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Expression of the A56 and K2 Proteins Is Sufficient To Inhibit Vaccinia Virus Entry and Cell Fusion▽†

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Many animal viruses induce cells to fuse and form syncytia. For vaccinia virus, this phenomenon is associated with mutations affecting the A56 and K2 proteins, which form a multimer (A56/K2) on the surface of infected cells. Recent evidence that A56/K2 interacts with the entry/fusion complex (EFC) and that the EFC is necessary for syncytium formation furnishes a strong connection between virus entry and cell fusion. Among the important remaining questions are whether A56/K2 can prevent virus entry as well as cell-cell fusion and whether these two viral proteins are sufficient as well as necessary for this. To answer these questions, we transiently and stably expressed A56 and K2 in uninfected cells. Uninfected cells expressing A56 and K2 exhibited resistance to fusing with A56 mutant virus-infected cells, whereas expression of A56 or K2 alone induced little or no resistance, which fits with the need for both proteins to bind the EFC. Furthermore, transient or stable expression of A56/K2 interfered with virus entry and replication as determined by inhibition of early expression of a luciferase reporter gene, virus production, and plaque formation. The specificity of this effect was demonstrated by restoring entry after enzymatically removing a chimeric glycophosphatidylinositol-anchored A56/K2 or by binding a monoclonal antibody to A56. Importantly, the antibody disrupted the interaction between A56/K2 and the EFC without disrupting the A56-K2 interaction itself. Thus, we have shown that A56/K2 is sufficient to prevent virus entry and fusion as well as formation of syncytia through interaction with the EFC.

Vaccinia virus (VACV) and other members of the *Poxviridae* are large double-stranded DNA viruses that replicate entirely within the cytoplasm of the cell (28). The primary infectious particle of VACV, termed the mature virion (MV), has a brick shape and consists of a lipid membrane with approximately 20 associated proteins surrounding an electron-dense core containing the viral genome plus enzymes and factors for early mRNA synthesis (11). Although the majority of MVs remains in the cytoplasm until cell lysis, some become enclosed by double membranes derived from trans-Golgi or endosomal cisternae (19, 45) and traffic on microtubules to the periphery, where they exit the cell with an additional membrane relative to the MV (30, 43). VACV MVs enter cells by fusion with the plasma membrane (2, 8, 9) as well as through a low-pH-dependent endocytic pathway (27, 48, 49). Genetic and biochemical studies have shown that virus entry and membrane fusion depend on a multiprotein entry/fusion complex (EFC) comprised of at least eight proteins, i.e., A16 (36), A21 (47), A28 (41), G3 (23), G9 (35), H2 (31, 39), J5 (40), and L5 (46); two EFC-associated proteins, F9 (5) and L1 (3); and another protein, I2, that has not yet been classified with regard to EFC association (32). Because the EFC proteins are present in the MV membrane, the outer membrane of the extracellular enveloped virion must be disrupted prior to fusion and entry. Disruption has been linked to binding of the extracellular enveloped virion to glycosaminoglycans on the cell surface (25) and can occur spontaneously on the surface of parental cells (20).

Fusion of VACV-infected cells can be triggered by brief low-pH treatment (12, 18) or by mutations in the viral A56R (21, 38) or K2L (24, 50, 55) gene. A56 and K2 can form a complex (A56/K2) in the plasma membrane of infected or uninfected cells (51). Syncytium formation is related to virus entry because of its dependence on production of extracellular virions and the EFC (4, 29, 40, 53). Moreover, A56/K2 binds to the EFC through the A16 and G9 subunits (53, 54). Very recently, cells infected with an A56 or K2 mutant were shown to have increased susceptibility to superinfection (52). Here we showed that expression of A56/K2 in uninfected cells was sufficient to prevent their fusion with cells infected with A56/K2 mutants of VACV. Furthermore, cells expressing A56/K2 exhibited resistance to VACV infection, suggesting that these proteins have dual roles in preventing superinfection and syncytium formation through interaction with the EFC.

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MATERIALS AND METHODS

Cell propagation. BS-C-1 (ATCC CCL-26) cells were maintained in minimum essential medium (MEM) with Earle's balanced salt supplement (Quality Biological, Gaithersburg, MD) containing L-glutamine and 10% fetal bovine serum (FBS). 293TT cells (7) were grown in Dulbecco's MEM (DMEM) (Quality Biological) supplemented with 10% FBS, 2 mM L-glutamine, and 400 μg/ml hygromycin (Invitrogen, Carlsbad, CA). HEK293 cells (ATCC CCL-1573) and 293EACK cells (described below) were grown in DMEM supplemented with

10% FBS and L-glutamine. In addition, 100 μg/ml of Zeocin (Invitrogen) was added to the medium of 293EACK cells.

Purification of VACV. HeLa S3 cells were infected with recombinant VACV at a multiplicity of infection of 3 and incubated for 2 days at 37°C. Infected cells were disrupted with a Dounce homogenizer, and MVs were pelleted twice through a 36% sucrose cushion as described previously (15). Purified MVs were used in all experiments.

Affinity purification of proteins. BS-C-1 cells (1.5×10^6) were mock infected or infected at a multiplicity of 1 PFU per cell with vA28iA56TAPG9-3XFlag in the presence of IPTG (isopropyl-β-D-thiogalactopyranoside) (54) or VACV WR. After 24 h, the cells were collected, washed once with 150 mM NaCl–50 mM Tris-HCl (pH 7.4), and then lysed while rotating for 1 h at 4°C with lysis buffer (1%Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4) supplemented with a complete protease inhibitor tablet (Roche Applied Sciences, Indianapolis, IN). A portion of the lysate was saved, and the remaining material was added to 30 μl of streptavidin-agarose slurry (Millipore, Billerica, MA). B2D10 monoclonal antibody (MAb) (38), provided by Inger Damon, Centers for Disease Control and Prevention, was added and the mixture incubated overnight. The agarose beads were washed six times with 1 ml of lysis buffer, and bound proteins were eluted with lysis buffer supplemented with D-biotin (USB Corporation, Cleveland, OH). Proteins in the eluate were concentrated by precipitation with trichloroacetic acid and resuspended in 1× sample buffer (Invitrogen) with 1× reducing agent (Invitrogen). Western blot analysis was done as previously described (53).

Codon optimization of A56 and K2. The codon usages and base compositions of the A56R and K2L genes of VACV WR (ATCC VR-1354, accession number AY243312) were optimized (Geneart, Regensburg, Germany) to improve RNA processing and translation. The codon-optimized A56R and K2L genes were PCR amplified (Accuprime Pfx; Invitrogen). During PCR amplification of the A56R gene, DNA encoding the V5 epitope was appended to the C terminus of the open reading frame. The A56V5 and K2 PCR products were inserted into the directional TOPO vector pcDNA 3.1 (Invitrogen) and sequenced.

Construction of a stable cell line expressing A56 and K2. The codon-optimized A56V5 and K2 genes were inserted into the vector pBudCE4.1 (Invitrogen) next to the human cytomegalovirus (CMV) immediate-early promoter and the human elongation factor 1α subunit promoter, respectively. Zeocin (300 μg/ml) was used to select stably transfected HEK293 cells. Multiple colonies were screened for coexpression of A56 and K2, and a single colony was cloned by limiting dilution to generate 293EACK13D.

Transfection. 293TT cells were trypsinized and resuspended at 6.6×10^5/ml in DMEM with 10% FBS, and approximately 300 μl was added per well of a 48-well plate and incubated overnight. Cells were transfected with 800 ng of total plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendation. Cotransfection of A56 and K2 was performed with 400 ng of the A56 plasmid and 400 ng of the K2 plasmid. For single gene transfections, 400 ng of A56, K2, or VSVG plasmid was supplemented with 400 ng of empty vector. The medium was changed at 24 h posttransfection, and the cells were incubated for an additional 24 h before analysis.

PLC treatment, antibody staining, and flow cytometry. Surface expression of A56 and K2 polypeptides was analyzed by staining with anti-A56 MAb 1H831 (provided by Alan L. Schmaljohn, U.S. Army Medical Research Institute for Infectious Diseases) or B2D10 (38) and anti-K2 MAb 4A11-4A3 (51). At 48 h after transfection, 293TT cells were treated with phosphatidylinositol-specific phospholipase C (PLC) (Sigma) at a final concentration of 0.5 U/ml for 2 h at 37°C and then stained with antibodies to A56 or K2. Antibody staining was performed as follows. 293TT cells were detached by gently pipetting and pelleted in a bench top centrifuge. Antibodies (anti-A56 or anti-K2) were diluted in 10% FBS–DMEM and incubated with the cells for 15 min. Cells were washed twice with 0.5 ml of Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+} (Quality Biological). Secondary antibody (Cy5-conjugated donkey anti-mouse [Jackson ImmunoResearch Laboratories, West Grove, PA]) was diluted in 10% FBS–DMEM and incubated with the cells for 15 min. The cells were washed twice with 0.5 ml of Dulbecco's phosphate-buffered saline, samples were examined with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), and data were analyzed using FlowJo software (TreeStar, San Carlos, CA).

Quantification of cell-cell fusion. BS-C-1 cells were seeded at 2×10^5 cells per well of a 24-well dish. The following day, cells were infected with vΔA56ΔK2 (53) at a multiplicity of infection of 5 in 2.5% Eagle's MEM. Separately, 293TT cells were cotransfected with plasmids encoding A56 and K2 or transfected individually with a plasmid expressing A56, K2, or VSV G glycoprotein fused to green fluorescent protein (GFP) or with an empty vector plasmid. Transfection mixtures also contained 100 ng of p11-FFluc plasmid or 1 μg of p11-*lacZ* plasmid. After 48 h, the transfected cells were resuspended at 5×10^5/ml in 10% FBS–DMEM with 40 μg cytosine arabinoside. The medium was removed from infected-cell monolayers after 16 h and replaced with 250 μl containing 1.25×10^5 transfected 293TT cells. The cells were cocultured for 18 h for β-galactosidase reporter or for 4 h for firefly luciferase (FFLuc) reporter, respectively. In situ staining for β-galactosidase was done as previously described (34). To measure FFLuc expression, cells were treated with cell culture lysis buffer (Promega, Madison, WI) for 10 min at room temperature. A 20-μl portion of the lysate was mixed with 100 μl of FFLuc assay substrate (Promega), and activity was quantified on a Berthold Sirius luminometer (Pforzheim, Germany).

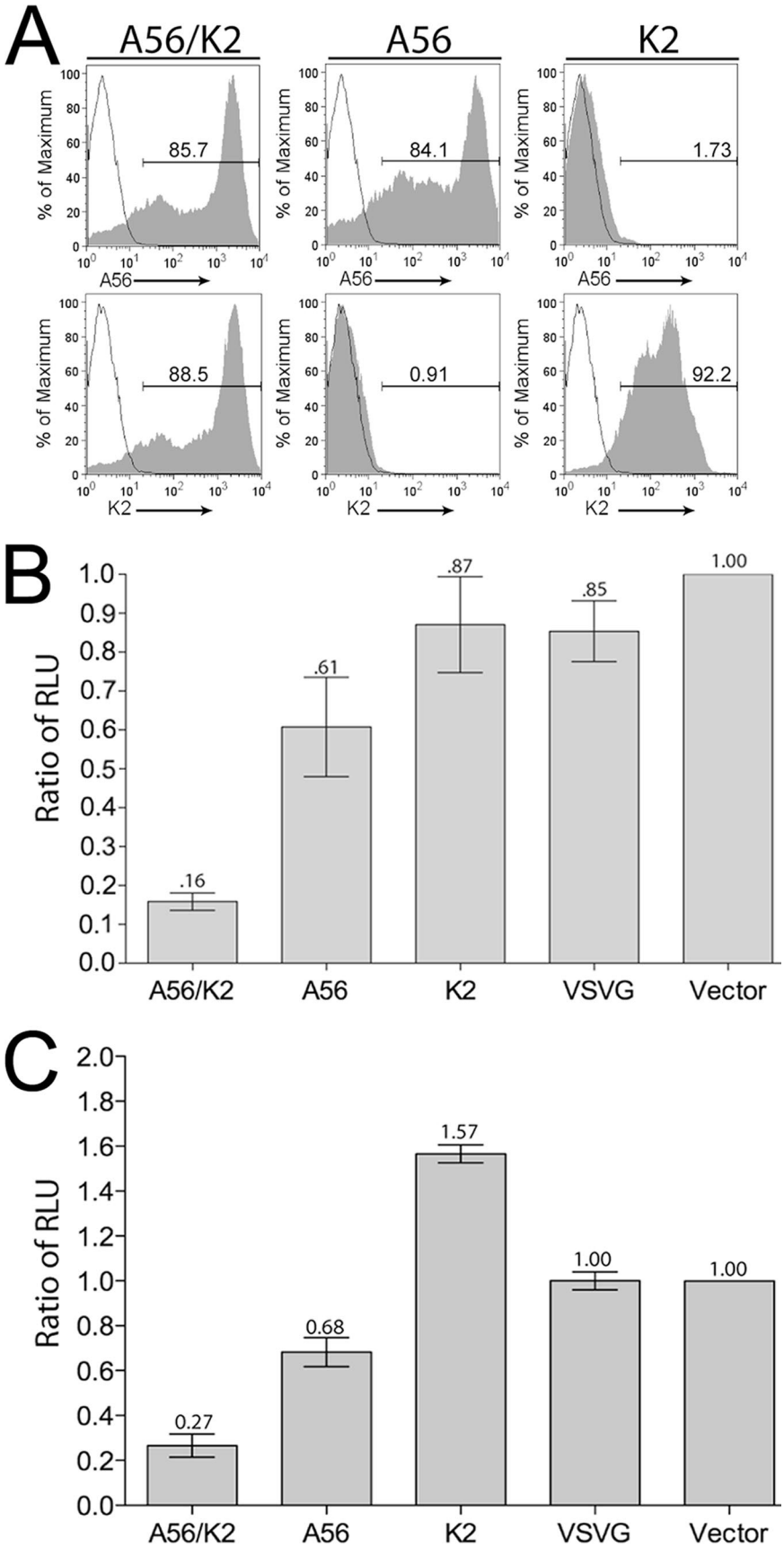
Quantification of virus entry. Purified vFire-WR (49) was diluted to 6×10^6 PFU/ml in DMEM with 10% FBS, and 50 μl of virus inoculum was added to 3×10^5 cells in individual wells of a 48-well plate. After 2 h, medium was removed and 100 μl of 1× cell culture lysis buffer was added and rotated for 10 min. Twenty microliters of lysate was mixed with 100 μl of FFLuc assay substrate, and expression was measured as described above.

Virus yields. At 18 h before infection, 2×10^5 HEK293 or 293EACK cells were plated per well of a 24-well plate and supplemented with final concentration of 10 to 25 μg/ml of T4 (14) or B2D10 MAb. Cells were infected with VV.NP-S-EGFP (1, 13, 33) at a multiplicity of 1.0 or 0.01 PFU per cell. After 24 h, fluorescent images were captured, and then the cells were harvested and virus titers were determined by plaque formation on BS-C-1 cells.

RESULTS

A56/K2 is sufficient to prevent fusion of uninfected and infected cells. Studies with mutated strains of VACV had previously indicated that expression of both A56 and K2 is required to prevent spontaneous fusion of infected cells at neutral pH. However, it was not known whether these two proteins are sufficient to prevent syncytium formation or whether additional viral proteins are needed, nor was it known whether uninfected cells could fuse with infected cells. In order to answer these and subsequent questions, we needed to express A56 and K2 on the surface of uninfected cells. This was accomplished by transfecting plasmids with the A56R and K2L open reading frames regulated by the CMV promoter individually or together. Expression of A56 and K2 on the cell surface was demonstrated by measuring the binding of specific antibodies with a flow cytometer (Fig. 1A). From 84 to 92% of the 293TT cells were transfected as judged by antibody binding, and similar amounts of A56 were present on the surface of cells in the presence or absence of K2. In contrast, K2 surface expression was much higher in the presence of A56 (mean fluorescence intensity of 1,862) than in its absence (mean fluorescence intensity of 326). This result confirmed the anchoring of K2 to the plasma membrane through its interaction with A56 (6, 51).

Luciferase expression was used to quantitatively measure cell fusion. A plasmid carrying the FFLuc gene controlled by a viral late promoter was transfected into uninfected 293TT cells along with A56 and K2 expression plasmids; alternatively, cells were transfected with FFluc and a single plasmid encoding A56, K2, or VSV G glycoprotein or empty vector plasmid. After 48 h, the transfected 293TT cells were added to a BS-C-1 monolayer that had been infected 16 h earlier with a syncytial VACV mutant, vΔA56ΔK2, that lacked both the A56R and K2L genes. The transfected and infected cells were cocultured in the presence of cytosine arabinoside to prevent further virus replication for 4 h prior to measuring FFLuc expression. Cells coexpressing the A56 and K2 polypeptides displayed only 16% of the FFLuc activity of cells transfected with the control vector plasmid, indicating reduced cell fusion (Fig. 1B). Expression of A56 or K2 individually resulted in FFLuc values of 61% and 87% of the control, respectively (Fig. 1B). The VSV G


A
A56/K2
A56
K2
% of Maximum
85.7
84.1
1.73
88.5
0.91
92.2
A56
K2
B
Ratio of RLU
.16
.61
.87
.85
1.00
A56/K2
A56
K2
VSVG
Vector
C
Ratio of RLU
0.27
0.68
1.57
1.00
1.00
A56/K2
A56
K2
VSVG
Vector

glycoprotein, used as a negative control, showed FFLuc values similar to those with K2 alone (Fig. 1B). Thus, the combination of A56 and K2 strongly inhibited cell-cell fusion, A56 alone had a partial effect, and K2 alone had no effect. Similar results were obtained using *lacZ* as a reporter gene and visualization of fused cells by in situ staining for β-galactosidase expression as described in Materials and Methods (data not shown).

A56/K2 inhibits virus entry. The fusion of infected cells in the absence of A56 or K2 requires the formation of extracellular virus particles and is thought to occur by a "fuse-back" or "superinfection" mechanism in which the components of the EFC and other proteins are first transferred from the MV membrane to the plasma membrane (29, 53). In a second step, the plasma membranes of multiple cells fuse, resulting in syncytia. Based on this model, we predicted that A56 and K2 would also prevent virus entry. Uninfected cells were transfected with plasmids in order to express both A56 and K2 or the individual proteins. As controls, cells were transfected with a plasmid expressing VSV G glycoprotein or the empty vector. MV entry was monitored by measuring FFLuc expression following infection with VACV vFire-WR, which expresses the FFLuc gene from an early viral promoter and has been used previously for such assays (48, 49). This sensitive and quantitative assay does not depend on virus uncoating, since early mRNA is synthesized within the virus core and secreted into the cytoplasm. Following transfection, the cells were infected with vFire-WR, and FFLuc expression was measured 2 h later. Data are presented as ratios of relative light units (RLU), with the value of the vector as the denominator. Expression of the control VSV G protein had no effect on levels of virus entry (Fig. 1C). Coexpression of A56 and K2 resulted in 27% of FFLuc expression compared to that with vector alone, and individual expression of A56 or K2 showed FFLuc values of 68% and 157%, respectively (Fig. 1C). The partial effect of A56 alone was similar to the effect of A56 in the syncytium assay (Fig. 1B). The increased FFLuc expression in cells transfected with the K2 plasmid was reproducible but unexplained. Importantly, cells coexpressing A56 and K2 showed the lowest FFLuc expression, consistent with binding to the EFC and inhibition of virus entry.

Removal of A56 and K2 from the cell surface restores virus entry. The results described above indicated that cells expressing A56/K2 were resistant to fusion with infected cells and virus entry. However, it was possible that expression or trafficking of A56/K2 in cells had an indirect effect on virus entry. To rule out such an effect, we needed a way of removing A56/K2 from the cell surface. Our strategy was to construct a chimeric form of A56 in which the transmembrane and cytoplasmic domains were replaced with a cleavable glycosylphosphatidylinositol (GPI) anchor. The functionality of A56-GPI was demonstrated by constructing the recombinant VACV vA56-GPI. Addition of the enzyme PLC to the medium of cells infected with vA56-GPI resulted in syncytium formation (see Fig. S1 in the supplemental material). This result indicated that both the transmembrane and cytoplasmic domains of A56 were dispensable and that the GPI anchor was susceptible to cleavage. Next, uninfected cells were transfected with plasmids expressing A56 and K2 or A56-GPI and K2 and then were mock treated or treated for 2 h with PLC. Surface staining of A56 and K2 was analyzed by flow cytometry. PLC treatment reduced surface levels of GPI-anchored A56 by 57% based on the values of the mean fluorescence intensities but had no effect on the native A56 (Fig. 2A). Because the K2 protein is anchored to the cell surface through an interaction with A56 (6, 51), PLC treatment resulted in concurrent removal of K2 from cells expressing the GPI-anchored form of A56 (Fig. 2A).

We next tested the effect of PLC treatment on virus entry into 293TT cells expressing A56/K2, A56-GPI/K2, or empty vector. Cells were mock treated or treated with PLC and then infected with vFire-WR, and FFLuc activity was measured after 2 h. Data are expressed as the ratio of RLU from six independent experiments. In the absence of PLC treatment, cells expressing A56/K2 or A56-GPI/K2 showed similar reduced levels of FFLuc expression, confirming that the transmembrane and cytoplasmic regions of A56 were not required for the biological role of A56 (Fig. 2B). PLC treatment of cells expressing A56-GPI/K2 increased FFLuc expression from 0.33 (without PLC) to 0.53 (with PLC). This highly significant difference ($P = 0.003$) represents a 60% increase, consistent with the removal of approximately 57% of the A56 by PLC. No change in FFLuc expression was observed after PLC treatment of cells expressing native A56 and K2 (Fig. 2B). These results indicated that partial removal of A56/K2 from the cell surface increased virus entry to a corresponding degree as measured by FFLuc expression. Nevertheless, the inability to express both A56 and K2 in all cells by transient transfection and to completely remove A56-GPI/K2 from the membrane led us to prepare cell lines that stably express A56 and K2.

Resistance of a stable cell line expressing A56 and K2 to virus infection. To further evaluate the effect of A56/K2 ex-

FIG. 1. Transient expression of A56 and K2 inhibits cell-cell fusion and virus entry. (A) Surface expression of A56 and K2. 293TT cells were transfected with empty vector plasmid or plasmids expressing A56 and K2 (A56/K2) or with single plasmids expressing A56 or K2, each under control of the CMV promoter. After 48 h, the unpermeabilized cells were stained with MAb to A56 (top row) or K2 (bottom row), followed by a secondary antibody conjugated to a fluorophore. Shaded gray areas represent A56 or K2 staining. The black line represents cells transfected with the empty vector, which were processed in parallel with the A56 and K2 samples and stained with both primary and secondary antibodies. Percentages of cell expressing A56 or K2 are indicated. (B) Inhibition of cell fusion. 293TT cells were transfected with an FFLuc reporter plasmid regulated by a VACV late promoter along with plasmids expressing A56 and K2 or with individual plasmids comprising empty vector, A56, K2, or VSV G, all under control of the CMV promoter. After 48 h, the cells were resuspended and added to the vΔA56ΔK2-infected cell monolayer. Cells were incubated together for 4 h and lysed, and FFLuc expression was measured. Data are provided as the ratio of the RLU obtained for each sample to the RLU obtained with vector. Ratios are indicated above the bars ($n = 4$ independent replicates; error bars represent standard errors of the means. (C) Inhibition of virus entry. Cells were transfected with plasmids expressing A56 and K2 or single plasmids expressing A56, K2, VSV G, or empty vector. After 48, the cells were infected with 1 PFU of vFire-WR per cell. At 2 h after infection, the cells were lysed and FFLuc expression was measured. Data are presented as ratios of RLU to that for empty vector. The numerical values of the ratios are indicated above the bars ($n = 3$ independent experiments; error bars represent standard errors of the means).

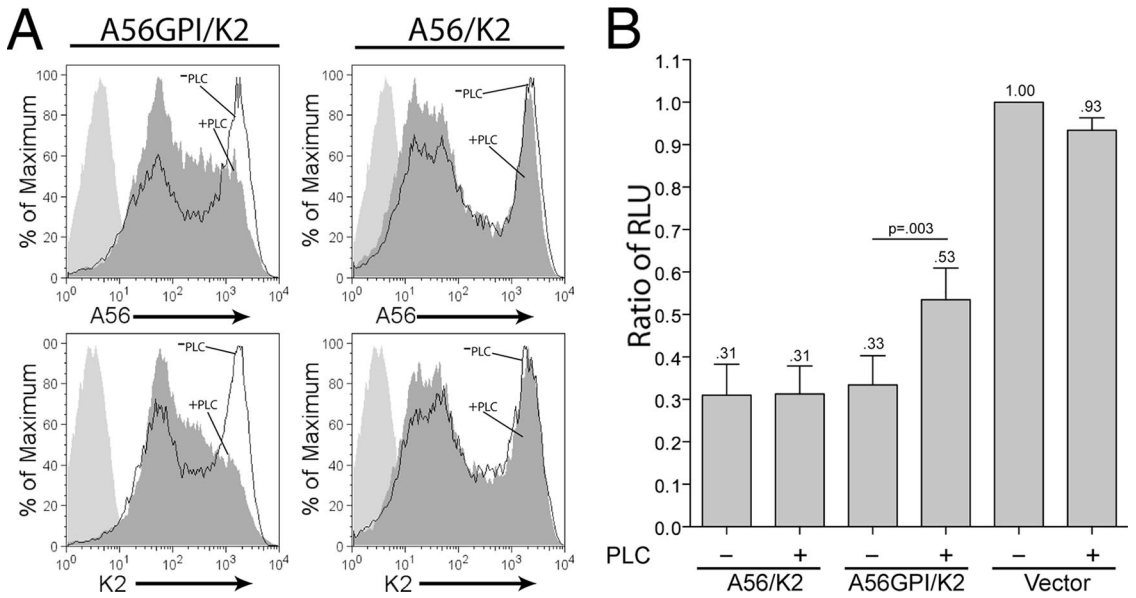


FIG. 2. Removal of GPI-anchored A56 from the cell surface restores virus entry. (A) Surface expression and removal of A56-GPI. 293TT cells were cotransfected with plasmids expressing A56 and K2 or with plasmids expressing A56-GPI and K2, each under control of the CMV promoter, or with an empty vector plasmid. After 48 h, cells were mock treated (−PLC; black line) or treated with PLC (+PLC; dark gray area) and then stained with A56 or K2 antibodies followed by secondary antibody conjugated to fluorophore prior to flow cytometry. The untreated vector control is indicated by the light gray area. (B) Expression of FFLuc. Cells were transfected as for panel A. After 48 h, the cells were mock treated or treated with PLC for 2 h and then infected with vFire-WR. After an additional 2 h, FFLuc expression was measured. Data are shown as ratios of RLU relative to that for the vector. The ratio numbers are indicated above the bars ($n = 6$ independent replicates; bars represent standard errors of the means; significance between A56-GPI/K2 with and without PLC, $P = 0.003$).

pression on virus entry, we introduced the genes encoding A56 and K2 into HEK293 cells to create the cell line 293EACK, which stably coexpresses A56/K2. Synthesis of A56 and K2 was confirmed by Western blot analysis of cell lysates (Fig. 3A). The minor band migrating faster than the major A56 band is likely due to decreased glycosylation. However, the minor band migrating faster than K2 was present in lysates of HEK293 cells and represents cross-reactivity. The cellular protein glyceraldehyde 3-phosphate dehydrogenase was used as a loading control. Specific cell surface expression of A56 and K2 in 293EACK cells was demonstrated by flow cytometry (Fig. 3B).

We next compared virus replication in 293EACK and HEK293 cells. Cells were infected with VV.NP-S-EGFP, which expresses GFP fused to the influenza virus nuclear protein, to visualize infected cells and determine virus yields. Only tiny green fluorescent foci consisting of individual or few infected cells developed on 293EACK monolayers by 24 h, whereas large plaques formed on the parental HEK293 cells (data not shown). Virus yield experiments were carried out at two multiplicities of infection. At 1 and 0.01 PFU per cell, the yields on 293 EACK cells were 3.7% and 0.98%, respectively, of that on HEK293 cells (Fig. 3C). To demonstrate that the failure to form normal plaques on 293EACK cells was specifically related to A56/K2 surface expression, we acquired an anti-A56 MAb (B2D10) that triggers fusion of cells infected with wild-type VACV (38). Addition of the B2D10 MAb allowed 293EACK cells to support plaque formation but had no effect on the size of plaques on HEK293 cells (data not shown). Furthermore, the B2D10 MAb increased the virus yield on 293EACK cells by 7- to 10-fold but had no effect on the virus yields in HEK293 cells (Fig. 3C). These results indicated that A56/K2 expression negatively affects virus replication, presumably by reducing virus entry.

Virus entry is reduced in 293EACK cells. We next examined virus entry into 293EACK and HEK293 cells by monitoring expression of FFLuc. Again we used the B2D10 MAb to show the specific involvement of A56. The 293EACK and HEK293 cells were incubated with 0 to 25 μg of B2D10 MAb per ml or 25 μg/ml of an isotype control antibody, T4, which is specific for the human immunodeficiency virus gp41 protein. FFLuc activity was measured at 2 h after infection with vFire-WR. The results are shown as ratios of FFLuc activities to the activity in HEK293 cells in the absence of MAb. Without B2D10 MAb, 293EACK cells had 9% of the FFLuc activity of the parental HEK293 cells (Fig. 3D). FFLuc activity increased to 61% in the presence of 10 μg/ml of B2D10 and to 90% with 25 μg/ml of B2D10. The 10-fold increase in luciferase activity in the presence of B2D10 MAb was similar to the 7- to 10-fold increase in virus titer (Fig. 3C). The control human immunodeficiency virus MAb had no effect on virus entry into 293EACK or HEK293 cells (Fig. 3D).

The B2D10 MAb inhibits association of A56/K2 with the EFC. Previously, we showed by affinity chromatography and mass spectroscopy that A56/K2 pulls down the EFC complex, which consists of at least eight proteins, through a direct interaction with the A16 and G9 components (53, 54). Neither A56 nor K2 alone stably interacted with the EFC. The ability of B2D10 to restore entry of VACV in 293EACK cells suggested that the MAb prevented or disrupted the interaction between A56 and K2 or between A56/K2 and the EFC. To examine these two possibilities, we carried out a pull-down experiment using cells infected with a recombinant VACV (vA28iA56TAPG9-3XFlag) (54) that ex-

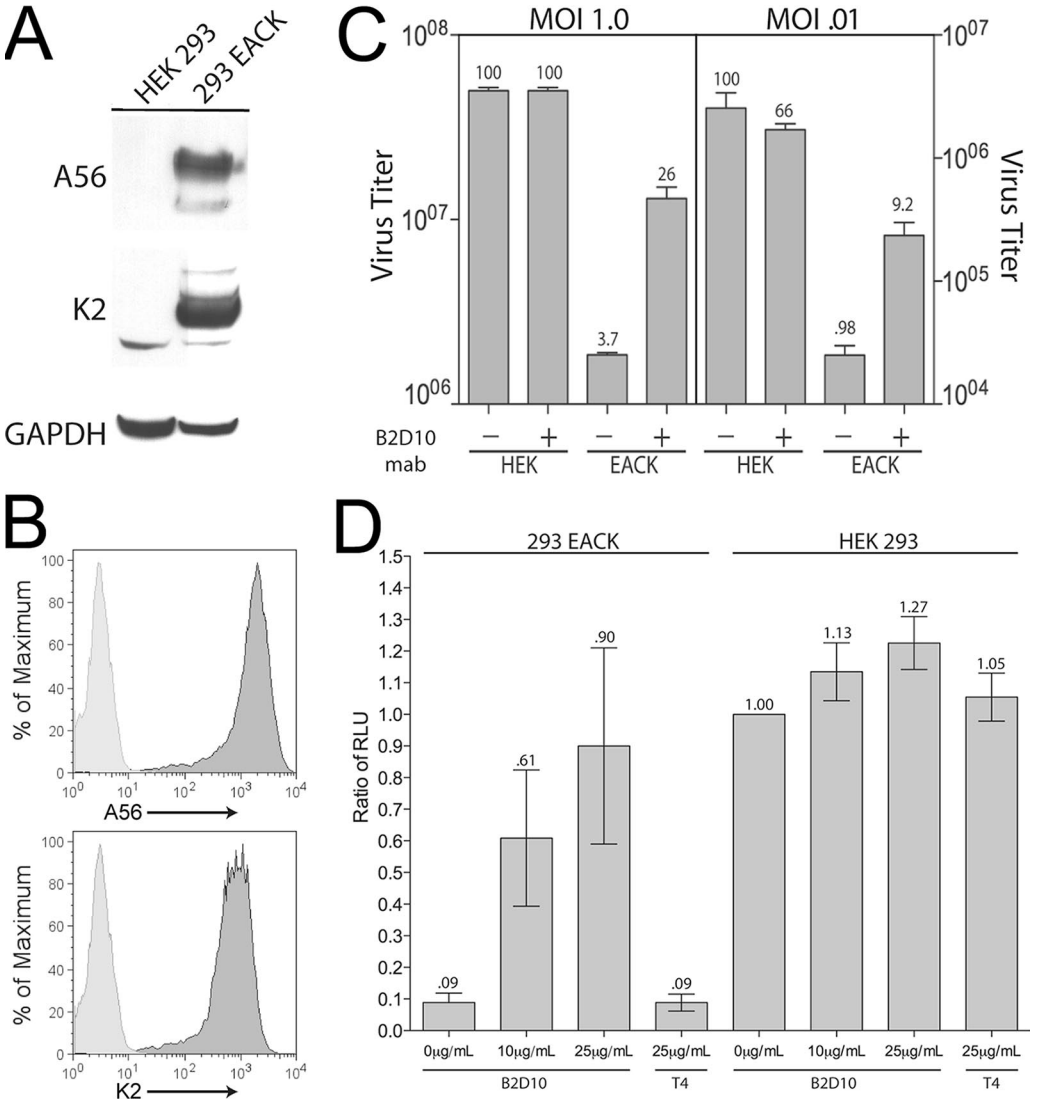


FIG. 3. Resistance to infection and virus entry of cell lines that stably express A56/K2. (A) Expression of A56 and K2. HEK293 and 293EACK cells were lysed, and proteins in the postnuclear supernatant were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to A56, K2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (B) A56 and K2 surface expression determined by flow cytometry. HEK293 cells (light gray) or 293EACK cells (dark gray) were incubated with anti-A56 or anti-K2 antibody, washed, stained with a secondary antibody conjugated to fluorophore, and then analyzed by flow cytometry. (C) Virus yields. HEK293 or 293EACK cells were infected with VV.NP-S-EGFP at a multiplicity of infection (MOI) of 1 or 0.01 PFU per cell in the presence (+) or absence (−) of 25 μg/ml of B2D10 MAb. After 24 h, the cells were harvested and virus titers were determined by plaque assay. Data are means of duplicates, with error bars indicating standard deviations. The numbers over the bars indicated the percent virus titer under the indicated condition relative to the titer after infection of HEK293 cells without antibody. (D) Cells (293EACK or HEK293) were incubated with 0 μg/ml, 10 μg/ml, or 25 μg/ml of B2D10 or T4 MAb. Cells were infected with 5 PFU per cell of vFire-WR. FFLuc expression was measured at 2 h after infection. Results are shown as the ratio of RLU under each condition to the RLU of HEK293 with no MAb. Ratio values are indicated above the bars ($n = 3$ independent replicates; error bars represent standard errors of the means).

presses a tandem affinity purification-tagged copy of A56, a 3XFlag-tagged copy of the G9 EFC protein, and an IPTG-inducible copy of the A28 EFC protein. The inducible nature of this recombinant was not relevant for this experiment; however, because of the epitope tags, the virus allowed us to determine the interaction between A56/K2 and G9, one of the two directly interacting components, and A28, a representative of the six other EFC components. BS-C-1 cells were infected with VACV WR or vA28iA56TAPG9-3XFlag in the presence of IPTG for 24 h. The cells were lysed with nonionic detergent in order to release A56/K2 and the EFC, which are topologically separated in infected cells. The postnuclear supernatant was mixed with streptavidin coupled to Sepharose, which specifically binds A56TAP, and then B2D10 or control T4 MAb was added. The bound proteins were eluted from the affinity resin, concentrated, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a membrane for Western blotting with antibodies to the A28 and G9 EFC proteins, A56 and K2, and the control protein glyceraldehyde phosphate dehydrogenase (Fig. 4). Pull-down of A28 and G9 with A56TAP was reduced in the

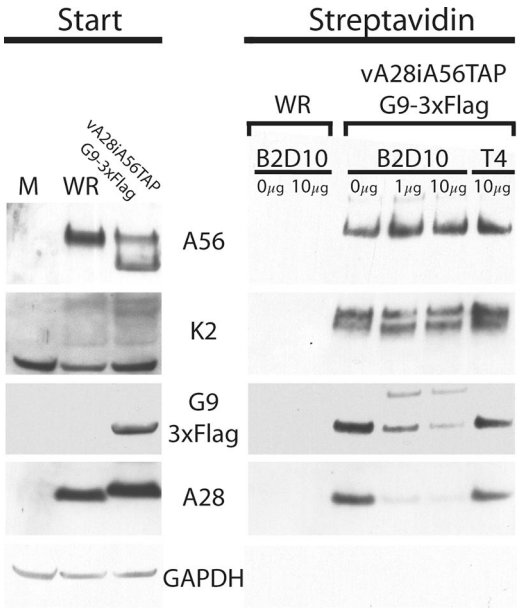


FIG. 4. Effect of B2D10 MAb on association of A56/K2 with the EFC. BS-C-1 cells were mock infected (M) or infected with VACV WR (WR) or vA28iA56TAPG9-3xFlag in the presence of IPTG. After 24 h, the cells were washed and lysed with nonionic detergent. A56TAP and associated proteins in the postnuclear supernatant were affinity purified by binding to streptavidin-Sepharose in the presence of the B2D10 or control T4 MAb as indicated. Affinity-purified samples (streptavidin) and starting material (start) were separated by SDS-PAGE and analyzed by Western blotting using antibodies to A56, K2, Flag, A28, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

presence of 1 μg and 10 μg of the B2D10 MAb but not with 10 μg of the control T4 MAb (Fig. 4). The VACV WR lysate served as a negative control for affinity purification of TAP-tagged A56 protein. Thus, prevention or disruption of the A56 interaction with the EFC was specific to the anti-A56 MAb. K2 copurified with A56, and this interaction was unaffected by B2D10 MAb. Analysis of the starting material prior to streptavidin binding showed expression of each of the proteins probed with antibodies (Fig. 4). The slower migration of A28 in vA28iA56TAPG9-3XFlag compared to VACV WR was due to a hemagglutinin epitope tag. The TAP tag may influence glycosylation of A56 protein, accounting for the faster-migrating form.

DISCUSSION

Many animal viruses induce infected cells to form syncytia. In the case of VACV, this phenomenon was associated initially with mutations in the hemagglutinin gene encoded by the A56R open reading frame (21, 38). Subsequently, additional syncytial mutants were associated with the K2L open reading frame (24, 50, 55). The finding that A56 and K2 proteins form a multimer on the surface of infected cells provided a link between the similar phenotypes of A56 and K2 mutations (51). A56 also interacts with C3, a complement regulatory protein, and O2, a nonessential glutaredoxin (17, 53). However, neither C3 nor O2 mutants exhibit a syncytial phenotype (22, 37). Nevertheless, A56 and K2 must be shown to be sufficient to prevent the spontaneous formation of syncytia by cells infected with VACV in order to eliminate the possibility that additional viral proteins are needed. In addition, recent evidence that A56/K2 interacts with the EFC and that the EFC is necessary for syncytium formation furnished a strong connection between virus entry and cell-cell fusion (53, 54). The main goals of the present study were to determine whether A56 and K2 were sufficient to prevent syncytium formation and virus entry by expressing the two proteins in uninfected cells.

First, we demonstrated that uninfected cells could fuse with cells infected with an A56/K2 deletion mutant and that this was inhibited by expression of A56 and K2 in the uninfected cells. A56 alone inhibited fusion to a lesser degree and K2 did not do so at all, which fits with previous data indicating that both proteins are needed to efficiently bind the EFC (53). Furthermore, we found that transient or stable expression of A56/K2 was sufficient to inhibit virus infection as determined by plaque assay and virus yield and entry as measured by expression of an FFLuc reporter. We ruled out the possibility that the effect was indirect and due to side effects of A56 and K2 expression. Specificity was demonstrated by showing that enzymatic removal of a GPI-anchored form of A56/K2 or binding of A56 with a specific MAb restored fusion and entry, as depicted in Fig. 5. The ability of A56/K2 to prevent virus entry suggests that it contributes to inhibition of superinfection. In a recent complementary study, Turner and Moyer (52) reported that cells infected with A56 or K2 VACV mutants were more susceptible to superinfection than cells infected with wild-type virus and that this could be inhibited by antibody to K2. In VACV-infected cells, superinfection exclusion probably has multiple causes and may occurs at several steps, including penetration, early gene expression, and uncoating (10).

The B2D10 MAb to A56 was previously shown to induce syncytia (38), although the mechanism was not defined. We considered two possible mechanisms: that the MAb disrupts either the interaction of A56 with K2 or the interaction of A56/K2 with the EFC. We found that the B2D10 MAb disrupted the interaction between A56/K2 and the EFC without disrupting the A56-K2 interaction. A MAb to K2 also induces syncytia (51) and suppresses the inhibitory action of A56/K2 expression (52). However, in that case the mechanism of action has not yet been determined.

Complementation studies had shown that the transmembrane domain of A56 is necessary to prevent spontaneous fusion of infected cells (51). Since the transmembrane domain of A56 is not needed for association with K2 or the EFC (53), this suggested that one role of the transmembrane domain is to anchor A56/K2 to the cell surface. We confirmed and extended this idea by constructing a recombinant VACV with the transmembrane and cytoplasmic domains of A56 replaced by a GPI anchor. The nonsyncytial plaques formed by cells infected with this recombinant virus resembled those formed by wild-type virus rather than those formed by an A56 deletion mutant. Furthermore, syncytia formed when the cells were treated with PLC.

The present study and that of Turner and Moyer (52) support a model for syncytium formation in which the first step is the fusion of extracellular virus with the cell, resulting in the deposition of the EFC and other viral proteins in the plasma membrane (29, 53). This step is necessary because the viral proteins do not traffic directly to the plasma membrane as occurs with the fusion proteins of most other viruses. The

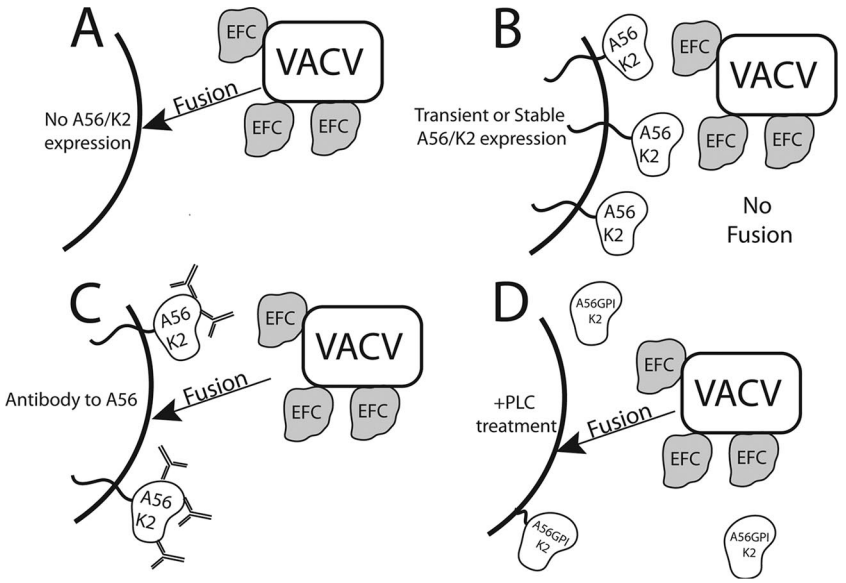


FIG. 5. Diagram depicting the correlation between A56/K2 surface expression and virus fusion and entry. (A) Uninfected cells not expressing A56/K2 or expressing A56 or K2 alone allow VACV fusion and entry. (B) Cells transiently or stably expressing A56/K2 exhibit resistance to VACV fusion and entry. (C) Binding of MAb to A56 allows VACV fusion and entry. (D) Removal of GPI-anchored A56 allows VACV fusion and entry.

second step is the fusion of infected cells with each other or, as we have shown here, with uninfected cells. The importance for virus fitness in preventing syncytium formation and superinfection is not yet understood. In animal models, deletion of A56 attenuated orthopoxvirus virulence (16, 26, 42), but deletion of K2 did not (24, 44).

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REFERENCES

1. **Anton, L. C., U. Schubert, I. Bacik, M. F. Princiotta, P. A. Wearsch, J. Gibbs, P. M. Day, C. Realini, M. C. Rechsteiner, J. R. Bennink, and J. W. Yewdell.** 1999. Intracellular localization of proteasomal degradation of a viral antigen. J. Cell Biol. **146:**113–124.
2. **Armstrong, J. A., D. H. Metz, and M. R. Young.** 1973. The mode of entry of vaccinia virus into L cells. J. Gen. Virol. **21:**533–537.
3. **Bisht, H., A. S. Weisberg, and B. Moss.** 2008. Vaccinia virus L1 protein is required for cell entry and membrane fusion. J. Virol. **82:**8687–8694.
4. **Blasco, R., and B. Moss.** 1991. Extracellular vaccinia virus formation and cell-to-cell virus transmission are prevented by deletion of the gene encoding the 37,000-dalton outer envelope protein. J. Virol. **65:**5910–5920.
5. **Brown, E., T. G. Senkevich, and B. Moss.** 2006. Vaccinia virus F9 virion membrane protein is required for entry but not virus assembly, in contrast to the related l1 protein. J. Virol. **80:**9455–9464.
6. **Brum, L. M., P. C. Turner, H. Devick, M. T. Baquero, and R. W. Moyer.** 2003. Plasma membrane localization and fusion inhibitory activity of the cowpox virus serpin SPI-3 require a functional signal sequence and the virus encoded hemagglutinin. Virology **306:**289–302.
7. **Buck, C. B., D. V. Pastrana, D. R. Lowy, and J. T. Schiller.** 2004. Efficient intracellular assembly of papillomaviral vectors. J. Virol. **78:**751–757.
8. **Carter, G. C., M. Law, M. Hollinshead, and G. L. Smith.** 2005. Entry of the vaccinia virus intracellular mature virion and its interactions with glycosaminoglycans. J. Gen. Virol. **86:**1279–1290.
9. **Chang, A., and D. H. Metz.** 1976. Further investigations on the mode of entry of vaccinia virus into cells. J. Gen. Virol. **32:**275–282.
10. **Christen, L., J. Seto, and E. G. Niles.** 1990. Superinfection exclusion of vaccinia virus in virus-infected cell cultures. Virology **174:**35–42.
11. **Condit, R. C.** 2007. Vaccinia, Inc.—probing the functional substructure of poxviral replication factories. Cell Host Microbe **2:**205–207.
12. **Doms, R. W., R. Blumenthal, and B. Moss.** 1990. Fusion of intra- and extracellular forms of vaccinia virus with the cell membrane. J. Virol. **64:** 4884–4892.
13. **Earl, P. L., J. L. Americo, and B. Moss.** 2003. Development and use of a vaccinia virus neutralization assay based on flow cytometric detection of green fluorescent protein. J. Virol. **77:**10684–10688.
14. **Earl, P. L., C. C. Broder, D. Long, S. A. Lee, J. Peterson, S. Chakrabarti, R. W. Doms, and B. Moss.** 1994. Native oligomeric human immunodeficiency virus type 1 envelope glycoprotein elicits diverse monoclonal antibody reactivities. J. Virol. **68:**3015–3026.
15. **Earl, P. L., and B. Moss.** 1998. Characterization of recombinant vaccinia viruses and their products, p. 16.18.1–16.18.11. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 2. Greene Publishing Associates, New York, NY.
16. **Flexner, C., A. Hugin, and B. Moss.** 1987. Prevention of vaccinia virus infection in immunodeficient nude mice by vector-directed IL-2 expression. Nature **330:**259–262.
17. **Girgis, N. M., B. C. Dehaven, X. Fan, K. M. Viner, M. Shamim, and S. N. Isaacs.** 2008. Cell surface expression of the vaccinia virus complement control protein is mediated by interaction with the viral A56 protein and protects infected cells from complement attack. J. Virol. **82:**4205–4214.
18. **Gong, S. C., C. F. Lai, and M. Esteban.** 1990. Vaccinia virus induces cell fusion at acid pH and this activity is mediated by the N-terminus of the 14-kDa virus envelope protein. Virology **178:**81–91.
19. **Hiller, G., and K. Weber.** 1985. Golgi-derived membranes that contain an acylated viral polypeptide are used for vaccinia virus envelopment. J. Virol. **55:**651–659.
20. **Husain, M., A. S. Weisberg, and B. Moss.** 2007. Resistance of a vaccinia virus A34R deletion mutant to spontaneous rupture of the outer membrane of progeny virions on the surface of infected cells. Virology **366:**424–432.
21. **Ichihashi, Y., and S. Dales.** 1971. Biogenesis of poxviruses: interrelationship between hemagglutinin production and polykaryocytosis. Virology **46:**533–543.
22. **Isaacs, S. N., G. J. Kotwal, and B. Moss.** 1992. Vaccinia virus complement-control protein prevents antibody-dependent complement-enhanced neutralization of infectivity and contributes to virulence. Proc. Natl. Acad. Sci. USA **89:**628–632.
23. **Izmailyan, R. A., C. Y. Huang, S. Mohammad, S. N. Isaacs, and W. Chang.** 2006. The envelope G3L protein is essential for entry of vaccinia virus into host cells. J. Virol. **80:**8402–8410.
24. **Law, K. M., and G. L. Smith.** 1992. A vaccinia serine protease inhibitor which prevents virus-induced cell fusion. J. Gen. Virol. **73:**549–557.
25. **Law, M., G. C. Carter, K. L. Roberts, M. Hollinshead, and G. L. Smith.** 2006. Ligand-induced and non-fusogenic dissolution of a viral membrane. Proc. Natl. Acad. Sci. USA **103:**5989–5994.
26. **Lee, S. L., J. M. Roos, L. C. McGuigan, K. A. Smith, N. Cormier, L. K. Cohen, B. E. Roberts, and L. G. Payne.** 1992. Molecular attenuation of

vaccinia virus: mutant generation and animal characterization. J. Virol. **66:** 2617–2630.
27. **Mercer, J., and A. Helenius.** 2008. Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. Science **320:**531–535.
28. **Moss, B.** 2007. Poxviridae: the viruses and their replication, p. 2905–2946. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, vol. 2. Lippincott Williams & Wilkins, Philadelphia, PA.
29. **Moss, B.** 2006. Poxvirus entry and membrane fusion. Virology **344:**48–54.
30. **Moss, B., and B. M. Ward.** 2001. High-speed mass transit for poxviruses on microtubules. Nat. Cell Biol. **3:**E245–E246.
31. **Nelson, G. E., T. R. Wagenaar, and B. Moss.** 2008. A conserved sequence within the H2 subunit of the vaccinia virus entry/fusion complex is important for interaction with the A28 subunit and infectivity. J. Virol. **82:**6244–6250.
32. **Nichols, R. J., E. Stanitsa, B. Unger, and P. Traktman.** 2008. The vaccinia I2L gene encodes a membrane protein with an essential role in virion entry. J. Virol. **82:**10247–10261.
33. **Norbury, C. C., D. Malide, J. S. Gibbs, J. R. Bennink, and J. W. Yewdell.** 2002. Visualizing priming of virus-specific CD8(+) T cells by infected dendritic cells in vivo. Nat. Immunol. **3:**265–271.
34. **Nussbaum, O., C. C. Broder, and E. A. Berger.** 1994. Fusogenic mechanisms of enveloped-virus glycoproteins analyzed by a novel recombinant vaccinia virus-based assay quantifying cell fusion-dependent reporter gene activation. J. Virol. **68:**5411–5422.
35. **Ojeda, S., A. Domi, and B. Moss.** 2006. Vaccinia virus G9 protein is an essential component of the poxvirus entry-fusion complex. J. Virol. **80:**9822–9830.
36. **Ojeda, S., T. G. Senkevich, and B. Moss.** 2006. Entry of vaccinia virus and cell-cell fusion require a highly conserved cysteine-rich membrane protein encoded by the A16L gene. J. Virol. **80:**51–61.
37. **Rajagopal, I., B. Y. Ahn, B. Moss, and C. K. Mathews.** 1995. Roles of vaccinia virus ribonucleotide reductase and glutaredoxin in DNA precursor biosynthesis. J. Biol. Chem. **270:**27415–27418.
38. **Seki, M., M. Oie, Y. Ichihashi, and H. Shida.** 1990. Hemadsorption and fusion inhibition activities of hemagglutinin analyzed by vaccinia virus mutants. Virology **175:**372–384.
39. **Senkevich, T. G., and B. Moss.** 2005. Vaccinia virus H2 protein is an essential component of a complex involved in virus entry and cell-cell fusion. J. Virol. **79:**4744–4754.
40. **Senkevich, T. G., S. Ojeda, A. Townsley, G. E. Nelson, and B. Moss.** 2005. Poxvirus multiprotein entry-fusion complex. Proc. Natl. Acad. Sci. USA **102:**18572–18577.
41. **Senkevich, T. G., B. M. Ward, and B. Moss.** 2004. Vaccinia virus entry into cells is dependent on a virion surface protein encoded by the A28L gene. J. Virol. **78:**2357–2366.
42. **Shida, H., Y. Hinuma, M. Hatanaka, M. Morita, M. Kidokora, K. Suzuki, T. Maruyama, F. Takahashi-Nishimaki, M. Sugimoto, R. Kitamura, T. Miyazawa, and M. Hayami.** 1988. Effects and virulence of recombinant vaccinia viruses derived from attenuated strains that express the human T-cell leukemia virus type 1 envelope gene. J. Virol. **62:**4474–4480.
43. **Smith, G. L., A. Vanderplasschen, and M. Law.** 2002. The formation and function of extracellular enveloped vaccinia virus. J. Gen. Virol. **83:**2915–2931.
44. **Thompson, J. P., P. C. Turner, A. N. Ali, B. C. Crenshaw, and R. W. Moyer.** 1993. The effects of serpin gene mutations on the distinctive pathobiology of cowpox and rabbitpox virus following intranasal inoculaiton of Balb/c mice. Virology **197:**328–338.
45. **Tooze, J., M. Hollinshead, B. Reis, K. Radsak, and H. Kern.** 1993. Progeny vaccinia and human cytomegalovirus particles utilize early endosomal cisternae for their envelopes. Eur. J. Cell Biol. **60:**163–178.
46. **Townsley, A., T. G. Senkevich, and B. Moss.** 2005. The product of the vaccinia virus L5R gene is a fourth membrane protein encoded by all poxviruses that is requried for cell entry and cell-cell fusion. J. Virol. **79:**10988–10998.
47. **Townsley, A., T. G. Senkevich, and B. Moss.** 2005. Vaccinia virus A21 virion membrane protein is required for cell entry and fusion. J. Virol. **79:**9458–9469.
48. **Townsley, A. C., and B. Moss.** 2007. Two distinct low-pH steps promote entry of vaccinia virus. J. Virol. **81:**8613–8620.
49. **Townsley, A. C., A. S. Weisberg, T. R. Wagenaar, and B. Moss.** 2006. Vaccinia virus entry into cells via a low-pH-dependent endosomal pathway. J. Virol. **80:**8899–8908.
50. **Turner, P. C., and R. W. Moyer.** 1992. An orthopoxvirus serpin-like gene controls the ability of infected cells to fuse. J. Virol. **66:**2076–2085.
51. **Turner, P. C., and R. W. Moyer.** 2006. The cowpox virus fusion regulator proteins SPI-3 and hemagglutinin interact in infected and uninfected cells. Virology **347:**88–99.
52. **Turner, P. C., and R. W. Moyer.** 2008. The vaccinia virus fusion inhibitor proteins SPI-3 (K2) and HA (A56) expressed by infected cells reduce the entry of superinfecting virus. Virology **380:**226–233.
53. **Wagenaar, T. R., and B. Moss.** 2007. Association of vaccinia virus fusion regulatory proteins with the multicomponent entry/fusion complex. J. Virol. **81:**6286–6293.
54. **Wagenaar, T. R., S. Ojeda, and B. Moss.** 2008. Vaccinia virus A56/K2 fusion regulatory protein interacts with the A16 and G9 subunits of the entry fusion complex. J. Virol. **82:**5153–5160.
55. **Zhou, J., X. Y. Sun, G. J. P. Fernando, and I. H. Frazer.** 1992. The vaccinia virus K2L gene encodes a serine protease inhibitor which inhibits cell-cell fusion. Virology **189:**678–686.