

Glycosylated and Nonglycosylated Complement Control Protein of the Lister Strain of Vaccinia Virus

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The vaccinia virus complement control protein (VCP) is a secreted viral protein that binds the C3b and C4b complement components and inhibits the classic and alternative complement pathways. Previously, we reported that an attenuated smallpox vaccine, LC16m8, which was derived from the Lister strain of vaccinia virus (VV-Lister), expressed a glycosylated form of VCP, whereas published sequence data at that time indicated that the VV-Lister VCP has no motif for N-linked glycosylation. We were interested in determining whether the glycosylation of VCP impairs its biological activity, possibly contributing to the attenuation of LC16m8, and the likely origin of the glycosylated VCP. Expression analysis indicated that VV-Lister contains substrains expressing glycosylated VCP and substrains expressing nonglycosylated VCP. Other strains of smallpox vaccine, as well as laboratory strains of vaccinia virus, all expressed nonglycosylated VCP. Individual Lister virus clones expressing either the glycosylated VCP or the nonglycosylated species were isolated, and partially purified VCP from the isolates were found to be functional equivalents in binding human C3b and C4b complement proteins and inhibiting hemolysis and in immunogenicity. Recombinant vaccinia viruses expressing FLAG-tagged glycosylated VCP (FLAG-VCPg) and nonglycosylated VCP (FLAG-VCP) were constructed based on the Western Reserve strain. Purified FLAG-VCP and FLAG-VCPg bind human C3b and C4b and blocked complement-mediated hemolysis. Our data suggest that glycosylation did not affect the biological activity of VCP and thus may not have contributed to the attenuation of LC16m8. In addition, the LC16m8 virus likely originated from a substrain of VV-Lister that expresses glycosylated VCP.

Vaccinia virus, a virus closely related to variola virus, the etiologic agent of smallpox, was used as the vaccine for the eradication of smallpox. The possibility of a reemergence of smallpox and concerns about zoonotic poxvirus diseases like cowpox and monkeypox (1–4) have fueled research into the development of new smallpox vaccines. The new smallpox vaccines are expected to be safer than the currently licensed smallpox vaccines in order to reduce or prevent the serious adverse events associated with the traditional smallpox vaccines (5).

LC16m8 is a live attenuated smallpox vaccine in development (6). The LC16m8 vaccine virus was derived by more than 45 passages and cold selection of the Lister/Elstree vaccine virus in primary rabbit kidney cells (7), with a truncation of the *B5R* gene identified as the main attenuating mutation (8). Previously, we reported that LC16m8 expressed a glycosylated form of the vaccinia complement control protein (VCP) (9), a 28-kDa protein encoded in the *C3L* open reading frame (ORF) (vaccinia virus Copenhagen designation). VCP is one of about 20 immunomodulatory proteins encoded in the vaccinia virus genome and is the major secreted viral protein detected in the culture medium of cells infected with a clonal vaccinia virus, DV-3, isolated from Dryvax (9). VCP is a homolog of the human regulators of complement activation proteins and has been shown to inhibit antibody-mediated complement-enhanced neutralization of vaccinia virus *in vitro* (10). Mechanistically, VCP functions to facilitate viral pathogenesis by inhibiting complement activation (11, 12) through enhancement of factor I-mediated inactivation of complement proteins C3b (13) and C4b and by accelerating the dissociation of the C3 convertase of the complement pathway (14, 15). The *C3L* gene is conserved among vaccinia virus strains, and homologs of *C3L* encoding inhibitors of complement enzymes (ICEs) have been described in other orthopoxviruses, including variola virus (VARV), monkeypox virus (MPXV), cowpox virus

(CPXV), and ectromelia virus (ECTV), where the encoded proteins are known as SPICE, MOPICE, CPV-IMP, and EMICE, respectively (16–19). Among the vaccine strains of vaccinia virus, a *C3L* homolog is present in Lister, Dryvax, ACAM2000, and LC16m8, but not in the modified vaccinia virus Ankara. Interestingly, the absence of the monkeypox virus inhibitor of the complement enzyme MOPICE (the VCP homolog found in MPXV) in the West African clade of MPXV is believed to contribute to its relative attenuation compared to that of the more virulent Central African clade of MPXV responsible for cases of human monkeypox in the Congo basin (20, 21).

At the time of the initiation of this study, all published sequences of vaccinia virus strains, including two Lister isolates (www.poxvirus.org), lack sequences for the N-glycosylation motif. Thus, we were interested in determining if glycosylation of VCP impairs its function, thereby contributing to the attenuation of LC16m8. We were also interested in determining the likely origin of the glycosylation motif in the LC16m8 VCP. A previous report (22) indicated that VV-Lister expresses glycosylated VCP that was biologically less active than nonglycosylated VCP expressed by vaccinia virus strain Western Reserve (VV-WR), suggesting a potential effect of glycosylation on biological activity. Here we report that VV-Lister expresses both glycosylated and

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nonglycosylated forms of VCP. We showed that the glycosylated VCP (L3-VCP) and nonglycosylated VCP (L5-VCP) expressed by the clonal isolates L3 and L5 of VV-Lister, respectively, bind the human C3b and C4b complement components and inhibit complement-mediated hemolysis. To confirm these findings, we constructed a recombinant vaccinia virus strain, WR, in which an N-linked glycosylation site was introduced by mutation at the same asparagine residue as that present in LC16m8 VCP. Glycosylation of the recombinant VCP was confirmed by digestion with peptide-N-glycosidase F (PNGase F). This posttranslational modification did not impede its ability to bind to the human C3b and C4b or its ability to inhibit complement-mediated cell lysis. DNA sequence analysis showed that the N-linked glycosylation site present in the L3 *C3L* ORF mapped to the same locus in the published sequence of LC16m8, suggesting that the LC16m8 virus could have originated from a lineage of VV-Lister expressing glycosylated VCP.

MATERIALS AND METHODS

Cells and viruses. Vaccinia virus strain Western Reserve (VV-WR) was a gift from Bernard Moss (NIAID/NIH). VV-WR and VV-Lister (ATCC VR-1549) were prepared by infection of BSC-1 cells (ATCC CCL-26) and purified by centrifugation through sucrose cushions as previously described (23) and the titer was determined by a plaque assay in BSC-40 cells (ATCC CRL-2761).

Mice. Male BALB/cByJ mice (4 to 5 weeks old) were obtained from The Jackson Laboratory (Bar Harbor, ME). They were housed in cages at a core facility in the Center for Biologics Evaluation and Research (CBER), where sterile feed and drinking water were provided. Mice were routinely cared for by the Division of Veterinary Services, CBER. Care and handling of animals were performed according to the guidelines provided by the Animal Research Advisory Committee of the National Institutes of Health, and the animal study protocol was approved by the Institutional Animal Care and Use Committee of the CBERS/FDA.

Isolation of VV-Lister virus clones. VV-Lister was diluted in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and plated on BSC-40 cells. Ten individual plaques of different growth phenotypes (small, medium, and large plaques) were selected, purified twice in BSC-40 monolayers, and tested for VCP expression by Western blotting.

Western blotting. Glycosylated and nonglycosylated VCP were resolved in NuPAGE 4 to 12% gel (Invitrogen), and proteins were transferred onto nitrocellulose membranes using iBlot (Invitrogen). Membranes were incubated in blocking buffer (4% nonfat milk-1% bovine serum albumin [BSA] in Tris-buffered saline [TBS]) for 30 min, followed by incubation with a 1:500 dilution of an in-house mouse anti-VCP polyclonal antiserum for 1.5 h. An enhanced chemiluminescence (ECL) horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (1:2,000) was used as the secondary antibody. Membranes were washed with TBS-Tween, and a 1:5 dilution of the SuperSignal West Dura chemiluminescent HRP substrate (Fisher Scientific, Rockford, IL) was added. After 5 min, a chemiluminescent image was captured using the Fujifilm LAS-3000 imaging system equipped with a charge-coupled device camera (Fujifilm Medical Systems, Stamford, CT).

Construction of recombinant VV-WR expressing FLAG epitope-tagged glycosylated and nonglycosylated VCP. The design of primers for the construction of VV-WR recombinants was based on the published sequence of vaccinia virus strain Western Reserve (GenBank accession no. NC_006998). A pair of primers corresponding to coordinates 18,066 to 18,091 and the reverse complement of coordinates 18,651 to 18,679 with the sequence corresponding to coordinates 1,667 to 1,695 of the plasmid pLW44 (a gift from Linda Wyatt and Bernard Moss, NIAID/NIH) appended to the 5' end were used to amplify a 611-bp left flanking sequence of the *C3L* ORF from VV-WR genomic DNA. A 599-bp right flanking

sequence of the *C3L* ORF was amplified with a pair of primers corresponding to coordinates 19,469 to 19,496 of WR DNA with the reverse complement of coordinates 976 to 1,002 of pLW44 appended on the 5' end and the reverse complement of coordinates 20,042 to 20,067.

A 723-bp fragment containing the sequence of the enhanced green fluorescent protein (eGFP) was amplified from plasmid pLW44. For this purpose, a pair of primers corresponding to the reverse complement of coordinates 1,666 to 1,695 of pLW44 with the sequence of coordinates 18,652 to 18,679 of the WR sequence appended to the 5' end and coordinates 976 to 1,003 of pLW44 with the reverse complement of coordinates 19,469 to 19,497 of the WR DNA sequence appended to the 5' end were used. The three PCR fragments were purified and used as the template for a second-step PCR, using the primer pair corresponding to coordinates 18,066 to 18,091 and the reverse complement of coordinates 20,042 to 20,067 of the WR DNA to amplify a 2-kbp fragment containing the right and left flanking sequences of the *C3L* gene, with the complete coding sequence of eGFP between the two *C3L* flanking sequences. This PCR product was ligated with the Zero Blunt TOPO vector (Invitrogen) to generate plasmid pT-rfGFP_{IF}. To generate a recombinant VV-WR deleted of the *C3L* gene, pT-rfGFP_{IF} was transfected into BSC-40 cells 2 h after the cells had been infected with VV-WR at a multiplicity of infection (MOI) of 0.1. Recombinant VV-WR expressing GFP (WR-gfpΔ*C3L*) underwent 6 rounds of plaque purification.

In making the recombinant VV-WR expressing FLAG-tagged VCP, a pair of primers corresponding to coordinates 18,083 to 18,110 of the WR DNA and the reverse complement of coordinates 18,646 to 18,670 with the sequence GACTATAAAGACGATGACGATAAGTAA encoding the octapeptide FLAG epitope appended to the 5' end were used to amplify the left flanking sequence (594 bp) of the *C3L* ORF. A 599-bp right flanking sequence of the *C3L* ORF was amplified with a pair of primers corresponding to coordinates 19,452 to 19,480 and the reverse complement of coordinates 20,042 to 20,067. An 816-bp fragment containing the sequence of the *C3L* ORF was amplified with a pair of primers corresponding to coordinates 18,689 to 18,704 with the sequence TTAATTATCGTC ATCGTCTTTATAGTC (reverse complement of the FLAG epitope sequence) appended to the 5' end and the reverse complement of coordinates 19,452 to 19,480 of the WR genome. The three fragments were purified and combined as the template in a second-step PCR using the pair of primers corresponding to coordinates 18,083 to 18,110 and the reverse complement of coordinates 20,042 to 20,067. The resulting ~2-kbp PCR product was ligated with the Zero Blunt TOPO vector to generate plasmid pT-rfC3L_{FLAG}. To generate a recombinant VV-WR expressing FLAG-tagged VCP, pT-rfC3L_{FLAG} was transfected into BSC-40 cells, 2 h after the cells had been infected with WR-gfpΔ*C3L* at a MOI of 0.1. Recombinant VV-WR expressing FLAG-tagged VCP (WR-C3LFLAG) underwent 6 rounds of plaque purification.

A similar strategy was used for the construction of a VV-WR recombinant (WR-C3LgFLAG) expressing a FLAG-tagged glycosylated form of VCP, with the exception of the generation of the FLAG-tagged *C3L* fragment that contained substitutions of nucleotides 18,909 and 18,910 (C and C) with nucleotide bases G and A, respectively, by site-directed mutagenesis. These base substitutions resulted in a change of the coding triplet base (GGT) for glycine-187 in the *C3L* gene to TCT encoding serine, thereby putting asparagine-185 in context for N-linked glycosylation. Both recombinant WR-C3LFLAG and WR-C3LgFLAG were prepared from BSC-40 infected cells and partially purified by centrifugation through a 36% sucrose cushion.

Expression and purification of VCP. BSC-40 cells in 850-cm² roller bottles were infected with vaccinia viruses (recombinant WR-C3LFLAG, WR-C3LgFLAG, WR-gfpΔ*C3L*, Lister clone L3 [L3], and Lister clone L5 [L5]) at a MOI of between 3 and 5 and incubated in reduced serum Opti-MEM medium (Invitrogen) for 24 h. The supernatant of infected cells was harvested and clarified by centrifugation at 3,000 rpm for 10 min at 4°C. The clarified supernatant was concentrated in Amicon 10,000 molecular weight cutoff (MWCO) columns (Millipore Corp., Billerica, MA)

and exchanged into sodium phosphate buffer (pH 7.0). The proteins were then partially purified by heparin affinity chromatography using HiTrap heparin columns (GE Healthcare, Piscataway, NJ). For the FLAG-tagged proteins (FLAG-VCP and FLAG-VCPg), a second-step purification was performed by FLAG affinity chromatography, and the proteins were eluted with the FLAG peptide (Sigma-Aldrich, St. Louis, MO). Purified proteins were concentrated in Amicon 10,000 MWCO columns, and protein concentrations were determined using the BCA assay kit (Thermo Fisher Scientific, Rockford, IL).

C3b and C4b complement binding ELISA. An enzyme-linked immunosorbent assay (ELISA) for the detection of VCP binding to human complement components C3b and C4b was performed as previously described (23, 24), with modifications. In brief, Immulon 2HB 96-well ELISA plates were coated with C3b or C4b (Complement Technology Inc., Tyler, TX) at 500 ng in phosphate-buffered saline (PBS) per well and stored at 4°C overnight. The wells were washed with PBS containing 5% BSA-0.1% Tween 20 for 2 h. BSA (catalog no. A7888) was obtained from Sigma-Aldrich. Plates were washed, and the test samples were added and serially diluted by 100- μ l serial transfers in diluent (wash buffer containing 4% BSA) and reincubated for 2 h. In assays containing L3-VCP and L5-VCP test samples with no FLAG tag, a 1:250 dilution of mouse anti-VCP polyclonal antiserum was added to the wells, and plates were incubated for 2 h. A 1:5,000 dilution of goat anti-mouse, HRP-conjugated antibody was used as the secondary antibody. In assays using VCP-FLAG and VCPg-FLAG, a 1:3,000 dilution of mouse anti-FLAG M2 peroxidase-conjugated monoclonal antibody (Sigma-Aldrich) was added to all wells, and plates were reincubated for 1 h. Plates were washed, and the 2,2'-azino-bis(3-ethylthiazolinesulfonic acid (ABTS)-hydrogen peroxide (H_2O_2) peroxidase substrate (KPL, Gaithersburg, MD) was used for the detection of antigen-antibody binding as described by the manufacturer. Absorbance (optical density) at 405 nm (OD_{405}) values were read in a Versa Max ELISA plate reader equipped with SoftMax Pro software (Molecular Devices, Sunnyvale, CA).

Hemolysis assay. The inhibition of complement-dependent hemolysis was performed as described previously (11) and as adapted in the laboratory of S. Isaacs at the University of Pennsylvania (personal communication), with minor modifications. Unless otherwise indicated, all reagents used in performing the hemolysis inhibition assay were obtained from Complement Technology Inc., and gelatin veronal buffer (GVB) was used as the diluent. Serial 2-fold dilutions of the test samples (VCP, VCPg, L3-VCP, and L5-VCP) and the negative controls (TBS and Δ VCP) were made in GVB in a 96-well round-bottom Immulon 1B plate (Thermo Scientific, Rochester, NY). The diluted samples were incubated with 1×10^8 cells/well of antibody-sensitized sheep erythrocytes (sRBC) in the presence of guinea pig serum that was diluted 1:25 in GVB. Assay wells containing only sheep erythrocytes and GVB were included as negative controls, and wells containing sheep erythrocytes, GVB, and guinea pig serum were included as lysed controls. Assay plates were incubated at 37°C for 30 min and cooled to room temperature. The plates were centrifuged at 1,000 rpm in a Sorvall Legend XTR benchtop centrifuge (Thermo Fisher Scientific, Pittsburgh, PA) for 10 min. The clarified supernatant (80 μ l from each well) was transferred into a flat-bottom Immulon 1B plate (Thermo Scientific, Rochester, NY), and the absorbance at 405 nm was read in a Versa Max ELISA plate reader, with the GVB/erythrocyte supernatant as the blank. The absorbance data were exported into Excel, and the percentage of hemolysis and the percentage of hemolysis inhibition were computed, using the mean OD_{405} value of the GVB/guinea pig serum/erythrocyte supernatants as 100% hemolysis.

Immunization and antibody ELISA. Groups of 5 mice were anesthetized with 2,2,2-tribromoethanol (Avertin) as previously described (22) and inoculated with 10^7 PFU (per mouse) of Lister clone 3 (L3) or clone 5 (L5) by tail scarification. Antiserum samples were obtained from mice 3 weeks after inoculation, and mice received boost inoculations (10^7 PFU/mouse) by subcutaneous injection. Antisera were collected 3 weeks after the booster inoculation. All serum samples were tested for VCP-specific

IgG by an ELISA as previously described (22), using purified VCP or VCPg as the antigen.

Statistical analysis. The analysis of differences in VCP-specific antibody responses between Lister clone 3-vaccinated and Lister clone 5-vaccinated mice was performed with SigmaPlot (Systat Software Inc.), using the unpaired Student *t* test or the Mann-Whitney rank sum test to compare the log-transformed values of the antibody titers. A value of $P < 0.05$ represents a significant difference.

Nucleotide sequence accession numbers. The GenBank nucleotide accession numbers for the *C3L* gene in VV-Lister clones L3, L5, L6, and L9 are [KC831551](https://www.ncbi.nlm.nih.gov/nucl/NC_020451.1), [KC831552](https://www.ncbi.nlm.nih.gov/nucl/NC_020452.1), [KC831553](https://www.ncbi.nlm.nih.gov/nucl/NC_020453.1), and [KC831554](https://www.ncbi.nlm.nih.gov/nucl/NC_020454.1), respectively.

RESULTS

Glycosylated and nonglycosylated forms of VCP are expressed by VV-Lister. All vaccinia virus strains tested, with the exception of the modified vaccine virus Ankara, expressed vaccinia VCP (not shown). Previously, we reported that the LC16m8 smallpox vaccine virus expresses a glycosylated form of VCP (9). Since LC16m8 was derived by passage of the Lister virus in primary rabbit kidney (PRK) cells, we were interested in determining whether the glycosylation of VCP in LC16m8 originated from the parent virus or was possibly due to an acquired mutation during the passage of VV-Lister in PRK cells. At the time of the initiation of this study, the nucleotide sequences of two VV-Lister isolates published by the Viral Bioinformatics Resource Center (VBRC), VBRC accession number VP0046057 of 8 September 2005 (VACV-Lister) and VBRC accession number VP0064359 of 20 March 2007 (VACV-Lister107) (www.poxvirus.org), indicated that neither of the Lister isolates has sequences encoding potential N-linked glycosylation motifs in the *C3L* ORF. In a preliminary experiment, VV-Lister VCP expressed in BSC-40 cell monolayers contained two molecular weight species of $\sim 28,000$ and $\sim 30,000$ (data not shown), indicating that there were two different forms of VCP expressed by VV-Lister. Since the band with a higher molecular weight was similar to the size of VCP detected in the culture medium of LC16m8-infected cells, we speculated that this band was likely a glycosylated species of the VV-Lister VCP.

In isolating the VV-Lister clones expressing the two species of VCP, 10 plaques were isolated from VV-Lister-infected cells. Each plaque was purified twice by passage in cell monolayers and tested for VCP expression by Western blotting. Of the 10 clonal isolates, 6 were found to have the double bands of VCP similar to the Lister virus stock, possibly due to insufficient purification of these isolates. However, the remaining 4 clones had single bands, three (clones L3, L6, and L9) of which expressed the higher molecular weight species and one (clone L5) that expressed the lower molecular weight species similar to DV-3, a clonal isolate from the Dryvax smallpox vaccine (Fig. 1A). Genomic DNA samples were extracted from the 4 single-band clones, and the *C3L* locus from each was amplified by PCR, cloned, and sequenced. The sequence data for all 4 clones were deposited into GenBank.

The sequence data confirmed the presence of sequences encoding a motif for the glycosylation of asparagine-185 of VCP, as found in LC16m8 in clones L3, L6, and L9. The sequence motif for potential N-glycosylation is absent in the *C3L* gene of clone L5. Although there were a few other amino acid differences among the 4 isolates, none of the 3 Lister isolates expressing the higher molecular weight species has any additional potential glycosylation site. Alignments of the translated amino acid sequences for residues 171 to 200 of Lister clone 3 and Lister clone 5 versus the amino acid sequence of the WR strain were generated using the

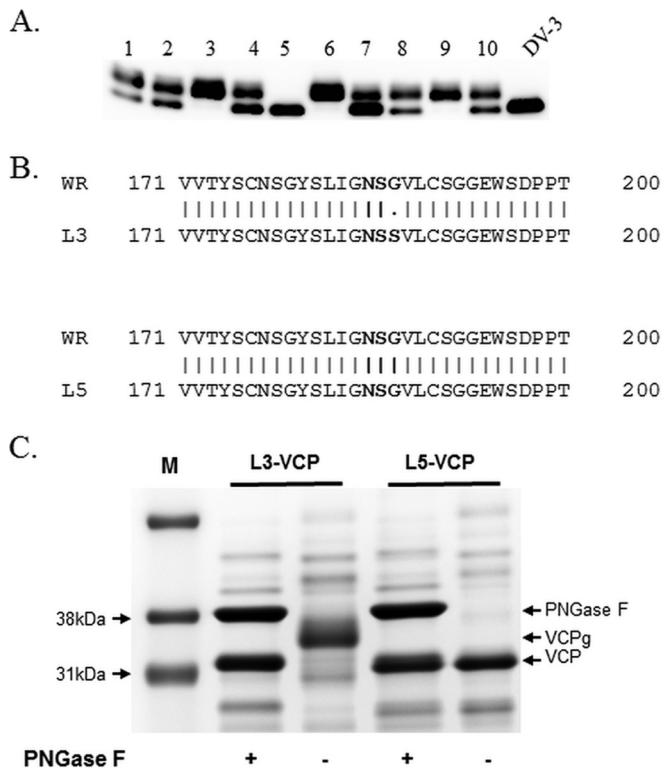


FIG 1 Expression of VCP from VV-Lister. (A) Ten plaque isolates of the vaccinia virus Lister strain were plaque purified in BSC-40 cell monolayers and tested for the expression of glycosylated or nonglycosylated VCP by Western blotting. DV-3 is a clonal isolate of Dryvax. (B) The amino acid sequence alignments of residues 171 to 200 of L3-VCP and L5-VCP versus those of residues of the WR strain, with the N-glycosylation site in L3 VCP are in bold. (C) Partially purified L3-VCP and L5-VCP were incubated in the presence (+) or absence (-) of PNGaseF, resolved by SDS-PAGE, and stained with SimplyBlue stain solution. Lane M, Rainbow high-molecular-weight protein markers.

EMBOSS-Lite sequence comparison tool (NCBI/NIH) and are shown in Fig. 1B. This set of data suggests that the original VV-Lister contains substrains expressing two species of VCP.

The clones L3 and L5, expressing the glycosylated and nonglycosylated forms of VCP, respectively, were selected for further characterization. VCP expressed by these two isolates, L3-VCP and L5-VCP, respectively, were partially purified on heparin columns from culture supernatants of infected cells. Both glycosylated and nonglycosylated VCP in Tris-buffered saline were stable for at least 6 months under storage at 4°C. In order to verify the glycosylation of L3-VCP, an aliquot of the protein was incubated at 37°C in the presence or absence of the deglycosylating enzyme PNGase F. The digested protein and untreated controls were resolved in a protein gel and stained (Fig. 1C). The L3-VCP that was incubated with buffer contained a band corresponding to the glycosylated protein. However, the lane containing the L3-VCP that was incubated with PNGase F had a single band corresponding to the lower molecular weight species of VCP (Fig. 1C), indicating that the protein had been deglycosylated and confirming that L3-VCP expressed by VV-Lister clone 3 is glycosylated. On the other hand, the lanes containing L5-VCP, whether or not incubated with PNGase F, contained a VCP band of the same molecular weight, indicating that incubation of L5-VCP with the enzyme did not result in a reduction in the molecular weight of the protein.

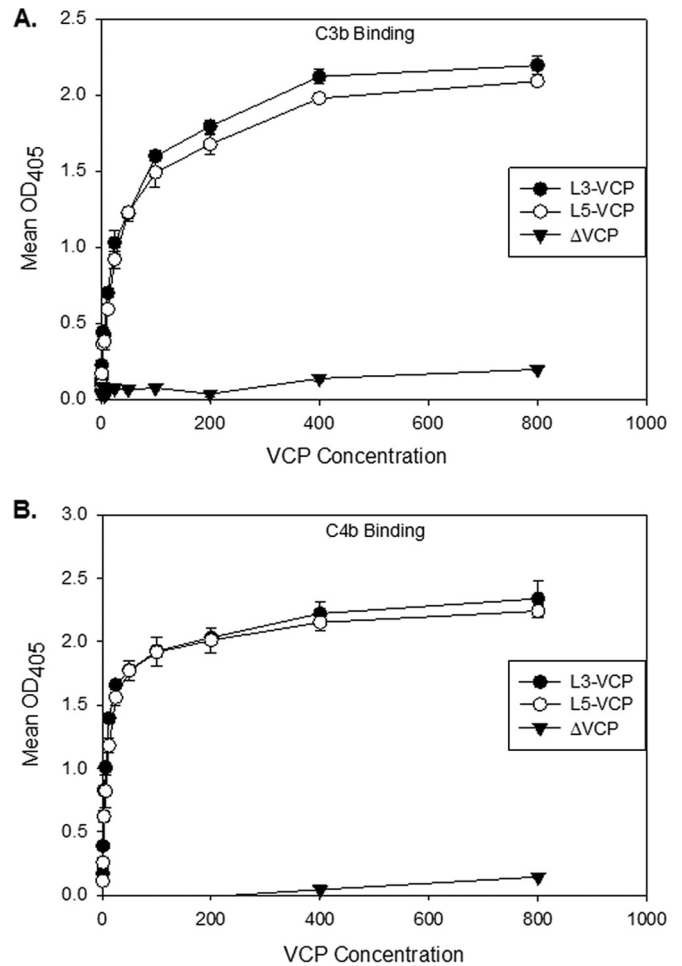


FIG 2 L3-VCP and L5-VCP binding to human C3b and C4b complement proteins. Human complement proteins C3b and C4b coated in the wells of Immulon 2HB plates were incubated with purified L3-VCP and L5-VCP or with TBS. C3b and C4b binding to L3-VCP or L5-VCP was detected by subsequent incubation with a mouse anti-VCP polyclonal antibody followed by incubation with an HRP-conjugated anti-mouse antibody. Each data point represents the mean OD₄₀₅ from duplicate assays for C3b binding (A), and C4b binding (B). The error bars represent the standard deviations for replicate samples. The data are representative of assays from two batches of L3-VCP and L5-VCP.

L3-VCP and L5-VCP bind to human C3b and C4b complement proteins. In order to test the biological activity of the glycosylated and nonglycosylated forms of VCP expressed by VV-Lister clones L3 and L5, respectively, BSC-40 cells were infected with VV-Lister clones L3 and L5 at a multiplicity of infection (MOI) of 3.0. Control cells were similarly infected with WR-gfpΔC3L, a recombinant vaccinia virus (WR strain) in which the C3L gene encoding VCP has been substituted with eGFP. Partially purified L3-VCP and L5-VCP proteins, along with a similarly prepared extract from WR-gfpΔC3L (ΔVCP), were tested by ELISAs for binding of the human C3b and C4b complement proteins. Fig. 2A and B shows plots of the absorbance (OD₄₀₅) values for C3b and C4b binding, respectively. Figure 2 shows that there was no reactivity with the ΔVCP extract, indicating that neither C3b nor C4b binds to the secreted cellular or viral proteins that might have copurified with VCP during partial purification on the heparin

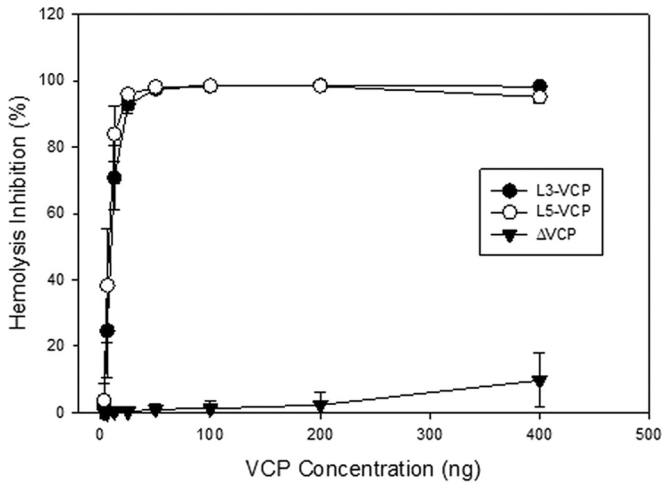


FIG 3 L3-VCP and L5-VCP inhibition of complement-mediated cell lysis. Serial 2-fold dilutions of L3-VCP, L5-VCP, or TBS were incubated with antibody-sensitized sheep red blood cells (sRBC) in the presence of guinea pig serum as the source of complement. sRBC without guinea pig serum served as the control. The OD₄₀₅ of clarified supernatants was determined. The error bars represent the standard deviations of duplicate assays. Each data point represents the mean from two experiments using two batches of L3-VCP and L5-VCP.

columns. However, both L3-VCP and L5-VCP bound C3b (Fig. 2A) and C4b (Fig. 2B) with similar affinity.

L3-VCP and L5-VCP block complement-mediated lysis of sheep erythrocytes. L3-VCP and L5-VCP, as well as the ΔVCP control preparation, were tested for biological activity by measuring *in vitro* the ability to block complement-mediated lysis of antibody-sensitized sheep erythrocytes. Serial 2-fold dilutions of the test samples were incubated with antibody-sensitized sheep red blood cells in the presence of guinea pig serum as a source of complement. The degree of hemolysis was measured spectrophotometrically, and the percentages of hemolysis inhibition were calculated. Figure 3 is a plot of the percentage of hemolysis inhibition of the test samples. Both L3-VCP and L5-VCP inhibited complement-mediated hemolysis but not the ΔVCP control preparation. This set of data suggested that both the glycosylated and nonglycosylated species of VCP expressed by the VV-Lister virus are biologically active with regard to the inhibition of complement-mediated cell lysis.

Construction of recombinant viruses expressing FLAG-tagged glycosylated or nonglycosylated VCP. Since the glycosylated and nonglycosylated VCP proteins expressed by the Lister isolates were not easily purified to homogeneity, we generated recombinant vaccinia viruses, based on the VV-WR strain, expressing FLAG-tagged versions of each protein. To obtain a glycosylated VCP similar in sequence to the VCP expressed in LC16m8 and VV-Lister, PCR site-directed mutagenesis was used to mutate the coding triplet nucleotides for glycine-187 to the coding triplet nucleotide for serine in VV-WR. This manipulation resulted in the generation of an N-linked glycosylation motif that makes asparagine-185 a potential glycosylation site (as present in LC16m8). The modification aligned perfectly to the corresponding sequence in L3. In addition, we appended the coding nucleotide sequence for the FLAG epitope octapeptide (DYKDDDDK) to the 3' end of the C3L gene encoding VCP to facilitate protein

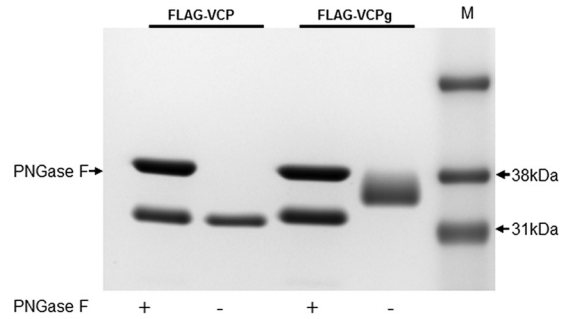


FIG 4 Expression of FLAG-VCP and FLAG-VCPg. BSC-40 cells were infected with recombinant VV-WR expressing FLAG-VCP or FLAG-VCPg. FLAG-tagged proteins were purified by affinity chromatography. Purified FLAG-VCP and FLAG-VCPg were incubated in the presence (+) or absence (-) of PNGase F, resolved in SDS-PAGE gel, and stained with SimplyBlue stain. Lane M, Rainbow protein molecular weight markers.

purification. The PCR product was amplified, along with nucleotide sequences upstream (594 bp) and downstream (599 bp) flanking C3L, and inserted into the recombinant WR-gfpΔC3L in which the sequence of C3L had been replaced with the sequence of eGFP to generate wrC3Lg-FLAG expressing the FLAG-tagged, glycosylated VCP (FLAG-VCPg). A similar strategy was used to produce wrC3L-FLAG, a recombinant VV-WR expressing the nonglycosylated, FLAG-tagged version of VCP (FLAG-VCP).

Recombinant FLAG-VCP and FLAG-VCPg were purified from BSC-40 cells infected with wrC3Lg-FLAG and wrC3L-FLAG, respectively, using a combination of heparin affinity and FLAG affinity chromatography. The purity of the proteins was confirmed by the detection of a single band of each protein in SDS-PAGE gel stained with SimplyBlue stain (not shown). To confirm glycosylation, aliquots of FLAG-VCP and FLAG-VCPg were treated with or without PNGase F, resolved by SDS-PAGE, and stained with SimplyBlue stain (Fig. 4). Untreated and PNGase F-treated FLAG-VCP migrated at a molecular weight of ~30,000, remaining unaffected by PNGase F treatment. FLAG-VCPg incubated with buffer only remained at the original size of ~34,000 (i.e., the band corresponding to FLAG-VCP). On the other hand, the PNGase F-treated FLAG-VCPg migrated at a molecular weight of ~30,000, which corresponded to the size of FLAG-VCP, indicating that FLAG-VCPg was deglycosylated and confirming that the recombinant protein was glycosylated.

FLAG-VCP and FLAG-VCPg bind to human C3b and C4b complement proteins. Purified FLAG-VCP and FLAG-VCPg were tested for binding to human C3b and C4b complement proteins. C3b- and C4b-coated ELISA plates were incubated with serial dilutions of FLAG-VCP or FLAG-VCPg or with Tris-buffered saline (TBS). In the C3b and C4b binding assays, both FLAG-VCP and FLAG-VCPg bind to C3b (Fig. 5A) and to C4b (Fig. 5B). The binding of FLAG-VCPg to C3b was weaker than that of FLAG-VCP, but binding of both proteins to C4b was similar. C3b and C4b binding was not detectable in TBS-coated wells. The relatively lower OD₄₀₅ values of the FLAG-tagged proteins (Fig. 5 versus Fig. 2) was probably due to the use of a monoclonal anti-FLAG antibody as the detection antibody in the former rather than to a reduction in activity caused by the FLAG tag. These results confirmed the binding of L3-VCP and L5-VCP to C3b and C4b complement proteins, indicating that the glycosylation of

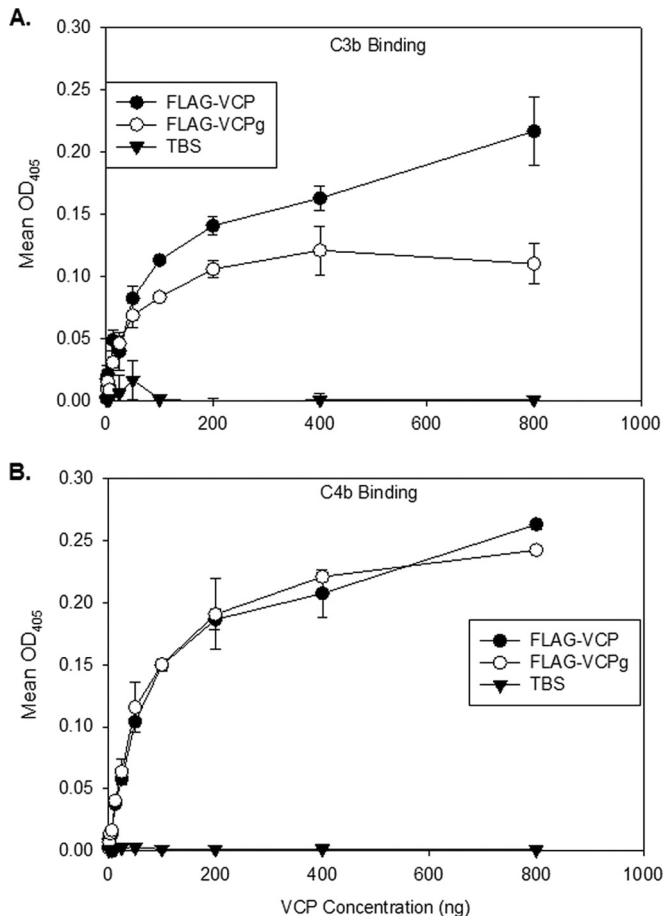


FIG 5 FLAG-VCP and FLAG-VCPg binding to C3b and C4b human complement proteins. Human C3b and C4b complement proteins on ELISA plates were incubated with purified FLAG-VCP, FLAG-VCPg, or TBS. C3b and C4b binding to FLAG-VCP or FLAG-VCPg was detected by subsequent incubation with a mouse anti-FLAG antibody conjugated to HRP. Each data point represents the mean OD₄₀₅ for duplicate assays for C3b binding (A) and C4b binding (B). Data are representative of two assays using two different batches of purified FLAG-VCP or FLAG-VCPg. The error bars represent standard deviations.

VCP has little or no impact on its interaction with the C3b and C4b complement proteins.

FLAG-VCP and FLAG-VCPg block complement-mediated lysis of sheep erythrocytes. The biological activity of FLAG-VCP and FLAG-VCPg was further evaluated in a hemolysis inhibition assay. In this assay, FLAG-VCP and FLAG-VCPg, with TBS as the control, were tested for the ability to inhibit complement-mediated lysis of antibody-sensitized sheep erythrocytes in the presence of guinea pig serum as a source of complement. Hemolysis was measured spectrophotometrically, and the percentages of hemolysis inhibition were calculated. **Figure 6** is a plot of the percentage of hemolysis inhibition of the test samples. Both FLAG-VCP and FLAG-VCPg inhibited the complement-mediated hemolysis but not the TBS control. The amount of FLAG-VCP needed to inhibit hemolysis was higher than that of FLAG-VCPg, with the 50% inhibitory concentrations (IC₅₀) determined to be 78.16 ng and 21.54 ng for FLAG-VCP and FLAG-VCPg, respectively, suggesting that the glycosylated protein was about 4-fold more active.

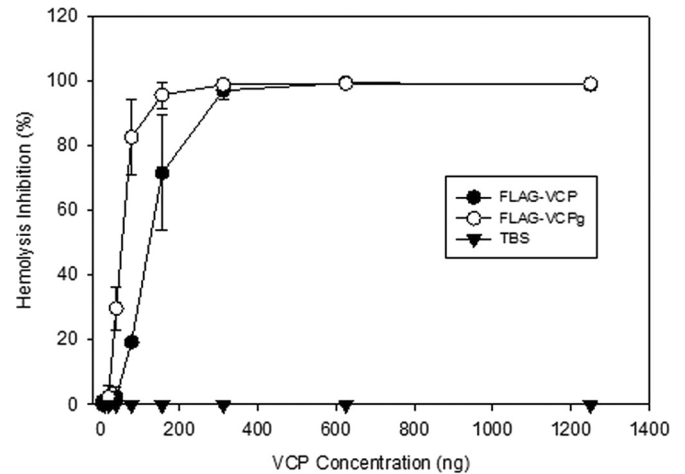


FIG 6 FLAG-VCP and FLAG-VCPg inhibition of complement-mediated cell lysis. Serial 2-fold dilutions of FLAG-VCP, FLAG-VCPg, or TBS were incubated with sRBC in the presence of guinea pig serum. sRBC without guinea pig serum served as a control. The OD₄₀₅ of clarified supernatants was determined. Each data point represents the mean from two experiments using two batches of FLAG-VCP and FLAG-VCPg.

Immunogenicity of VCP and VCPg. VCP is known to block antibody-dependent complement-mediated vaccinia virus neutralization, thereby contributing to virulence (10). Thus, anti-VCP immune responses should abrogate the contribution of VCP to virulence. Previously, we reported that immunization of mice with purified VCP protein from a Dryvax background (9) elicited robust antibody responses in mice, but mice were not protected. In order to assess the effect of glycosylation on the immunogenicity of VCP, we used the clonal isolates of VV-Lister (i.e., clones L3 and L5) to immunize mice. The elicited antibody response was measured using FLAG-VCP and FLAG-VCPg as antigens. The measured absorbance values (OD₄₀₅) of serially diluted antiserum samples for FLAG-VCP antigen and FLAG-VCPg antigen are presented in **Fig. 7A** and **B**, respectively. When FLAG-VCP was used as the antigen and at 3 weeks after the priming vaccination, the VCP-specific antibody response elicited against Lister clone 3 was higher than the response to Lister clone 5, but following the booster vaccination the antibody response was higher in the clone L5 group. Using FLAG-VCPg as the antigen produces higher antibody responses in the L3 antisera, after both the prime and booster immunizations. At the two antibody measurement time points, the differences in antibody responses were not statistically significant between the group of mice vaccinated with Lister clone L3 and Lister clone L5, irrespective of whether FLAG-VCP ($P = 0.081$, 1st blood sample; $P = 0.766$, 2nd blood sample) or FLAG-VCPg ($P = 0.081$, 1st blood sample; $P = 0.766$, 2nd blood sample) was used as the coating antigen. However, the level of antibody detected with FLAG-VCPg as the antigen was statistically significantly higher ($P = <0.001$) than the level of antibody detected with FLAG-VCP as the antigen for each test serum sample. Together, the data suggest that both Lister clone 3 and Lister clone 5 elicited a robust VCP-specific antibody response in mice. Thus, the glycosylation of VCP has no effect on its immunogenicity as determined by the antibody response.

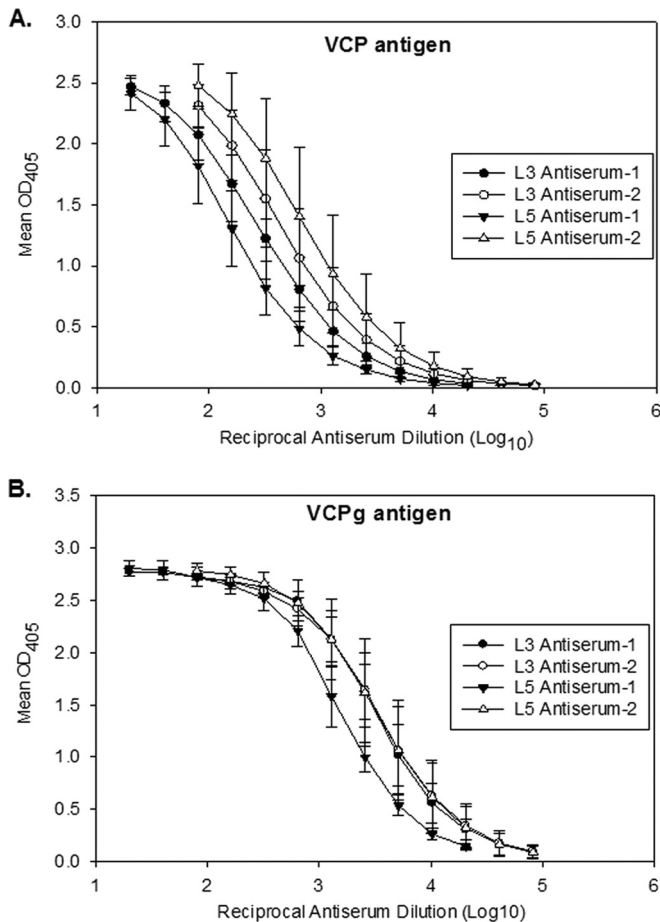


FIG 7 Immunogenicity of glycosylated and nonglycosylated VCP. Groups of 5 mice were vaccinated with 10^7 PFU of VV-Lister clone L3 or VV-Lister clone L5 by prime/booster at an interval of 3 weeks. Antisera obtained 3 weeks after the priming doses (L3 Antiserum-1 and L5 Antiserum-1) and 3 weeks after the booster (L3 Antiserum-2 and L5 Antiserum-2) were tested for reactivity with purified FLAG-VCP (A) or FLAG-VCPg (B) by IgG ELISA. Each data point represents the mean OD₄₀₅ value for all mice per treatment group, and the error bars represent standard deviations.

DISCUSSION

Poxviruses, including vaccinia and variola, encode immunomodulatory proteins that modify or interfere with the host innate immune responses to viral infection. The vaccinia complement control protein (VCP) is similar to a family of proteins with characteristic sequence consensus repeat domains that are regulators of complement activation (25). VCP has been shown to block the innate response to vaccinia virus infection by inhibiting both the classic and alternative complement pathways (13, 15). Mechanistically, VCP causes the decay or inactivation of the C3 convertase enzyme (13, 26), thereby preventing the formation of a membrane attack complex required for effector function. Girgis et al. (27) also reported that VCP can modulate the adaptive arm of the immune response by reducing the humoral and cellular immune responses to vaccinia virus, thereby acting as a virulence factor.

Previously, we reported that the VCP is a dominant viral protein detected in the culture supernatant of cells infected with vaccinia virus (9), and all vaccinia virus strains originating from the New York City Board of Health strain, including Dryvax,

ACAM2000, VV-WR, and IHD-J, expressed nonglycosylated VCP (data not shown). We also observed that while a Dryvax clonal isolate (DV-3) expressed an $\sim 28,000$ VCP, LC16m8 expressed glycosylated VCP that resolved at a molecular weight of $\sim 30,000$. The presence of a potential N-linked glycosylation site in the *m8025L* gene (homolog of the *C3L* ORF of the Copenhagen strain of vaccinia virus) of LC16m8 has been confirmed (28). We were interested in determining the likely origin of the N-glycosylation motif in the LC16m8 VCP and whether the glycosylation of VCP interferes with its known biological function, thereby possibly contributing to the attenuation of LC16m8. Glycosylation is known to alter a protein's biological functions. For instance, O-linked glycosylation of the membrane cofactor protein, a member of the human regulators of complement activation, was shown to have an enhancer effect on its ability to bind the C4b complement protein (29). In addition, it has been shown that blocking VCP activity with monoclonal antibodies significantly reduced virulence in a rabbit intradermal challenge model (30).

Interestingly, we detected both glycosylated and nonglycosylated VCP in culture supernatant of cells infected with VV-Lister. Two VV-Lister isolates, L3 and L5, expressing glycosylated and nonglycosylated VCP, respectively (Fig. 1), were further investigated. Considering the isolation of the vaccinia virus Lister substrains expressing two variants of VCP, one glycosylated and the other nonglycosylated, it is conceivable that other genetic differences exist at other vaccinia gene loci. These differences could have arisen due to the passage of vaccinia virus strains in different species of large animals in the preparation of smallpox vaccines during the 20th century. A similar heterogeneity of Dryvax, the U.S.-licensed smallpox vaccine that was used during the smallpox eradication campaign, was demonstrated by the differences in virulence of clonal isolates during the development of ACAM2000, the currently licensed smallpox vaccine in the United States (31).

The glycosylated L3-VCP and nonglycosylated L5-VCP from the Lister strain were functionally identical as determined by binding to complement components C3b and C4b (Fig. 2), as well as by their ability to inhibit complement-mediated hemolysis (Fig. 3). The inability of Δ VCP (derived from cells infected with VV-WR deleted of *C3L*) to inhibit hemolysis compared to that of the clonal Lister supernatants (Fig. 3) or a heparin-purified supernatant of wild-type VV-WR (not shown) indicates that the inhibition of hemolysis measured in our assay was due to the activity of L3-VCP and L5-VCP.

Although Kotwal (22) reported that the glycosylated VCP (Lis-VCP) was 4-fold less active but more immunogenic than the nonglycosylated VCP of VV-WR, our data indicate that L3-VCP and L5-VCP are not different in their inhibition of cell lysis or in their immunogenicity in mice. It is possible that the 242-amino acid Lis-VCP, which has two N-glycosylation sites (22), is more heavily glycosylated and thus less efficient in inhibiting cell lysis but more immunogenic than the 244-amino acid L3-VCP containing a single N-glycosylation site described in our work, further underscoring the heterogeneity of vaccinia virus strains.

Each of the four Lister isolates in our study encodes a 244-amino acid VCP polypeptide. To confirm our data from the use of the partially purified L3-VCP and L5-VCP proteins, recombinant WR vaccinia viruses expressing FLAG-tagged proteins (FLAG-VCP and FLAG-VCPg) were produced and characterized (Fig. 4). Both FLAG-VCP and FLAG-VCPg were capable of binding human C3b and C4b complement proteins (Fig. 5), albeit the FLAG-

VCPg was less efficient in binding C3b. FLAG-VCP and FLAG-VCPg inhibited complement-mediated lysis of antibody-sensitized sheep red blood cells (Fig. 6), but FLAG-VCP was less efficient in inhibiting cell lysis. This set of data confirms that the glycosylation of VCP has little or no effect on the protein's interaction with human C3b and C4b complement or the inhibition of complement-mediated cell lysis. Both VV-Lister clones L3 and L5 elicited robust VCP-specific antibody responses in mice (Fig. 7). These antisera had no inhibitory effect on the ability of FLAG-VCP or FLAG-VCPg to block complement-mediated hemolysis (data not shown), but we cannot rule out the possibility that the concentration of antibody specific to the complement-interacting domains of VCP was low in the polyclonal antiserum preparations and thus insufficient to block VCP interaction with complement components. Thus, although VCP provokes a robust antibody response, the elicited response did not block VCP activity.

In summary, we have shown that glycosylation does not impair the biological activity of VCP, as determined by binding of C3b and C4b complement proteins and the ability to block complement-mediated cell lysis. Taken together, our data suggest that the LC16m8 vaccine virus may have arisen from a substrain of VV-Lister that encodes glycosylated VCP and the glycosylation of VCP may not have contributed to the attenuation of LC16m8.

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