus cationic polymer (Applied Biosystems, Foster City, CA). Adsorbed protein was subjected to Edman degradation, using an Applied Biosystems 473A Protein Sequencer (19).

Hemolytic assay

Inhibition of the classical and alternative pathway-mediated hemolytic activity was determined as previously described (22). In brief, inhibition of the classical pathway was determined by incubating various concentrations of complement control proteins (rVCP, factor H, and sCR1) with 7 μ l of normal human serum (diluted 1:10 in GVB²⁺) and 5 μ l of E_S coated with Abs (1×10^9 /ml). The total volume of the reaction mixture was adjusted to 250 μ l by adding GVB²⁺ and incubated at 37°C for 1 h. The reaction was stopped by placing the reaction mixture at 0°C followed by centrifugation. The percentage of lysis was determined by measuring the OD of 200 μ l of supernatant at 405 nm. To determine the influence of the various complement control proteins on the alternative pathway of complement, 10 μ l of rabbit E (E_R) (1×10^9 /ml in GVB) were mixed with 8 μ l of undiluted normal human serum and 5 μ l of MgEGTA (0.1 M) in the presence of various concentrations of rVCP, factor H, or sCR1. The volume of the reaction mixture was adjusted to 100 μ l with GVB. The reaction mixture was incubated at 37°C for 20 min and the reaction was stopped by adding 200 μ l of GVBE. After centrifugation, the absorbance of 200 μ l of the supernatant from each sample was determined at 405 nm. The percentage of lysis was normalized by setting 100% lysis to be equal to the degree of lysis occurring in the absence of inhibitory proteins. The effect of complement control proteins on the alternative pathway-mediated lysis of E_S was

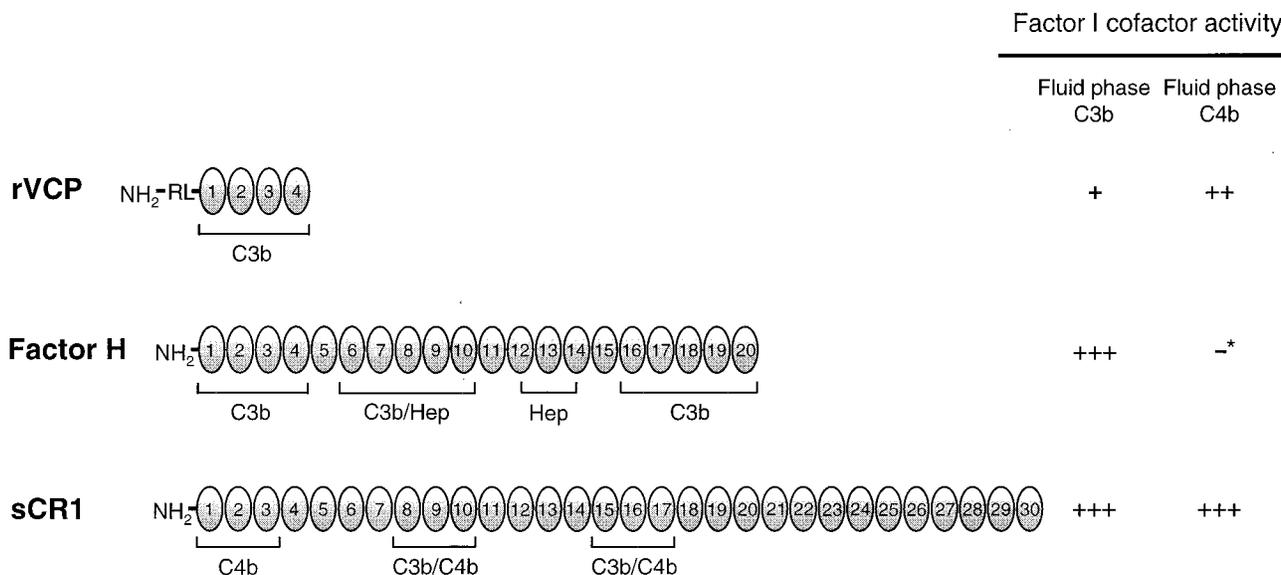


FIGURE 1. Schematic representation of rVCP, factor H, and sCR1. A full-length VCP cDNA was subcloned into the pHIL-S1 expression vector, transformed, and expressed in yeast cells as described in *Materials and Methods*. In the construct, cleavage of the signal peptide leaves two additional amino acids at the N terminus: arginine (R) and leucine (L). The SCR domains of each protein are numbered, and the binding domains for C3b, C4b, and heparin (Hep) are marked (28, 37–41). The extent of cofactor activity of rVCP relative to that of factor H and sCR1 is shown. The relative activity is based on results of experiments described in Figures 5 and 6. *, Factor H does not support factor I-mediated C4b cleavages (4).

measured as described below. Various concentrations of complement control proteins were mixed with 10 μ l of 0.1 M MgEGTA, 40 μ l of factor H-depleted human serum, and 10 μ l of E_s (1×10^9 /ml in GVB) in a final volume of 100 μ l of GVB. The reaction mixture was incubated at 37°C for 30 min and stopped by adding 200 μ l of GVBE. After centrifugation, hemolysis was determined at 405 nm.

ELISA measurement of rVCP binding to C3 and C3 fragments

To measure the binding of rVCP to C3 and C3 fragments, wells of ELISA plates (Nunc, Naperville, IL) were coated overnight at 4°C with 100 μ l of VCP at 60 μ g/ml. After coating, wells were saturated with blocking buffer at 22°C for 30 min and washed three times with PBS containing 0.05% Tween-20. Twofold dilutions of C3 and C3 fragments were added to the wells and incubated at 22°C for 1 h, and then the wells were washed. Bound C3 and C3 fragments were detected by incubating the wells at 22°C for 1 h with 100 μ l of 2 μ g/ml polyclonal rabbit anti-C3b Ab, followed by a 1:1000 dilution of peroxidase-conjugated goat anti-rabbit IgG Ab (Bio-Rad, Hercules, CA). Unbound Ab and conjugate were washed, 2,2'-azino-bis(3 ethylbenzthiazoline-6-sulfonic acid) (ABTS) was added, and the OD was measured at 405 nm. The anti-C3b Ab, peroxidase conjugate, and C3 and C3 fragments were diluted in PBS, pH 7.4, containing 10% blocking buffer.

Measurement of factor I cofactor activity of rVCP

A time course of the cleavage of C3b by factor I in the presence of rVCP, factor H, or sCR1 was performed in 10 mM phosphate buffer, pH 7.4, containing 145 mM NaCl. In a typical assay, 33 μ g of C3b were mixed with 11 μ g of rVCP, factor H, or sCR1 and 0.5 μ g of factor I in a total volume of 143 μ l and incubated at 37°C. Samples (13 μ l) were removed at different time intervals and mixed with sample buffer containing DTT, boiled for 3 min, and subjected to electrophoresis on a 9% SDS-PAGE gel. Cleavage products were visualized by staining the gel with Coomassie blue. The gel was scanned for densitometric analysis and the percentage of each C3 fragment generated was calculated. To rule out the presence of contaminating proteases, experiments were also performed in the presence of a mixture of antiproteases containing chymostatin (100 μ g/ml), pepstatin (11 μ g/ml), antipain (100 μ g/ml), and leupeptin (2.5 μ g/ml). Controls were formed by incubating C3b with cofactors (rVCP, factor H, or sCR1) in the absence of factor I.

The rate of C4b cleavage by factor I in the presence of rVCP and sCR1 was determined as follows. In this assay, 27 μ g of C4b was mixed with 9 μ g of rVCP or sCR1 and 1 μ g of factor I in 135 μ l of PBS. The reaction mixture was allowed to incubate at 37°C. Samples (15 μ l) taken out at different time intervals were mixed with sample buffer containing DTT,

subjected to electrophoresis on a 9% SDS-PAGE gel, and analyzed for cleavage products as described above for C3b.

Separation of C3b, iC3b₁, and C3b cleaved by factor I between Arg¹²⁸¹-Ser¹²⁸² and Arg¹²⁹⁸-Ser¹²⁹⁹ (iC3b₂) by anion-exchange chromatography

C3b and its inactivated forms, iC3b₁ and iC3b₂, were separated by anion-exchange chromatography using a Mono Q HR 5/5 column (Pharmacia). C3b (1 mg) was mixed with 300 μ g of rVCP and 16 μ g of factor I in a total volume of 1.3 ml and incubated at 37°C for 10 h. This resulted in degradation of C3b into iC3b₁ and iC3b₂. To better indicate all three species on chromatogram, 0.6 mg of C3b was added to the mixture. The reaction mixture containing all the three fragments of C3 was diluted in buffer A (10 mM sodium phosphate, pH 7.9) and passed over the column, which had been preequilibrated in buffer A. The column was washed with 5 ml of 20% buffer B (10 mM sodium phosphate, 500 mM NaCl, pH 7.9) to wash the rVCP off the column (rVCP elutes between 10 and 20% of buffer B). Bound proteins were then eluted with a linear gradient of 55 ml from 20 to 60% of buffer B. All the protein-containing fractions were analyzed by SDS-PAGE.

Detection of C3 convertase formation

The ability of C3b, iC3b₁, and iC3b₂ to interact with factor B to form C3 convertase, C3b,Bb was analyzed as follows: C3b or its inactivated forms, iC3b₁ and iC3b₂ (0.5 μ g each), were mixed with factor B (0.6 μ g) and factor D (4 ng) in 25 μ l of PBS, pH 7.4, containing 5 mM MgEGTA. The reaction mixture was incubated at 37°C for 30 min and run on a 7.5% SDS-PAGE gel under reducing conditions. The gel was stained with silver stain to visualize the proteins. The presence of Bb and Ba fragments indicated the formation of C3 convertase. Controls were incubated in GVBE to inhibit the factor B cleavage.

Results

Expression, purification, and characterization of rVCP

In the present study, we have expressed a functionally active rVCP using the yeast expression system (Fig. 1). This expression system produced a high yield of rVCP; the recombinant protein was secreted into the medium at a level of 50 to 150 μ g/ml, as determined by SDS-PAGE and Western blot analysis. The culture supernatant containing the expressed protein was

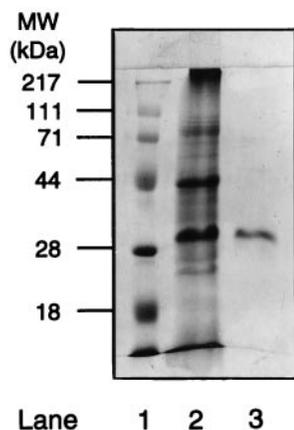


FIGURE 2. SDS-PAGE analysis of purified recombinant VCP. Culture supernatant of *Pichia pastoris* encoding rVCP and purified rVCP were subjected to electrophoresis through a 12% SDS-PAGE gel under reducing conditions and stained with Coomassie blue. Lane 1, m.w. markers; lane 2, culture supernatant; lane 3, purified rVCP.

precipitated by ammonium sulfate and further purified by anion-exchange chromatography on a DEAE-Sephacel column. These two methods concentrated the protein and removed most of the yeast proteins, in particular the yeast metal-binding proteins, which impart yellow and green colors to the culture supernatant. Following these steps, the sample was passed over the Mono Q anion-exchange column and eluted with a linear salt gradient. The eluted protein was homogeneous and >95% pure as determined by SDS-PAGE analysis (Fig. 2).

To confirm the identity of rVCP, the purified protein was subjected to automated Edman degradation. The predicted N-terminal sequence of the first 10 residues of the expressed VCP is R-L-C-C-T-I-P-S-R-P. The amino acid sequence of rVCP corresponded to the predicted sequence (Table I). The sequencing data also confirmed that the signal peptide was cleaved in the mature protein. The first two amino acids, RL, are the result of cloning VCP into the *Pichia* vector. The size of the expressed protein was analyzed by SDS-PAGE (Fig. 2) and mass spectrometry (Table I). The calculated molecular mass of rVCP was 26,906 Da, but the protein migrated as a single band of 30,000 Da. However, this apparent overestimation by SDS-PAGE is not due to glycosylation, because the primary sequence lacks glycosylation sites (10). Thus, mass spectrometry was used to determine the accurate mass of the protein. The mass 27,164 Da was closer to the calculated mass of the protein and is within the range of estimated error (error <1%) of this method (Table I). Western blot analysis of rVCP and VCP produced by vaccinia virus-infected cells revealed that rVCP and VCP were recognized by anti-VCP Abs and were identical in size (data not shown).

Table I. Molecular mass and N-terminal analysis of rVCP

Molecular mass (Da) as determined by	
Formula ^a	26,906
SDS-PAGE ^b	30,000
Mass spectrometry ^c	27,164
N-terminal sequence ^d	R-L-X-X-T-I-P-S-R-P

^a Based on the primary sequence (10).

^b Calculated from Figure 1.

^c Determined as described in *Materials and Methods*.

^d Amino acids were not detected at positions 3 and 4. The primary sequence contains cysteines at these positions.

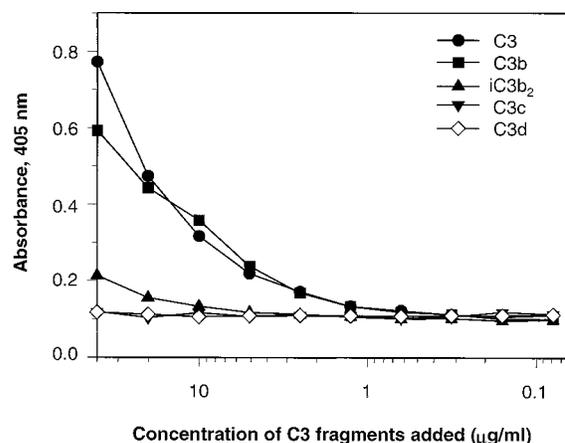


FIGURE 3. Binding of rVCP to C3 and C3 fragments. An ELISA plate was coated with purified rVCP, saturated with blocking buffer, washed, and incubated with a twofold dilution of C3 and C3 fragments (C3b, iC3b₂, C3c, and C3d). The binding of C3 and C3 fragments to rVCP was detected by a rabbit anti-C3b Ab and a peroxidase-coupled anti-rabbit Ab. Color was developed with ABTS peroxidase substrate.

Binding of rVCP to C3 fragments

A previous study (13) had shown that VCP secreted by vaccinia virus-infected cells binds to C3b. To test whether rVCP is biologically active and has retained its ligand binding activity, we performed a direct ELISA binding assay. Our results showed that rVCP, like virally secreted VCP, bound to C3b (Fig. 3). In this study, we also characterized the binding of rVCP to various physiologic fragments of C3. When we measured its binding to C3, iC3b₂, C3c, and C3d, we found that unlike C3b, the expressed rVCP did not bind to C3c and C3d. Surprisingly, rVCP did bind to C3 and weakly to iC3b₂. It is likely that the observed binding to C3 was due to the presence of C3(H₂O), since this preparation contained 61% native C3 and 39% C3(H₂O), but we cannot rule out the possibility that VCP binds to native C3. Additional experiments described below suggest that the binding we observed to iC3b₂ is not physiologically significant.

Inhibition of complement activation by rVCP, CRI, and factor H

We compared the effects of rVCP, factor H, and sCRI on the classical and alternative pathways of complement by evaluating their ability to inhibit the complement-mediated lysis of E (Fig. 4). We chose to use hemolytic assays because these assays have been used in the past to quantitate the effect of complement control proteins and inhibitors. Therefore, the IC₅₀ values we obtained would be useful for comparison purposes. In our assays, rVCP inhibited lysis of E_R and E_S by the alternative and classical pathways with IC₅₀ values of 3.4 μM and 0.1 μM, respectively (Fig. 4). The concentrations of factor H and sCRI required to inhibit the alternative pathway-mediated lysis of E_R were 0.54 μM and 2.7 nM, respectively (Fig. 4A). The concentrations of factor H and sCRI required to inhibit the classical pathway were 0.42 μM and 0.21 nM, respectively (Fig. 4C). On a molar basis, rVCP was four times more active in inhibiting the classical pathway than was factor H. These results suggest that although rVCP is a poor inhibitor of the alternative pathway when compared with factor H; it is more potent than factor H in inhibiting the classical pathway.

It is well known that E_R spontaneously activate the alternative pathway because the C3b attached to E_R have a relatively low

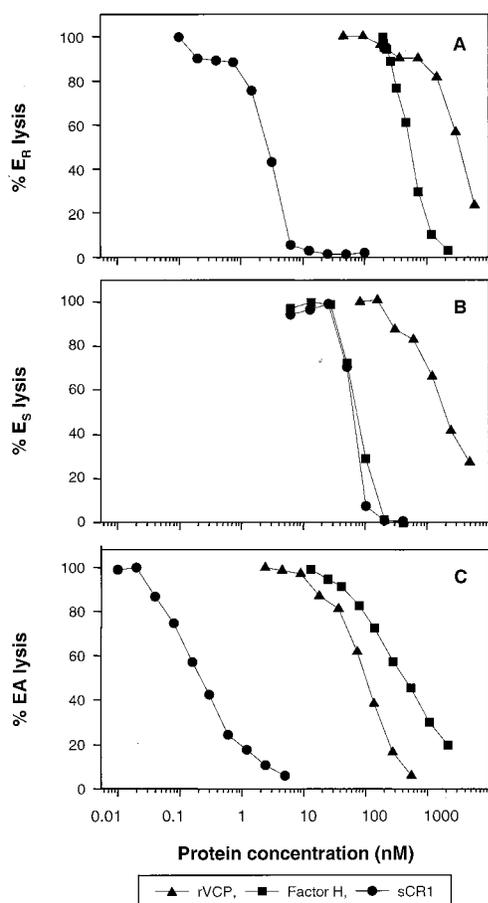


FIGURE 4. Inhibition of alternative and classical pathway-mediated lysis of E by rVCP, factor H, and sCR1. All three proteins were compared for their relative effect on the alternative pathway-mediated lysis of E_R (A) and E_S (B), and the classical pathway-mediated lysis of Ab-coated E_S (C) as detailed in *Materials and Methods*.

affinity for factor H in comparison to C3b attached to E_S . To determine whether the alternative pathway regulatory activity of rVCP is different for E_R and E_S , we measured the effect of rVCP on alternative pathway-mediated lysis of E_S and compared it with factor H and sCR1 (Fig. 4B). As expected, the amount of factor H required to inhibit the alternative pathway-mediated lysis of E_S was significantly less (7.7-fold) than that required to inhibit lysis of the E_R . In contrast, a less than 2-fold difference was observed for rVCP ($IC_{50} = 1.9 \mu M$). The IC_{50} value of sCR1 for E_S lysis was similar to that of factor H.

Comparison of the cofactor activity of rVCP, factor H, and sCR1

Complement control proteins inhibit complement activation by supporting the proteolytic inactivation of C3b and/or C4b by factor I. We further analyzed the interaction of rVCP with C3b and C4b by assessing its ability to act as a cofactor in factor I-mediated cleavage of C3b and C4b. The cofactor activity of rVCP for C3b was compared with that of two physiologic regulators of complement, factor H and CR1. These assays allowed us to analyze the complete time course of C3b inactivation by the three proteins. While previous studies have compared the interaction of factor H or CR1 with C3b, a comparative time course of C3b inactivation has not been reported to date. In this assay, the appearance of 68-kDa and 46-kDa fragments indicated the first factor I-mediated cleavage of C3b between Arg¹²⁸¹-Ser¹²⁸² and generation of $iC3b_1$;

the appearance of a 43-kDa fragment indicated the second cleavage between Arg¹²⁹⁸-Ser¹²⁹⁹ and generation of $iC3b_2$; and the appearance of 25-kDa and C3dg fragments indicated the third cleavage between Arg⁹³²-Glu⁹³³ and generation of C3c and C3dg. The first and second cleavages occurred immediately (within the first 5 min) in the presence of both factor H and sCR1 (Fig. 5). The third cleavage was slow in the presence of sCR1 and did not occur in the presence of factor H under these conditions. The third cleavage was observed in the presence of factors H and I only when the reaction was performed overnight in half-ionic-strength buffer (data not shown). These results suggest that factor H is as efficient as sCR1 in inactivating fluid-phase C3b to $iC3b_2$. However, this similarity did not hold true for activator-bound C3b, since 200-fold higher concentrations of factor H than of sCR1 were needed to inhibit E lysis when C3b was bound to E_R (Fig. 4A).

Our data indicated that rVCP also acts as a cofactor in factor I-mediated cleavage of C3b (Fig. 5). However, it behaved differently from factor H and sCR1 in two ways. 1) The cleavage pattern of C3b was different from that obtained in the presence of factor H and sCR1, and 2) the rate of C3b cleavage was slower than that observed in the presence of factor H and sCR1. It is clear that in the presence of rVCP, C3b was predominantly cleaved at the first site, leading to the generation of $iC3b_1$. The second cleavage was much slower, occurring only after 4 h instead of 5 min. Furthermore, rVCP failed to support the third cleavage, even when the reaction mixture was incubated overnight at 37°C in half-ionic strength buffer (data not shown). Cofactors incubated with C3b in the absence of factor I showed no cleavage products (data not shown). Whether the predominant species generated ($iC3b_1$) is inactive or is able to participate in the formation of C3 convertase was not clear from these experiments. Additional experiments (described below) were performed to determine the role of $iC3b_1$ in complement activation.

The factor I cofactor activity of rVCP for C4b was also determined in a fluid phase assay and compared with that of sCR1 (Fig. 6). The α' chain of C4b is cleaved by factor I between Arg¹³¹⁸-Asn¹³¹⁹ (first cleavage) and Arg⁹³⁷-Thr⁹³⁸ (second cleavage) (23). In this assay, generation of 71-kDa and 16-kDa fragments would indicate the first cleavage and formation of $iC4b$. However, the 71-kDa and 16-kDa fragments comigrate with the β -chain and the dye front, respectively, and thus were not seen in the gel. The appearance of 25-kDa and C4d fragments indicated the second cleavage and generation of C4c and C4d. The results depicted in Figure 6 show that similar to sCR1, both of the cleavages of the α' chain of C4b also occurred in the presence of rVCP, resulting in the generation of C4d. These data suggest that rVCP acts as a factor I cofactor for inactivation of C4b.

Does $iC3b_1$ form C3 convertase?

The ability of rVCP to act as a cofactor in the factor I-mediated cleavage of C3b to $iC3b_1$ allowed us to isolate and determine the role of this C3b species in complement activation. rVCP was incubated for 10 h with C3b and factor I, the products of the reaction were mixed with C3b and separated on a Mono Q HR 5/5 column. The three C3 forms (C3b, $iC3b_1$, and $iC3b_2$) bound to the anion-exchange column and eluted according to the number of cleavages in the molecule (Fig. 7). The identity of each C3 form was confirmed by analyzing the samples on SDS-PAGE under reducing conditions.

The ability of $iC3b_1$ to form C3 convertase was examined in a fluid-phase assay. C3 fragments generated and purified as described above were incubated with factors B and D in the presence of MgEGTA at 37°C for 30 min (Fig. 8). The generation of Bb and Ba fragments indicated the formation of C3 convertase, C3b,Bb. As expected, C3b participated in the formation of C3 convertase (Fig. 8,

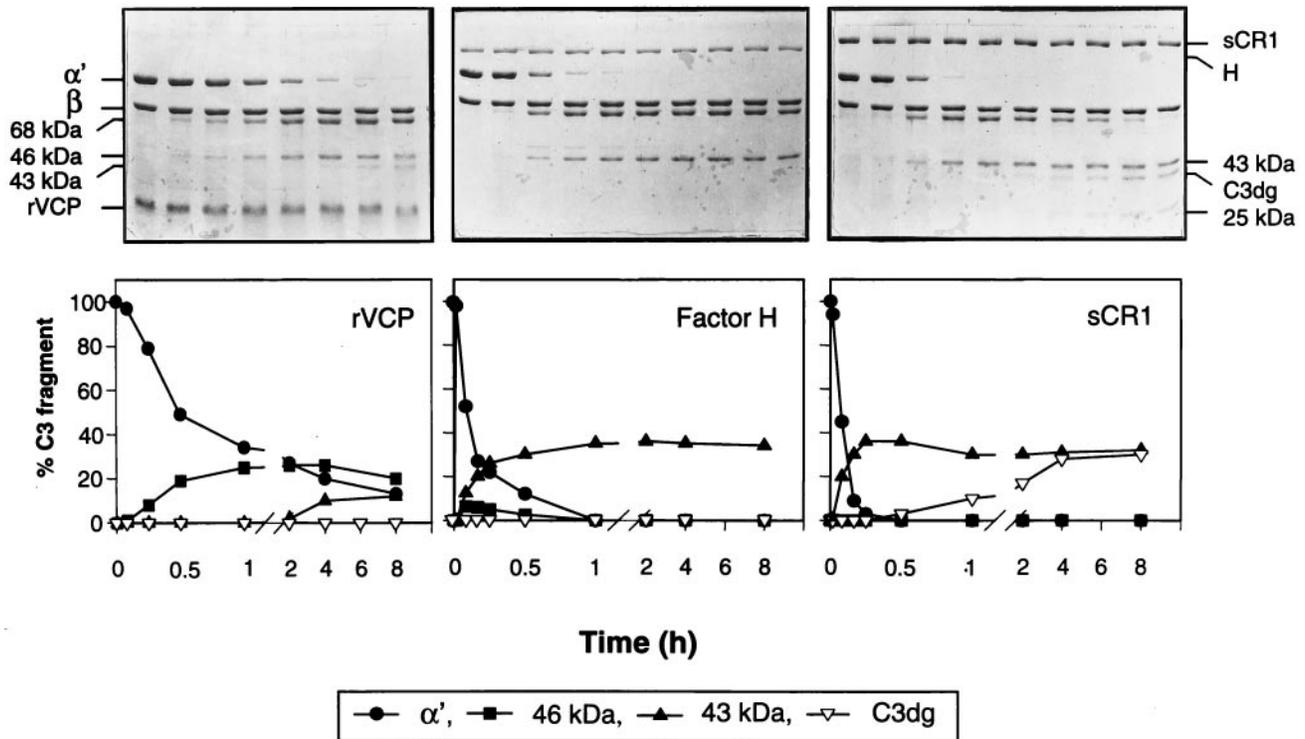


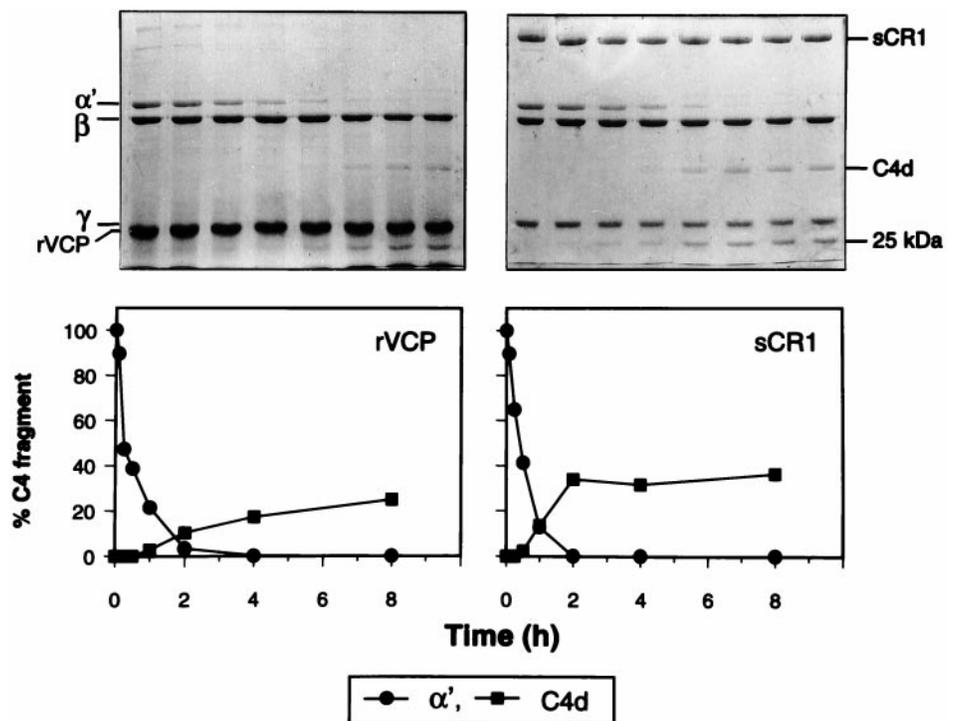
FIGURE 5. Time course of factor I cofactor activity of rVCP, factor H, and sCR1 for C3b. Cofactor activity was measured by incubating C3b with rVCP, factor H, or sCR1 in the presence of factor I. The reactions were performed at 37°C in 10 mM sodium phosphate, pH 7.4, containing 145 mM NaCl. Samples were removed at different time intervals, as shown in the lower panels, and the reaction was stopped by adding SDS-PAGE sample buffer containing DTT. Cleavage of C3b was examined by subjecting samples to electrophoresis on 9% SDS-PAGE gels (upper panel). Cleavage products were visualized by staining the gels with Coomassie blue, and the intensity of selected bands were determined by densitometric analysis and graphed (lower panel). The appearance of 46-kDa, 43-kDa, and C3dg fragments indicate the generation of iC3b₁, iC3b₂, and C3c and C3dg, respectively.

lane 2), while iC3b₂ failed to do so (Fig. 8, lanes 4 and 5). It is important to note that iC3b₁ was unable to participate in the formation of C3 convertase (Fig. 8, lane 3), suggesting that the single cleavage in the C3b molecule converts the molecule into an inactive species that no longer supports complement activation.

Discussion

Previous in vivo studies have established that VCP, a major secretory product from vaccinia virus-infected cells, is a virulence factor. It has been shown that vaccinia virus mutants that do not express VCP are

FIGURE 6. Time course of factor I cofactor activity of rVCP and sCR1 for C4b. C4b was incubated 37°C with rVCP or sCR1 in the presence of factor I. Samples were removed at different time intervals and the reaction was stopped by adding SDS-PAGE sample buffer containing DTT. Cleavages of C4b were examined by analyzing samples on 9% SDS-PAGE gels (upper panel). Cleavage products were visualized by staining the gels with Coomassie blue, and the intensity of selected bands was determined by densitometric analysis and graphed (lower panel). The appearance of 25-kDa and C4d fragments indicates the generation of C4c and C4d.



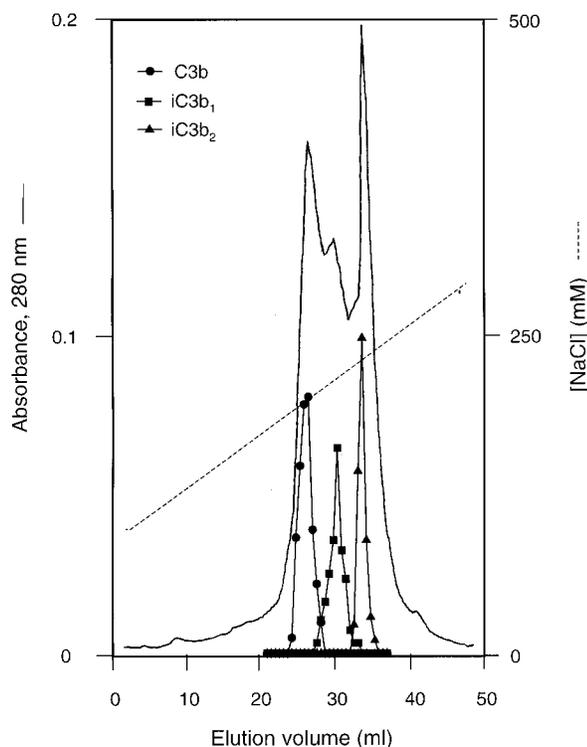


FIGURE 7. Separation of C3b, iC3b₁, and iC3b₂ by anion exchange chromatography. The sample containing a mixture of C3b, iC3b₁, and iC3b₂ was diluted in Mono Q buffer A and loaded onto the column. The column was washed and proteins were eluted with a linear salt gradient. Fractions containing protein were analyzed on 9% SDS-PAGE gels under reducing conditions. The gels were visualized by silver stain and subjected to image analysis to obtain data points for graphical representation of the results. The amount of C3b, iC3b₁, and iC3b₂ was determined by measuring the intensity of the α' , 46-kDa, and 43-kDa bands, respectively.

attenuated in rabbits and guinea pigs (12). The cowpox virus homologue of VCP has been shown to modulate the inflammatory response to infection in mice (24). *In vitro*, VCP has been shown to inhibit the formation and accelerate the decay of the classical and alternative pathway C3 convertases (13); however, the detailed mechanism by which VCP inactivates complement remains unclear. The present study was undertaken to characterize the interaction of VCP with C3 and C4 and to determine how it relates to other known regulators of complement activation. We present evidence that, unlike other regulators of complement (factor H and CR1), VCP serves as a factor I cofactor primarily for the first cleavage of C3b. This cleavage leads to generation of iC3b₁, which is unable to participate in the formation of the alternative pathway C3 convertase (C3b,Bb).

In this study, we expressed an rVCP to evaluate its effect upon the complement system. The *Pichia* expression system used in this study provided large quantities of rVCP. Although other expression systems have been used to express of SCR-containing proteins (25–28), the yeast expression system used in this study provided a 10- to 30-fold higher yield than had previously been reported. To purify the rVCP, we have used a combination of ammonium sulfate precipitation and anion-exchange chromatography steps. The ammonium sulfate precipitation step followed by anion-exchange chromatography on a DEAE-Sephacel column removed most of the yeast proteins. Thus, these two steps should be useful in purifying other yeast-expressed proteins, since yeast proteins bind tightly to the DEAE-Sephacel column and elute only at high salt concentration (2 M) or low pH (pH 2.3).

The expressed VCP was functionally active, as judged by its binding to C3b (Fig. 3) and inhibition of complement-mediated E lysis (Fig. 4). To characterize the ligand-binding properties of rVCP, we studied its interaction with various C3 fragments (Fig. 3). Binding was observed with C3 and C3b but not with C3c or C3d. A weak binding was also observed with iC3b₂. These results suggest that the affinity of the binding site for small C3 fragments (C3c or C3d) is very low and was therefore below the detection limit of our assay system. Another possibility is that the binding site on C3 is conformational and is lost after the cleavage of C3b to C3c and C3d. Further studies are needed to address this issue and to localize the binding site of VCP on C3.

Recently, Subramanian et al. (29) have demonstrated that tyrosine and aspartic acid at positions 37 and 79 determine the binding specificity of SCRs 1 to 3 of CR1 to C3b/C4b. Because VCP binds to both C3b and C4b (13), we aligned its sequence with SCRs 1 to 3 of CR1 to determine whether VCP contains tyrosine and aspartic acid at comparable positions. We found that VCP contains arginine and glycine instead of tyrosine and aspartic acid. Thus, the presence of tyrosine and aspartic acid at positions 37 and 79 does not, by itself, always dictate the binding specificity for C3b/C4b.

VCP has previously been shown to inhibit the classical pathway-mediated lysis of E_s (11); however, it was not clear whether it also inhibits the alternative pathway in whole human serum. In the present study, we show that rVCP inhibits the classical as well as alternative pathway-mediated lysis of E in normal human serum (Fig. 4). It is clear from the data that the concentration of rVCP required to inhibit the classical pathway (Fig. 4C) is about 34-fold lower than that required to inhibit the alternative pathway-mediated lysis E_R (Fig. 4A). It is possible that the higher concentrations of rVCP required to inhibit E_R lysis are due to reduced affinity of rVCP for the C3b bound to E_R. This phenomenon is well documented for factor H activity on C3b bound to E_R vs E_S (30, 31). However, this seems unlikely for rVCP, as its alternative pathway regulatory activity is nearly same for E_R and E_S (Fig. 4, A and B).

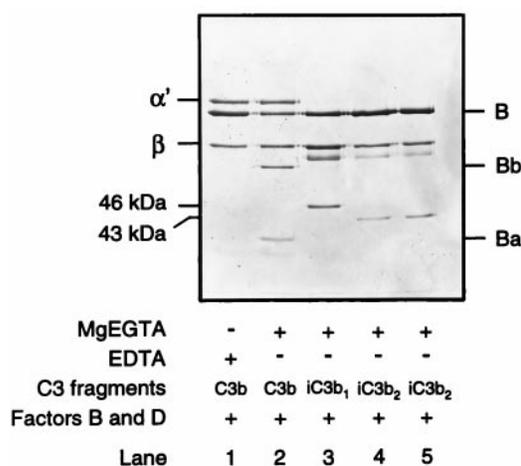


FIGURE 8. Analysis of the formation of fluid-phase C3 convertase by C3b, iC3b₁, and iC3b₂. C3 fragments (C3b, iC3b₁, or iC3b₂) were incubated with factors B and D in the presence of MgEGTA at 37°C for 30 min. The reaction was terminated by adding SDS-PAGE sample buffer, and the formation of C3 convertase was determined by the presence of Bb and Ba fragments of factor B on a 7.5% SDS-PAGE gel. Controls contained EDTA, which inhibits the formation of C3 convertase (lane 1). C3b was included as a positive control, and iC3b₂ generated using rVCP and factor I (lane 4) and factors H and I (lane 5) were used as negative controls.

These results suggest that, unlike factor H, rVCP does not discriminate between activator (E_R)- and nonactivator (E_S)-bound C3b. Thus, based on the present and previous (13) observations, the greater effect of VCP on the classical pathway could be attributed to its effect on C3 as well as C4. This dual effect of VCP on C3 as well as C4 could benefit the virus by preventing its damage by host complement in the absence as well as the presence of a specific Ab response.

VCP is composed entirely of four SCR domains and is much smaller in size than factor H (composed of 20 SCRs) or CR1 (composed of 30 SCRs). This difference led us to ask: Is VCP as effective as other physiologic regulators of complement in inhibiting complement activation? The data shown in Figure 4 demonstrate that it is less effective than CR1 in inhibiting both pathways of complement and than factor H in inhibiting the alternative pathway. However, it is noteworthy that rVCP was four times more effective than factor H in inhibiting the classical pathway, due to its dual action on C3 and C4. Thus, it seems that the virus encodes a small protein that reflects the limited size of its genome, but at the same time this protein is able to regulate multiple components of the complement system, a situation that produces a cumulative effect on the classical pathway activation that results in a higher inhibitory effect than that of factor H. This phenomenon could be essential for the survival of the virus. Recently it has become clear that this strategy of complement evasion is not unique to the vaccinia virus and that other viruses also encode proteins similar to human complement control proteins (8).

While prior work (13) suggested that VCP had factor I cofactor activity, this activity was not conclusively established, as the resulting C3b/C4b cleavage products had not been identified. To characterize the cofactor activity of VCP, we have studied the factor I-mediated cleavages of C3b and C4b in the presence of VCP. Although no differences in the cleavage pattern of C4b was observed (Fig. 6), to our surprise, we found that rVCP primarily supports the cleavage of C3b to iC3b₁ (Fig. 5); before this study, it had not been clear whether iC3b₁ could participate in the formation of C3 convertase, C3b,Bb. While iC3b₁ is also generated in the presence of other cofactors (factor H, CR1, and MCP), the second cleavage occurs immediately after the first, and therefore it had been difficult in the past to isolate and purify this cleavage product. Generation and accumulation of iC3b₁ in the presence of VCP allowed us to study this second form. We used anion exchange chromatography to separate iC3b₁ from C3b and iC3b₂ (Fig. 7). This method provided sufficient resolution to yield monodisperse populations of each of the C3 cleavage products. Incubation of iC3b₁ with factors B and D in the presence of MgEGTA showed that iC3b₁ is unable to form the alternative pathway of C3 convertase and is biologically inactive (Fig. 8). These data suggest that the first cleavage by itself is sufficient to inactivate C3b, and second cleavage is not necessary, as has been previously thought. Our data also reinforce the belief that accessibility to different cleavage sites by factor I is dependent upon the cofactor involved (4).

The data obtained in the present and previous studies make it clear that VCP effectively regulates complement by inactivating C3 and C4 and helps the vaccinia virus to evade the host immune response. It is reasonable to assume that at the site of infection, VCP effectively inhibits complement activation and the generation of C3a and C5a. As a result, it would protect the virus from complement-mediated damage and also reduce the specific inflammatory response against the virus. However, it would be advantageous for the virus to encode a membrane-bound complement regulatory protein, which could inhibit the complement activation focused on the viral surface and the surface of infected cells. Vac-

cinia virus encodes a second protein (the gene product of B5R) (32), which is present on the outer envelope of the virus (33–36) and shows sequence similarity to complement control proteins (32–34). The role of this protein in complement inactivation, however, remains unknown. Further characterization of this protein would provide valuable insight into other essential aspects of viral pathogenesis.

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