THE VACCINIA VIRUS A56/VCP COMPLEX: MECHANISM OF FORMATION AND IMPLICATIONS FOR VIRULENCE

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Dedication

This thesis is dedicated to my daughter, Claire Eloise DeHaven.

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I would first like to thank Stuart, my mentor, for a wonderful and rewarding graduate school experience. I always felt valued and supported in your lab, and I'm proud of the work we did together. I'm only sorry I couldn't ever repay you with a decent March Madness bracket.

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Abstract

THE VACCINIA A56/VCP COMPLEX: MECHANISM AND IMPLICATIONS FOR PATHOGENESIS

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Vaccinia replication is complex, as it involves both producing new infectious particles as well as a myriad of proteins dedicated to combating the host's immune response. Although these proteins are often called "non-essential", they are frequently needed for the virus to achieve maximum virulence in vivo. Two of these proteins are the vaccinia complement control protein (VCP) and A56, the vaccinia hemagglutinin. VCP had been previously described as a virulence factor that was secreted from infected cells and blocked the activation of the complement cascade; A56 was known to bind another viral protein, K2. We have found that VCP and A56 interact on the surface of infected cells, and that this interaction is important for full vaccinia virulence in vivo. The first part of this thesis focuses on showing a direct interaction between A56 and VCP on the surface of infected cells, and that the N-terminal cysteine of VCP is needed for this interaction. The next section establishes that this interaction occurs via an intermolecular disulfide bridge between the two proteins in a transfection model, and also extends this phenomenon to VCP homologs from other poxviruses. Mutagenesis shows that VCP binds to the 3rd cysteine (residue 162) of the ectodomain of A56. We also begin to show that viruses that cannot form the A56/VCP complex are attenuated in vivo, using a virus where the N-terminal cysteine of VCP is mutated. In the last section, we create a virus

with a recombinant A56 protein that cannot bind VCP. We show that this virus is attenuated in intranasal and intradermal models of infection in mice; infections done in C3-knockout mice suggest that this attenuation is complement dependent. This work shows that VCP is not only important as a secreted protein, and that the A56/VCP complex is important for the virus to achieve maximum pathogenesis in vivo. These results also provide insight into the contributions of the A56 protein to vaccinia virulence in an infection, and for the first time tests a site-directed A56 mutant in vivo.

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CHAPTER ONE: The vaccinia virus, the host complement system, and poxvirus regulators of complement activation

Introduction

The Poxviridae family includes some of the most important pathogens in human history. The variola virus is the causative agent of smallpox and was responsible for hundreds of millions of deaths worldwide (30). Fortunately, after a successful vacciniation campaign with the closely related vaccinia virus (VACV), smallpox was declared eradicated in 1980. Today, VACV is still widely studied for a variety of reasons. It serves as model for other, more virulent poxviruses, such as variola and monkeypox viruses. It can also be used to study the immune response to an infection and can be used as a recombinant vaccine against other infectious diseases. It even shows some promise as an oncolytic cancer vector (70). Vaccinia, variola, cowpox, monkeypox viruses and the mousepox-causing ectromelia virus are all orthopoxviruses and are highly related. Their genomes are highly conserved, and proteins in one virus often have the same activity in others (65).

VACV devotes a large part of its genome to non-essential virulence genes, which combat the hosts' immune response (65). One of these is VCP, the vaccinia complement control protein (59). It is able to interfere with the host complement cascade at multiple steps, and is important for full virulence in vivo (23, 37, 50). VCP was initially characterized as a secreted protein. Here, we will show that it also is expressed on the surface of infected cells, through an interaction with another viral protein, A56. We will also show that this interaction occurs with other orthopoxvirus species' proteins, and that this interaction is needed for maximum virulence in vivo.

The vaccinia virus

VACV is the most studied poxvirus and serves as its model orthopoxvirus. Structurally, it is a large DNA enveloped virus, with two infectious forms. Internally, the virus is composed of a dumbbell-shaped core with two lateral bodies on either side. These structures are enveloped by one or more lipid bilayers, as there are two forms of infectious VACV. The first and most abundant is mature virs (MV), which has a single lipid bilayer studded with non-glycosylated viral proteins (82). The other infectious form is enveloped virus (EV). These virions are MV particles with an additional viral membrane, one that contains a different set of glycosylated viral proteins as well as host proteins (115). Notably, the entry/fusion machinery of VACV is only on the MV membrane (83), so the outer envelope on EV must dissolve before entry of EV can occur (63). This has led to the hypothesis that the EV membrane's functional purpose is immune evasion. That is, it "hides" neutralizing antibody targets from the host. EV particles are important for spread within the host, as mutations that interfere with EV formation and release significantly attenuate the virus (75, 92, 122, 138).

The genome of VACV is linear and covalently closed at both ends. There are inverted terminal repeats at either end which flank the large number of open reading frames (ORF) found in VACV (7). Historically, genes in VACV are often named for where they lie on a HindII restriction digest map (28). For VACV (Copenhagen strain) this digest results in 15 fragments (named A-O in order of decreasing size); each ORF on a fragment is named with sequential numbers and its orientation (38). Thus, A56R is the 56th ORF in the HindIII A fragment, and it reads to the right (R). There is also gross organization of the VACV genome. The genes in the center of the genome tend to be

necessary for replication and well conserved; conversely, those on either arm often encode virulence factors. These genes are not necessary for replication in tissue culture and vary more widely among viral isolates and species (73).

Vaccinia virus lifecycle

VACV entry into a cell is complex and can occur via multiple routes. An entry receptor for VACV has not been identified, and the virus can enter almost any type of cell. VACV has highly complex entry/fusion machinery, rather than a single protein, like most other viruses. The entry fusion complex is a large complex of 9 proteins (83). Mature virus (MV) particles have been observed entering cells via membrane fusion, endocytosis, and macropinocytosis (17, 79, 121, 137). In addition, EV particles must shed their outer membrane to expose the entry/fusion complex (63).

Once inside the cell, the virus uncoats and gene expression begins, although some early transcription starts before uncoating. Unlike nearly all other DNA viruses, VACV replicates in the cytoplasm of infected cells, in a distinct cellular environment called the viral factory (54). This means that the virus must encode and package its own enzymatic replication machinery. VACV gene expression can be separated temporally into three classes: early, intermediate, and late genes. Early gene expression occurs before viral DNA replication, and includes proteins necessary for DNA replication, as well as proteins that combat the host's immune response (104). By a mechanism that is still not completely understood, VACV DNA initiates replication by self-priming in the inverted terminal repeats (82); this results in long DNA concatamers which are cleaved by a viral resolvase enzyme into single-genome units (22, 34).

DNA replication is followed by intermediate and late gene expression. There are only a small number of intermediate genes, and several of these are transcription factors for late gene expression. The late genes include structural proteins needed for viral morphogenesis and transcription machinery to be packaged into viruses. During morphogenesis, MV particles are formed; although they make up the majority of the virus produced, they only leave the cell upon lysis or necrosis. A small proportion of these particles travel on microtubles away from the viral factory, where they gain two additional lipid membranes from endosomal or Golgi apparatus (102, 120). This form of virus, called intracellular enveloped virus (IEV), is then transported to the cell surface on microtubules (43). Once there, the outermost membrane fuses with the cellular membrane to expose an EV particle. Initially, the EV particle remains attached to the surface of the cell and is call the cell associated EV (CEV) (4, 5). While some CEV is released from the cell, in other cases the virus drives actin polymerization underneath the particle, causing the CEV to be protruded out from the cell in an actin tail; these tails can project the virus into neighboring cells (33, 88).

Vaccinia virus infection and the complement system

In the course of an infection, the VACV encounters and must contend with the host's immune system. It is well established that after initial challenge in both humans and animal models robust B- and T-cell responses develop (141) and are protective against subsequent infection (41, 53). This can extend to different poxviruses; the most famous case is of course scarification with VACV protecting against smallpox (30). However, other arms of the immune system are also important for protection, and one of these is the complement system.

The complement cascade

"Complement" is a highly complex and organized system of more than 30 proteins that makes up one of the earliest acting components of the immune system. Within minutes of an infection, it starts to label an invading pathogen as "non-self" and begins a chain reaction that can lead to the release of chemotaxic molecules and to the formation of lytic pores. The complement cascade made up of mostly sequentially acting proteins named C1 to C9 as well as other accessory proteins. The cascade is centered around the multifunctional C3 protein (134, 135).

There are three different activation pathways for complement: classical, alternative, and mannose-binding lectin. While they have different activators and initial steps, all three merge with the formation of C3 convertases and can terminate in the formation of the membrane attack complex (MAC), a multi-protein pore-forming structure that destroys membrane integrity and can cause cell lysis of pathogens and infected cells. The classical pathway was the first to be discovered. In this pathway, complement component C1 recognizes antibody bound to a foreign object or pathogen. Lectin binds carbohydrate residues found on pathogens. The alternative pathway, by contrast, does not have a specific activator. It starts with spontaneous cleavage of C3 to its active form of C3b. All three pathways converge on formation of a C3 convertase, a catalytic complex that creates more activated C3. When classical and mannose-binding lectin pathways are activated, C2 and C4 molecules are cleaved into their active fragments, C4b and C2a. They combine to form the classical C3 convertase, C4bC2a. The alternative pathway convertase consists of C3 bound to activated factor B, C3bBb. As more and more C3b is created by these complexes, some C3b binds to the C3

convertases, changing them to C5 convertases, referred to as C3bBbC3b or C4bC2aC3b. These new enzymatic complexes cleave C5 into C5a and the larger C5b, which then recruits C6 through C9, which form the membrane attack complex. C6 binds C5b and recruits C7; C7 and C8 have hydrophobic sites that allow membrane insertion. C8 also recruits 10-16 subunits of C9, which forms the actual lytic pore.

There are several receptors for complement components on immune system cells. Several of these respond to C3b, such as complement receptor 1 (CR1), CR2, CR3 and CR4. There are also receptors for the cleaved fragments of C3a and C5a, known as C3a receptor (C3aR) and C5aR. These receptors can mediate phagocytosis of opsonized particles, chemotaxis of inflammatory cells to the site of an infection and initiation of the adaptive arm of the immune system (26).

Host regulators of complement

Complement has the ability to damage not only pathogens but also the host's own cells. To prevent this from occurring, a protein family called regulators of complement activity (RCA) inhibits the complement cascade. These include both membrane-bound and soluble inhibitors of complement. There are two main ways to deactivate the complement cascade. The first is the Factor I mediated degradation of activated C3 and C4 molecules to inactive forms. Factor I activity requires co-factor proteins. Proteins with cofactor activity include CR1, factor H, C4-binding protein, factor H like protein 1 (FHL-1) and membrane cofactor protein (MCP). Other proteins have decay accelerating activity (DAA), which is the ability to dissociate C3 convertase complexes. Proteins with DAA include CR1, factor H, FHL-1 and decay-accelerating factor (DAF) (134, 135).

Importantly, these proteins all share the same structural building block, known as a short consensus repeat (SCR). These are small domains (60 to 70 amino acids) that contain two disulfide bridges. The SCR domains are found in repeats (from 4 to 30). Several pathogens encode their own complement regulatory proteins, some of which are made up of SCR domains. Although it does not contain SCR domains, the host protein CD59 is also an RCA, and prevents formation of the membrane attack complex.

Viruses and complement

Historically, viruses were not thought of as a main target of complement. However, many studies have now shown that complement acts against viral pathogens, and that viruses have responded by evolving defenses against the complement system (61). Complement is able to lyse both viral particles and infected cells; this has been shown in many viruses, such as HIV, orthopoxviruses, herpesvirus, and influenza virus (10). Complement molecules are also able to opsonize viral particles through C3, C4 and C5 deposition, which can lead to neutralization and/or phagocytosis by immune cells.

A deficient complement response, often shown experimentally with knockout mice, can also affect the activity of the adaptive immune system. C3 knockout (C3-/-) mice challenged with influenza virus exhibit impaired T-cell responses (56). Similar results were seen in complement-deficient mice infected with West Nile Virus and LCMV and vaccinia (37, 77, 118).

More recently, studies have shown that complement is important for survival from poxvirus infections. For instance, a fully functional complement system is needed for normally resistant mice to survive infection with ectromelia virus. C3-/- mice suffered higher mortality rates than wild type mice in footpad, ear pinnea, and intranasal models

of infection (84). These higher rates were also seen with both C4-/- and fB -/- mice. In addition, complement is likely important during a secondary poxvirus infection. A recent study by Benhnia, et al characterized a monoclonal anti-VACV Ab that fixed complement (8). This Ab provided greater in vitro virus neutralization and in vivo protection against challenge than other, similar antibodies that were not complement-fixing.

Viral inhibition of complement

To respond to host complement, viruses have devised ways to interfere with the complement system during an infection. Many microorganisms such as bacteria and fungi actually incorporate host RCAs and several viruses have also evolved to do this (61). These include poxviruses, paramyxoviruses and several retroviruses. VACV has been shown to incorporate DAF, MCP and CD59 into EV particles, making that form of the virus relatively resistant to complement neutralization (129). Similarly, HTLV-1 incorporates DAF and CD59, HIV incorporates DAF, CD59, and factor H, and SIV incorporates DAF, CD59, and MCP into their envelopes during viral budding (72, 81, 103, 117). The paramyxoviruses simian virus 5, and the mumps virus incorporate MCP into virion particles, as well (52).

In addition to these host proteins, some viruses encode their own regulators of complement. These can either be evolutionarily related to host complment control proteins, or without obvious homology. Many poxviruses encode an RCA homolog (10). Such a protein was first characterized in VACV and named the vaccinia complement control protein (VCP). It has been studied extensively and will be reviewed in greater detail below. Two other viral families also encode complement inhibitors,

herpesviruses and flaviviruses. Both HSV-1 and HSV-2 encode a transmembrane protein, gC, that binds C3b and protects virions from complement-mediated neutralization (32, 42, 44). Interestingly, gC has no homology to host RCA proteins. Herpesvirus Samiri and HHV-8, on the other hand, encode transmembrane complement control proteins that contain SCR domains (1, 2, 99). The flavivirus protein NS1 binds to the surface on infected cells and has also recently been shown to be a complement inhibitory protein (6, 20). Like gC, it has no homology to host RCA proteins.

The vaccinia complement control protein

VCP is a 263 amino acid protein (243 residues after cleavage of its signal sequence) located in the C3L ORF of VACV (59). It is the most abundant viral protein secreted from vaccinia-infected cells. Structurally, VCP is made up of four SCR domains (59). VCP has high levels of homology to host RCA proteins, including C4-binding protein, MCP and DAF (57). Electrostatic modeling predicts that the first SCR has a net positive charge, while SCRs two and three have a negative charge (107). VCP also contains an unpaired N-terminal cysteine just prior to the start of the first SCR domain. This cysteine can mediate homodimerization of the protein, which occurs with a minority of VCP molecules (68). When purified, these homodimers have a higher level of complement regulatory activity than monomers.

VCP is able to block the complement cascade at multiple steps of both the classical and alternative pathways. VCP can act as a cofactor in the in factor I mediated cleavage of both C3b and C4b (76, 100). VCP also possesses decay accelerating activity, the ability to disassociate both C3 and C5 convertases (76). The varying activities of

VCP can be attributed to specific SCR domains in the protein. C3 and C4 binding along with cofactor activity occur in the first three SCR domains. Classical pathway decay-accelerating activity occurs in SCR domains 1 and 2; alternative pathway DAA happens in domains 2 to 4 (86). VCP also has two heparin binding sites, one each in SCRs 1 and 4 (86, 116). These have been shown to mediate cell-surface binding of recombinant protein (68, 116).

Initially, VCP was shown to have complement regulatory activity by its ability to block complement mediated lysis of sheep erythrocytes in vitro (57). VCP prevents complement-mediated neutralization of virus particles (50), and some anti-VCP antibodies can prevent this from occurring (48). In vivo, VCP is a virulence factor; it is not necessary for replication, but knockout viruses are attenuated. This was first shown in rabbit and guinea pig models, where a VCP-knockout (ko) virus made smaller lesions than wild-type virus (50). VCP-ko viruses are also attenuated in a mouse intradermal model of infection. This attenuation was linked to an increase in the hosts' adaptive immune response (37).

Other poxvirus complement control proteins

The vaccinia complement control protein has homologs in many other orthopoxviruses. Proteins that have been characterized include the smallpox inhibitor of complement enzymes (SPICE), MoPICE from monkeypox virus, EMICE from ectromelia virus, and inflammation modulatory protein (IMP) from cowpox virus. These proteins share a very high level of amino acid similarity with VCP, with up to 95% homology. The small differences between these proteins has been used to learn about the

structure/function relationships in poxvirus complement inhibitors, and also about poxvirus evolution.

One of the closest homologs to VCP is SPICE, from smallpox. It contains the same four SCR domains as VCP (74) and differs in only 11 amino acids (98). Intriguingly, these changes have a large effect on the functional profile of SPICE. VCP has activity against a wide range of host complement proteins, but SPICE is a specialist. While less effective than VCP against most species' complement systems, against human complement it has a much higher activity than VCP (98, 107, 142). Electrostatic modeling has shown that the charge is markedly different between VCP and SPICE. VCP has a moderate positive charge in SCR1, and a net negative charge in SCR2 and 3. SPICE, in contrast, has a large positive charge in SCR1 and almost no charge in SCRs 2 and 3 (107). These changes may account for some of the functional difference between the two proteins. Substitution mutagenesis has begun to shed light on the specific residues responsible. The four changes H98Y, S103Y, E108K and E120K seem to be most important in conferring the highly human-active and human-specific profile of SPICE (107, 142). Another mutated VCP with only two changes (Q77H and E120K) also had a regulatory profile similar to SPICE (69). In addition to being secreted, recombinant SPICE can also be attached to the cell surface by binding to host glycosaminoglycans. It does this through a series of heparin binding sites found on the protein (69). On the surface of infected cells, it retains its ability to cleave C4b and also prevents the deposition of C3 (67).

The monkeypox homolog to VCP, MoPICE, is interesting in several respects. While there are two main strains of monkeypox, West and Central African, only Central

African isolates possess the ORF for MoPICE (19). Central African strains are also more virulent and the presence of MoPICE has been proposed as one of the reasons for this difference (19). The MoPICE ORF also encodes a protein that is shorter than the ones that encode VCP and SPICE due to an early stop codon in the fourth SCR domain. This results in a truncated protein that expresses only the first three SCRs. Despite the truncation it maintains the ability to bind C3 and C4 and has cofactor activity in vitro; however, there is no measurable decay-accelerating activity (68). Interestingly, this truncation results in a free C-terminal cysteine. MoPICE molecule form homodimers via this cysteine; the N-terminal residue in MoPICE is a tyrosine.

EMICE is the ectromelia (mousepox) virus version of VCP. It has been shown to protect MV particles from complement neutralization and infected cells from the alternative pathway of complement activation (85). It can be expressed on the surface of cells through both an interaction with ectromelia A56 (24) and recombinant protein has been shown to bind cells through host glycosaminoglycans (85).

The cowpox virus homolog of VCP is known as IMP, the inflammation modulatory protein. In contrast to in vivo work with VACV lacking expression of VCP, cowpox viruses lacking IMP are more virulent in mouse models, due to increased inflammation and immune cell infiltration (45, 58). This is different than with other PICES, which are either thought to be or have been shown to contribute to poxvirus virulence in vivo. These results may be a result of the specific model; however, it does show that poxvirus inhibitors of complement have an effect on the host's immune system. While most of the previous work studied PICES as secreted proteins, in the next chapters

we will investigate the mechanism and consequences of VCP and other PICE surface expression.

Chapter Two:

The vaccinia virus A56 protein: A multifunctional transmembrane glycoprotein that anchors two secreted viral proteins

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Abstract

The vaccinia virus A56 protein was one of the earliest described poxvirus proteins with an identifiable activity. While originally characterized as a hemagglutinin protein, A56 has other functions as well. A56 is capable of binding two viral proteins, a serine protease inhibitor (K2) and the vaccinia virus complement control protein (VCP), and anchoring them to the surface of infected cells. This is important; while both proteins have biologically relevant functions at the cell surface, neither one can locate there on its own. The A56/K2 complex reduces the amount of virus from superinfecting an infected cell and also prevents syncytia formation of infected cells; the A56/VCP complex can protect infected cells from complement attack. A56R is a non-essential gene and can be used as an insertion point for foreign genes and has been deleted in some viruses in clinical development as oncolytic agents. Deletion of the A56R gene results in varying affects on vaccinia virus virulence, which are strain and infection model dependent.

Introduction

Orthopoxviruses are some of the most complex viruses infecting humans and include variola virus, the causative agent of smallpox, and vaccinia virus, which is used as a live-virus vaccine. Although smallpox has been eradicated, vaccinia virus (VACV) is still studied as a model organism to understand basic aspects of poxvirology and as a vaccine-vector to immunize against infectious agents and as a candidate oncolytic agent. While VACV is a relatively large virus, the genome is still small compared to host cells. For this reason, VACV encodes a number of multi-functional proteins, one of which is A56.

There is a long history of studies with VACV looking at what is now called the A56 protein, which is found on the membrane of infected cells and some virus particles (Fig. 2.1). Because of its hemagglutination activity, the protein was one of the first VACV proteins identified and studied (87) and was called VACV hemagglutinin (HA) from the 1940s until near the end of the 20th century. The presence of a VACV protein with hemagglutination activity was believed to be important because several other viruses such as influenza, measles, and mumps contained proteins with hemagglutination activity. The VACV protein responsible for this activity was first isolated in 1977 (46).

The ability to track the protein by hemabsorption provided a means to show that it was on the infected-cell membrane (11) and not on mature virus (MV) (Fig. 2-1). HA was also found on extracellular virus (EV) (94). While a biologically relevant function could not be ascribed to VACV's hemagglutination activity, in the course of studying HA, many important discoveries were made like the identity and location of the HA gene (108) and the apparent size of HA as an 85-kDa glycoprotein (111). The A56 gene has two separate promoters for early and late gene expression. Interestingly, late expression produces a smaller, 68-kDa form of A56 (14), although whether this form has biological significance is not known. Both the 85 and 68-kDa forms of A56 can be seen via western blot at late times. Based on the sequence, A56 contains putative sites for N- and O-glycosylation (14, 90, 111). When glycosylation is inhibited, A56 migrates at a weight of 58-kDa, which is different than the 35-kDa calculated weight (110). Much of this work has been summarized in a review by Hisatoshi Shida (109).

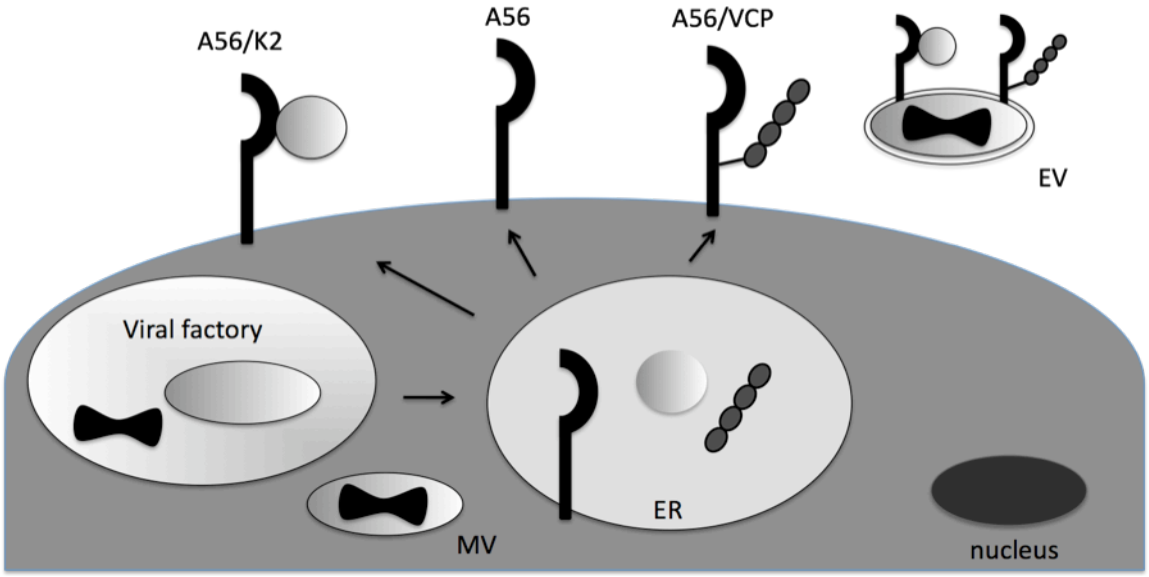

Fig. 2.1. Diagram of the location of A56 in a VACV-infected cell. A56, K2, and VCP have signal sequences that result in their insertion into the lumen of the endoplasmic reticulum (ER), where presumably initial protein-protein interactions take place, followed by trafficking through the secretory pathway. As a transmembrane protein, A56 is found on the infected cell surface, where it is present as a monomer, as well as in complex with K2 and VCP. A56/K2 (and possibly A56/VCP) is also found on extracellular virus (EV). Also shown is the viral factory producing mature virus (MV).

A schematic diagram of A56 is shown in Fig. 2.2. The N-terminal region of A56 has homology with the immunoglobulin (Ig) superfamily. In this Ig-domain, it was hypothesized that the two cysteines form an intramolecular disulfide bridge. Computer modeling shows that the first two cysteines of A56 are in close proximity and are predicted to form an intramolecular bridge (Fig. 2.3). Between the Ig-domain and the transmembrane domain are two tandem repeat motifs (51), which do not share homology to other proteins. An intracellular domain of ~13 amino acids is present at the C-terminus and may be responsible for trafficking A56 out of the ER and into the Golgi (113). Analysis of mutants produced by chemical mutagenesis (113) revealed that the hemabsorption and the cell-cell fusion regulatory properties of A56 are separate (105). While gene knockout viruses cannot perform either function, one point mutant (Glu121 to Lys) was found to be hemabsorption-negative, but could still prevent syncytia formation. Similarly, a virus that contained a Cys103 mutation formed syncytia, but hemabsorption was unaffected. This suggested that while both hemagglutination and syncytia-prevention functions can be traced to the Ig-domain, there are distinct residues that impact these functions.

A56 inhibits spontaneous cell-cell fusion of VACV-infected cells by forming a complex with K2

The ability to hemagglutinate was not the only early function ascribed to A56. Cells infected with some VACVs can result in cell-cell fusion, and in 1971 this was directly linked to a lack of A56 (47). Interestingly, the loss of another viral protein, K2, also causes infected cells to fuse (62, 127, 147). A56 and K2 was found to form a complex on

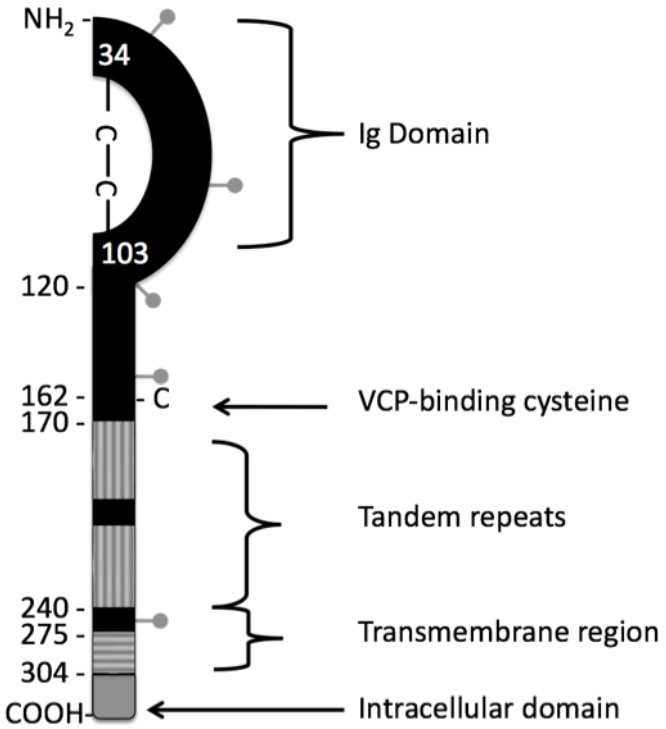

Fig. 2.2: Schematic map of the domains of A56. After a cleaved signal sequence (residues 1-19), there is an Ig-domain (residues 20-120), which includes a predicted intramolecular disulfide bridge (cysteines 34 and 103), a stalk region (121-275) containing tandem repeats (170-240), a transmembrane domain (276-303), and a cytoplasmic tail (residues 304-315). K2 binding appears to occur with the Ig-domain; cysteine 162 forms a disulfide bridge with a free cysteine on VCP. Also shown are locations of the predicted N-linked glycoslyation sites (lollipops) at residue 37, 69, 112, 161, and 254.

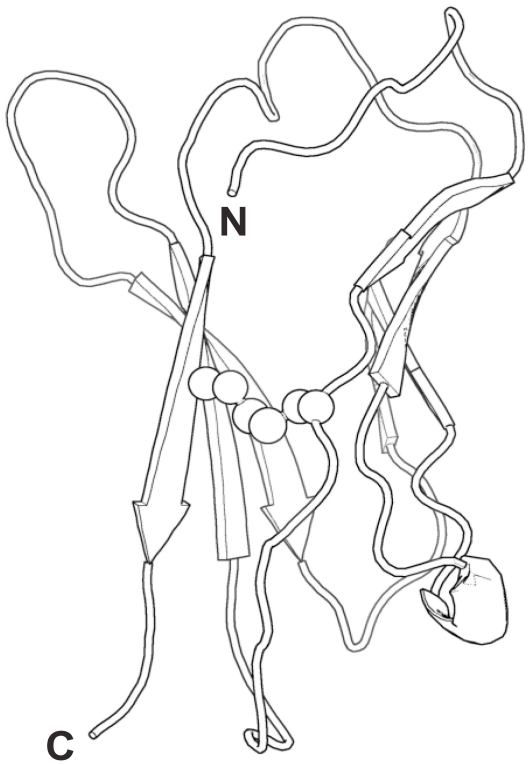

Fig. 2.3: Predicted structure of the N-terminus of A56. Homology modeling of the Ig-domain of A56 predicts a disulfide bridge between cysteine residues shown as spheres. The N-terminal domain of A56 was used as a query sequence for five iterations of PSI-BLAST (3) searching of the NCBI RefSeq database (96). The best scoring protein from this query with an atomic structure is the T-cell receptor alpha chain IgG domain from Cf34 (PDB 3FFC, Chain D (39)), with an E score of 9.02e-27 and 22% sequence identity. The A56 sequence was threaded onto this three-dimensional structure using the homology modeling program MODELLER 9v8 (29). The model was rendered using the program PYMOL.

the surface of infected cells, and that this complex is responsible for preventing syncytia (125). K2 is also found on EV particles, as observed by electron microscopy and proteomics (15, 71). K2, also known as serine protease inhibitor (SPI)-3, is one of a family of poxvirus proteins with homology to serpin proteins (13). K2 (SPI-3) has in vitro protease-inhibition activity (124, 136), but this activity is not required to prevent syncytia (126).

The mechanism by which the A56/K2 complex inhibits spontaneous fusion of infected cells was not clear until the discovery that the virus-encoded multi-subunit entry fusion complex (EFC) found on the MV membrane (106) interacted with the A56/K2 complex (131). A56/K2 interacts with A16 and G9 proteins, two proteins that are part of the EFC (133). These findings led to the hypothesis that A56/K2 on infected cells could prevent re-infection of already infected cells. Support for this was obtained when VACVs with deletions of A56 or K2 were used to infect cells followed by superinfection with VACV with a luciferase reporter gene. Superinfection of cells that were infected with virus expressing A56/K2 had significantly lower luciferase levels than cells infected with a virus expressing a mutated A56/K2 (128). Also, transfecting A56 and K2 into cells is sufficient to diminish both infection and cell-cell fusion (132). The A56/K2 complex preventing superinfection by incoming MV particles should not be confused with the ability of another set of poxvirus proteins (A33 and A36) that can repel EV, a process that speeds up viral spread (25).

III. Cell surface expression of VCP through interactions with A56

K2 is not the only viral protein that directly interacts with A56. A new interaction was recently discovered between A56 and VCP. VCP is a 35-kDa protein that was previously characterized as the major secreted protein from VACV-infected cells (50, 57, 59). VCP has the ability to inhibit complement activation (e.g., (9, 57, 68, 69, 76, 80, 86, 97, 98, 100, 107)). VCP-deletion viruses are mildly attenuated in vivo (24, 50), possibly due to an improved adaptive immune response in the absence of complement inhibition (37). Besides being secreted, VCP is expressed on the infected cell surface (36). Surface expression required the presence of A56 and the free N-terminal cysteine on VCP (36). Furthermore, this free cysteine on VCP forms a covalent bond with the cysteine 162 on A56 (24). The A56/VCP complex can protect infected cells from complement-mediated lysis (24, 36), and surface-bound VCP may be important for full virulence in vivo (24, 36).

The interaction between VCP and A56 is not limited to VACV. The smallpox homolog of VCP can also bind to VACV-A56 when plasmids expressing both proteins are transfected into cells. Also, immunofluorescence staining of ECTV-infected cells shows surface expression of its complement control protein (CCP). Interestingly, in transfection studies, ECTV-CCP does not interact with VACV-A56, but is able to bind ECTV-A56 (24). This suggests co-evolution of these proteins may have occurred. The monkeypox virus (MPXV) CCP does not contain a free N-terminal cysteine; however, due to a mutation that results in early termination, there is an unpaired C-terminal cysteine. While this C-terminal cysteine allows MPXV-CCP to form disulfide-bonded homodimers (68), it appears not to allow efficient interaction with MPXV-A56 for surface expression (24). By transient tansfection and immunofluorescent staining of

MPXV-infected cells, some MPXV-CCP surface staining is seen, but at a much lower level than seen with the other poxvirus CCPs (24). Proteomics on MV and EV particles from VACV and MPXV found VCP on VACV virions, and that more VCP was found on EV than MV (71). Conversely, MPXV virions had no MPXV-CCP (71). This is consistent with the finding that VCP, but not MPXV-CCP, binds to A56 efficiently and also likely suggests the presence of A56/VCP on EV particles (Fig. 2.1). Much of the work summarized in this section will be explained in greater detail in chapters 3,4 and 5 of this thesis.

A56 and VACV virulence

The contributions of A56 to VACV virulence have not been fully elucidated. Virulence levels appear to vary widely according to the route of infection and the strain of the parental virus. Work with the NYCBH strain showed significant attenuation with an A56R gene knockout (ko) virus (64). Intracranially, the A56R-ko had an LD50 of 1.6×10^5 pfu versus 1.9×10^2 pfu for the wildtype virus. Intranasally, the difference was even more pronounced, with and an LD50 of A56R-ko of $>1\times10^8$ pfu versus 2.5×10^4 pfu for the wildtype virus. This degree of attenuation was not as clear with other strains of VACV. In studies with a Lister-based oncolytic virus containing directed deletions of the thymidine kinase (J2L) and F14.5 genes, the addition of an A56R deletion resulted in evidence of further attenuation. Infection of nude mice with the parental J2L/F14.5-deletion virus resulted in survival of >100 days, but all mice eventually died by day 120. Mice infected with the virus with the additional A56R-ko survived for the length of the study, 135 days (145, 146). However, the mice in this experiment had xenograft tumor

implants, making it difficult to ascertain whether death was from tumor growth or viral replication. An A56-ko in VACV strain Western Reserve (WR) showed a small amount of attenuation when given intracranially in young mice (31). In this model, mice infected with an A56R-ko died more slowly (first death at five days, last death at 17 days) than with a wildtype virus (100% death at 8 days). No attenuation was seen when inserting a foreign gene in the place of A56R in the LC16mO or LO strains when given by either intracranial or intraperitoneal routes (112).

Deletion of the VACV proteins that bind A56 results in different levels of in vivo consequence. K2-deletions in strain WR do not appear to be attenuated, with no difference in survival after intranasal infection (62). Similarly, intranasal infection of mice with a K2-ko cowpox or wildtype viruses had nearly identical LD50s (5.3×10^5 pfu and 5.1×10^5 pfu, respectively) (119). As previously mentioned, VCP is needed for full VACV (strain WR) virulence, as VCP-ko are attenuated when given intradermally to rabbits and guinea pigs (50), and when given to mice intradermally or intranasally (24, 37). The specific contribution of surface expression of VCP to virulence is not yet known.

Other uses of A56

Because A56R is a non-essential gene for VACV replication, it has been used as a region in the genome for insertion of foreign genes. The hemagglutinin properties of A56 allow for identification of recombinant plaques with foreign genes inserted into A56R in the absence of a selection marker. That is, the addition of chicken erythrocytes to an infected cell monolayer will cause wildtype plaques to appear red, while

recombinants with deletion of A56R will remain white (89, 113). Another application of A56 mutants has been in the development of VACV as an oncolytic agent. GLV-1h68, which includes the deletion of A56R along with a series of other gene deletions, was able to reduce the size of breast cancer tumors in a nude mouse model (146). This virus has since been successfully used to shrink tumors in several other xenograft mouse models (35, 66, 139, 143, 144).

Conclusions

The history of A56 can be divided into two acts. In the first act, HA was discovered and many basic characteristics were described. After a number of years of diminished work, the last decade has seen a burst of studies that focus on A56. During this second act, A56 interactions with other viral proteins and their relevant biological functions were discovered. A56 is found in multiple locations during an infection (Fig. 2.1), and its interactions with other viral proteins produce multiple effects. While A56 is a "non-essential" protein, it is an important one: through its interaction with K2 it is involved in preventing re-infection of already infected cells, which may promote viral spread. Through its interactions with VCP, it is involved with defending infected cells from the host's immune response. Research on A56 has also been incorporated into new oncolytic agents and new panels of mutant viruses are in progress that may further elucidate the role A56 plays in pathogenesis.

CHAPTER THREE:

VCP is expressed on the cell surface by using its first SCR domain to interact with the viral A56 protein

Portions of this chapter were published in:

"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".

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Abstract

The vaccinia virus complement control protein was originally characterized as the major protein secreted from infected cells. Although it lacks a transmembrane domain, we observed that VCP is also expressed on the surface of infected cells. In order to characterize this finding, we studied a panel of recombinant viruses that produced tagged and/or mutated versions of VCP. We found that VCP co-purifies with the viral protein A56, a transmembrane protein found at the cell surface. We also confirmed that the N-terminal free cysteine is critical for surface expression of VCP, and that this expression occurs even if only the first 85 amino acids (19 a.a. signal peptide + 66 residues) are present.

Introduction

Vaccinia virus (VACV) encodes many non-essential proteins, some of which can be characterized as virulence factors that affect pathogenesis (40, 104). One of these is VCP, the vaccinia complement control protein. VCP shares homology to host regulators of complement, and is made up of short consensus repeat (SCR) domains (59). It is able to block activation of complement at multiple steps (57, 76, 86, 100), preventing complement-mediated virus neutralization (50) and affecting both innate and adaptive immune responses (37).

VCP has been well characterized as the major secreted protein from VACV-infected cells (59), and important for full virulence in vivo in multiple animal models (24, 37, 50). Our lab made the observation that VCP is also expressed on the surface of infected cells. Since some host complement regulatory proteins are active on the cell

surface, we hypothesized that surface expression of VCP may have functional consequences. VCP lacks a transmembrane domain or any described binding partners, so we were interested in whether VCP bound to any viral or cellular proteins at the cell surface. Prior research in our lab showed two important findings: first, that the free N-terminal cysteine of VCP is critical for cell surface expression, and that the viral protein A56 is also required for surface expression (36). Cells infected with recombinant VACVs with the free cysteine of VCP mutated to a threonine (vvVCPmut) or a virus lacking A56 (vvA56-ko) still produce VCP, but cannot express it on the cell surface (36). A56 is an Ig-like protein that has a transmembrane domain (51); it interacts with another vaccinia protein called K2 at the surface of infected cells (125).

To investigate VCP surface expression, we used two sets of viruses. The first set consists of adding a His-tag to both wild-type and the cysteine-mutated VCP. The second set consists of truncated VCP proteins, where only the first SCR domain is present. Here, we demonstrate that VCP forms a high-molecular weight complex with the viral A56 protein by both native Western blotting of infected cell lysates and also affinity pulldown analysis. We also show that only the first SCR of VCP is required for this interaction. These viruses furthered our understanding of the mechanism of VCP cell surface expression, and the relationship between VCP and A56.

Methods

Virus construction

vv-VCPwt-His (vv91i) and vv-VCPmut-His (vv205a1-1) were designed to overexpress His-tagged forms of VCPwt and VCPmut, respectively. The viruses were generated by

homologous recombination of plasmid constructs into the parental virus vSIGK-1 (57). This parental virus has a *gpt* selection cassette replacing the entire VCP ORF (as well as a portion of the flanking regions). His-tagged forms of VCP under the control of a VACV synthetic strong early-late promoter (18) were then inserted into the thymidine kinase locus, and recombinant viruses were isolated by selection with bromodeoxyuridine and screening with β-galactosidase. After three rounds of plaque purification, isolated plaques were expanded and the sequences of the recombinant viruses were confirmed using PCR and Western blotting. The plasmids used for homologous recombination were constructed by adding a 6×-His tag to the carboxy terminus of the VCP ORF using PCR primers to make VCPwt-His (36) and by cloning this PCR product into pSC65 (18). The resulting plasmid also served as the template to generate a His-tagged VCP containing the cysteine-to-threonine mutation. This mutation was introduced using QuikChange site-directed mutagenesis (Stratagene) with the same primers used in the generation of for vv-VCPmut (36). The plasmid was sequenced to confirm the mutation and to ensure that no other unintended mutations were generated during PCR amplification. Also used in these studies are the previously constructed viruses vvVCP-SCR1 (original name v200b2-1), vvVCPmut-SCR1(v210k2-1), vvVCP-SCR1+cys (v183b2-1) and vvVCPmut-SCR1+cys (v184m3-1). These viruses were generated from the VCP-knockout virus vSIGK-3, which contains a gpt selection cassette interrupting the VCP open reading frame. The plasmid pGK35 (57) contains a truncated VCP ORF under the natural VCP promoter and expresses the first SCR followed by 5 amino acids before a frame shift mutation results in a stop codon. This plasmid was used along with parental virus vSIGK3 to generate vvVCP-SCR1+ cys. To make vvVCP-SCR1, site directed mutagenesis was used to place

a stop codon before the C-terminal cysteine (primers used were: for: 5`--CTC TTT AAT CAA TGT ATT AAA TAA TGA TGC CCA TCG CCT CGA G—3`; rev: 5`-C TCG AGG CGA TGG GCA TCA TTA TTT AAT ACA TTG ATT AAA GAG—3`). The resulting plasmid was called pTM234. For vvVCPmut-SCR1+cys and vvVCPmut-SCR1, the previously described N-terminal Cys to Thr primer set was used to mutate pGK35 and pTM234, respectively. After infection with vSIGK3 and tranfection with these newly constructed plasmids, viruses were plaque purified by reverse gpt selection (49).

Immunofluorescence

RK-13 cells were infected at an MOI of 1 with indicated viruses in 8-well chamber slides. At 24 hours post-infection, media was removed and the cells were washed with PBS and then fixed with 1% paraformaldehyde. To permeablize the cells, cells were incubated with 0.1% Triton X-100. After blocking in 1% BSA, the primary antibody (the anti-VCP monoclonal 3B1 (48)) was added at 10 μg/ml for one hour at room temperature. After three washes with PBS, a FITC anti-mouse secondary antibody was added at a dilution of 1:5000 for another hour. After three more washes with PBS, DAPI was added to the cells and a coverslip was mounted for viewing.

Western blotting

For nonreducing, nondenaturing (native) gels, cells were infected in 2.5% MEM and cell lysates were harvested as described above and lysed in RIPA buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris) containing 1× protease inhibitor (Sigma). Native samples

were diluted in sample buffer containing 0.1% SDS and no reducing agents without boiling, and proteins were resolved on a 10% Tris-glycine gel and Western blotted for VCP using the mouse anti-VCP MAb 3F11 (48) at a concentration of 5 µg/ml. For reducing and denaturing gels samples were diluted in sample buffer containing 1.0% SDS + beta-mercaptoethanol, boiled and resolved on a 10% Tris-glycine gel; membranes were probed for VCP using the mouse anti-VCP MAb 5F1 (48) at a concentration of 5 µg/ml in blocking buffer for 2 h at room temperature. . After being washed with PBS containing 0.02% Tween 20, horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz) was diluted 1:2,000 in blocking buffer and incubated with the membranes for 2 h. The membranes were then washed, and proteins were detected by chemiluminescence using the enhanced-chemiluminescence Western blotting detection kit (GE Healthcare Biosciences). Blots were imaged using an LAS-1000 Pus gel documentation system (Fujifilm).

A56 pulldown

BSC-1 cells in T-75 flasks were infected in 2.5% MEM at an MOI of 1 for 48 h. Cells were harvested, washed once in ice-cold buffer (50 mM Tris-HCl [pH 7.4] and 150 mM NaCl), and lysed in lysis buffer (50 mM Tris-HCl [pH 7.4], 1% Triton X-100, 150 mM NaCl, and 1× protease inhibitor cocktail [Sigma]). Cells lysates were clarified by centrifugation and incubated with nickel-agarose beads for 2 h at 4°C. Samples were loaded onto a column, washed three times with lysis buffer containing 20 mM imidazole, and bound proteins were eluted with lysis buffer containing 500 mM imidazole. Proteins eluted from the column were separated by SDS-polyacrylamide gel electrophoresis

(PAGE) under reducing and denaturing conditions and transferred to nitrocellulose. Membranes were probed for His-tagged VCP and for A56. To probe for His-tagged VCP, membranes were incubated with a primary anti-6x-His tag MAb (Qiagen) diluted 1:200. The membrane was then washed and incubated with a horseradish peroxidase-conjugated secondary goat anti-mouse IgG antibody diluted 1:3,000 (Santa Cruz). Membranes were also probed for A56 using polyclonal rabbit anti-A56 peptide antiserum (131) diluted 1:1,250. The membranes were then washed and incubated with a secondary donkey anti-rabbit antibody conjugated to horseradish peroxidase (GE Healthcare Biosciences). Proteins were detected with SuperSignal West Pico chemiluminescence reagent and imaged using an LAS-1000 Pus gel documentation system (Fujifilm).

Results

Characterization of a high molecular weight complex containing VCP

Previous data suggested that A56 is necessary for the localization of VCP to the cell surface (36); we hypothesized that there is a specific interaction between VCP and A56. When lysates from cells infected with vv-VCPwt were resolved under nonreducing, nondenaturing (native) conditions and probed for VCP, three distinct bands were present (Fig. 3.1, first lane). These bands include an ~30-kDa band representing the VCP monomer, an ~50-kDa band representing the VCP dimer, and a third high-molecular-mass band. The presence of the high-molecular-mass band suggests that VCP interacts with another protein. To determine if A56 was required for the formation of the high-molecular-mass complex, lysate from cells infected with vv-A56ko was resolved under native conditions and probed for VCP (Fig. 3.1, third lane). In the absence of A56, we

found the ~30-kDa and ~50-kDa bands, representing monomeric VCP and dimeric VCP, respectively, but the high-molecular-mass band was no longer present. This suggests that the high-molecular-mass complex does not form in the absence of A56. Furthermore, when lysates from cells infected with vv-VCPmut were probed for VCP, only monomeric VCP was detected, demonstrating the importance of the unpaired cysteine residue on VCP for dimer formation, as well as the formation of the higher-molecular-mass complex (Fig. 3.1, second lane).

His-tagged VCP is still expressed at the surface of infected cells

In order to generate reagents to show a physical interaction between A56 and VCP, we created two tagged VCP-producing viruses: vvVCP-His and vvVCPmut-His. Both of these contain C-terminal 6x Histidine tags, as diagrammed in Figure 3.2A. In order to see if tagging VCP interferes with surface expression, infected cells were prepared for immmunofluorescence staining with an anti-VCP antibody. Under permeablized conditions, both VCP-His and VCPmut-His are produced and can be seen intracellularly in permealblized cells. Importantly, under non-permeablized conditions VCP-His, but not VCPmut-His is seen at the cell surface (Fig 3.2B). This mirrors what is seen with un-tagged proteins, and shows that the His-tag does not alter cell-surface expression.

VCP interacts directly with A56

To determine if VCP and A56 interact in infected cells, we used viruses encoding His-tagged forms of VCP (vv-VCPwt-His) or VCPmut (vv-VCPmut-His) to pull down A56.

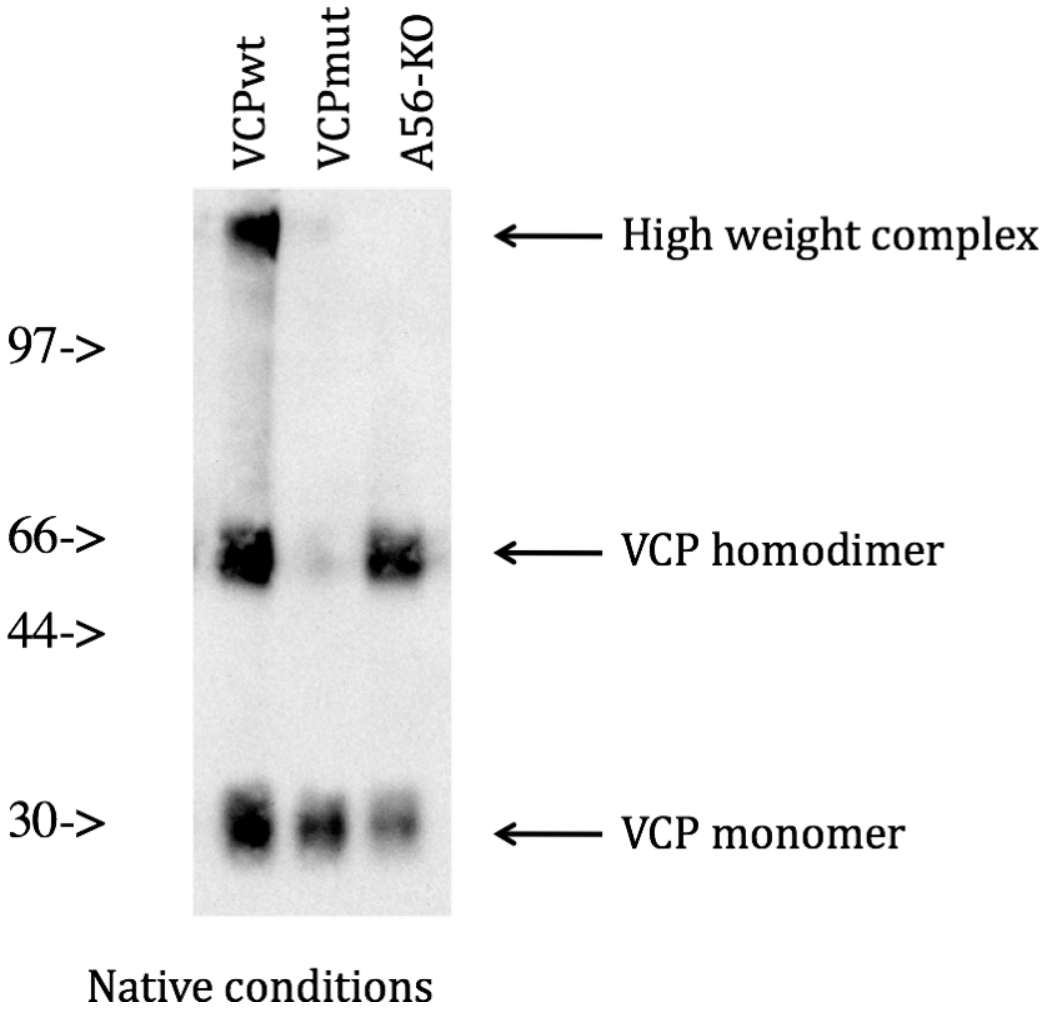

Fig. 3.1: VCP forms a high weight complex under native conditions. (A) BSC-1 cells were infected overnight with the indicated virus at an MOI of 5. Infected cells were then harvested and washed with and lysed in RIPA buffer. Lysates were run on a 10% precast gel under native (nonreducing, nondenaturing) conditions. Membranes were transferred to nitrocellulose and probed for VCP using MAb 5F1.

Lysates from cells infected with vv-VCPwt-His or vv-VCPmut-His incubated with nickel-agarose. Following extensive washing, proteins were eluted with imidazole, separated using SDS-PAGE, and probed for A56 (Fig. 3.3). We found that A56 was present only in eluates from vv-VCPwt-His-infected cells, not vv-VCPmut-His-infected cells, indicating that A56 interacts with vv-VCPwt-His but not vv-VCPmut-His. Taken together, these results indicate that VCP and A56 interact in infected cells. Of note, at the same time we were performing this work, Wagenaar and Moss used tandem affinity purification and mass spectrometry to identify proteins that interact with A56 (131). Their study identified VCP as one of many proteins that copurifies with A56, lending support to our finding that these two proteins interact.

Truncated forms of VCP are expressed on the cell surface (unpublished data)

The N-terminal cysteine of VCP has been established as critical for cell-surface expression of VCP and presumably interacting with A56 (36). However, it was initially unknown what other parts of the protein may play a role in this interaction. A previously-made mutant virus, vvSIGK-8 (49), was able to shed some light on this question. vvSIGK-8 was previously isolated by Stuart Isaacs and Girish Kotwal from vSIGK-3, a recombinant virus that had a gpt cassette inserted within a 70 base pair deletion within VCP. The removal of the gpt cassette from vvSIGK-3 resulted in vvSIGK-8, a virus that still did not express a full length VCP protein. However, on closer inspection, the removal of the gpt cassette resulted in an ORF that expressed 70 amino acids (SCR1 plus a few residues of SCR2). Antibodies that recognized the first SCR revealed that this fragment of VCP was found to be both secreted from cells (48)

A

B

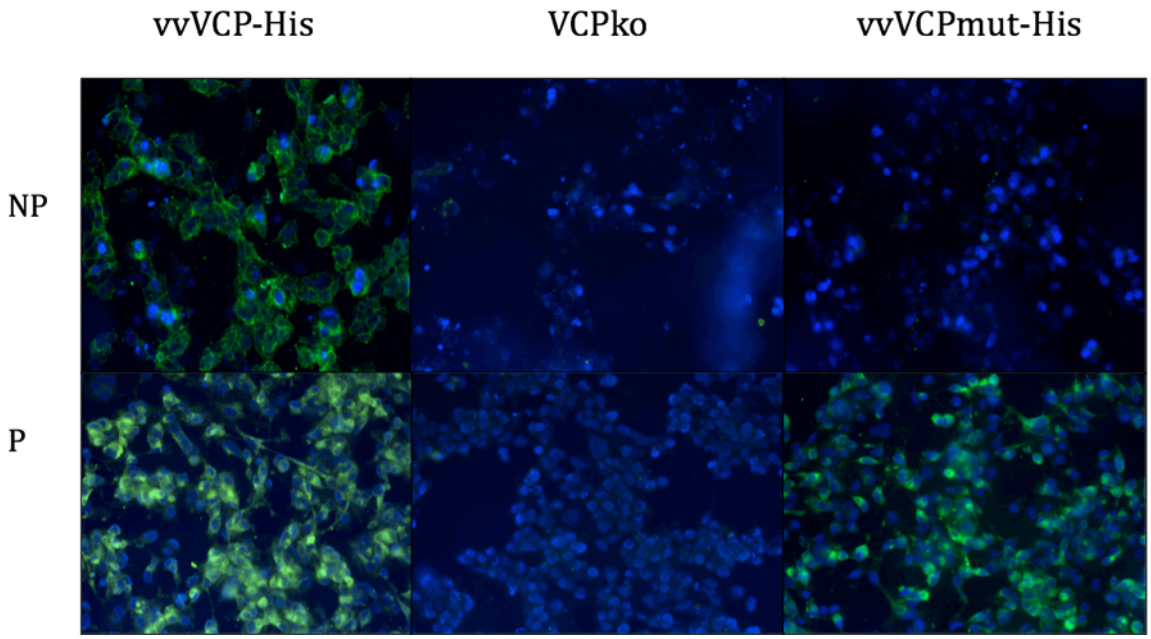

Fig. 3.2: His-tagged VCP is still expressed at the cell surface. (A) Schematic diagram of wild-type VCP tagged with a 6x-His tag; also shown is VCPmut (Cys20Thr) with the same tag. (B) Immunofluorescence of infected RK-13 cells under either permeablized (P; for internal staining) or non-permeablized (NP; for cell-surface staining only) conditions. VCP is stained green, and the cell nuclei are stained blue with DAPI.

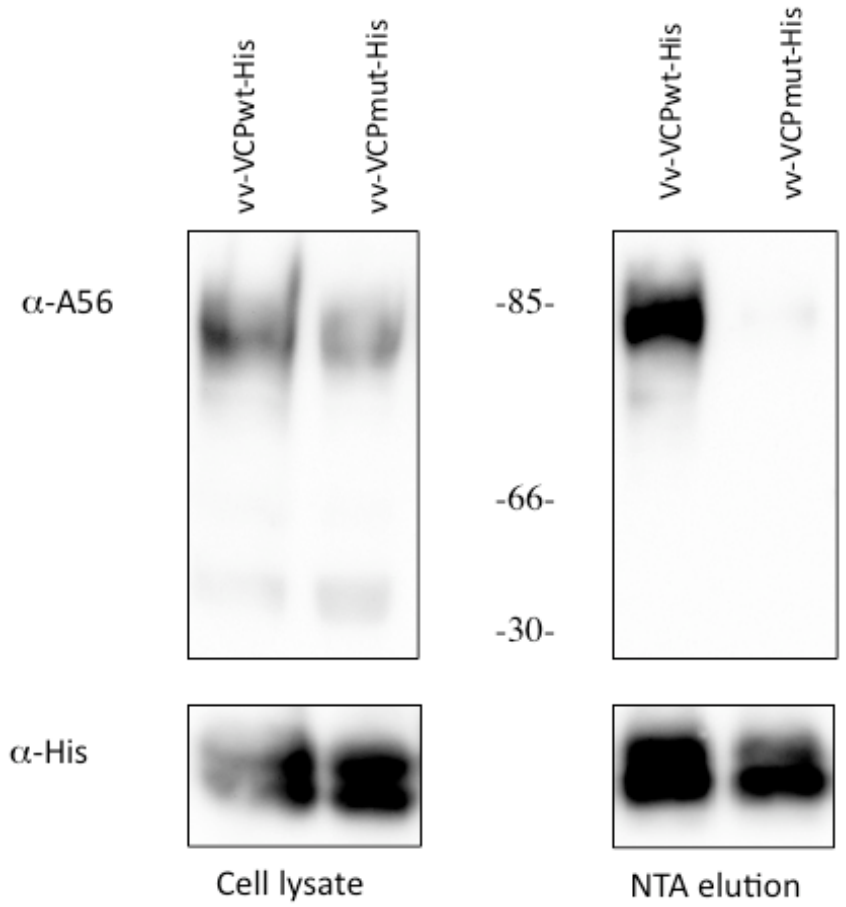

Fig. 3.3: VCP and A56 interact under native conditions. BSC-1 cells were infected for 48 h with the indicated viruses expressing His-tagged versions of VCP at an MOI of 5. Lysates were incubated with nickel-agarose. Proteins were eluted with imidazole, separated via SDS-PAGE under reducing, denaturing conditions; and probed for the presence of A56 using an anti-A56 rabbit polyclonal antibody (top) or probed for VCP using an anti-His antibody (bottom). Molecular mass markers (in kDa) are indicated along the left-hand side of the gels. α, anti.

and on the cell surface (Isaacs, unpublished). However, because of the way the mutation in vSIGK-8 was made, the expressed first SCR also contained a C-terminal cysteine (a remnant of the beginning of the second SCR) and thus it was not clear if surface expression was due to the abnormal expression of a protein with both a N- and C-terminal cysteine (Fig 3-4A, vvVCP-SCR1+cys, a virus that was re-generated similar to vSIGK-8). To further investigate this, we made additional VCP mutants, each containing only the first SCR either of the wild-type protein (vvVCP-SCR1) or VCPmut (vvVCPmut-SCR1) (Fig. 3.4A). Interestingly, we found that VCP-SCR1 is still expressed on cell surface, while vvVCPmut-SCR1 was not. This means that only the N-terminal ~60 amino acid residues are necessary for the VCP/A56 interaction to occur. We also recreated the original truncated VCP from vvSIGK-8. This virus is called VCP-SCR1+cys, as the extra residues from SCR2 results in an additional C-terminal free cysteine (Fig 3.4A). As with the original virus, this form of VCP is expressed on the cell surface (Fig 3-4B, vvVCP-SCR1+cys). Mutating the N-terminal cysteine of this truncated VCP yielded surprising results. When this residue is mutated to a threonine in this protein, cell surface expression remains, presumably through the unpaired C-terminal cysteine (Fig. 4a and b, vvVCPmut-SCRcys). In contrast, mutating the N-terminal cysteine from VCP-SCR1 (VCPmut-SCR1) abrogates surface expression (Fig. 3.4B, 5th panel), because this truncated form of VCP has no unpaired cysteines. This also reinforces the idea that only the first SCR and a free cysteine are necessary for this interaction.

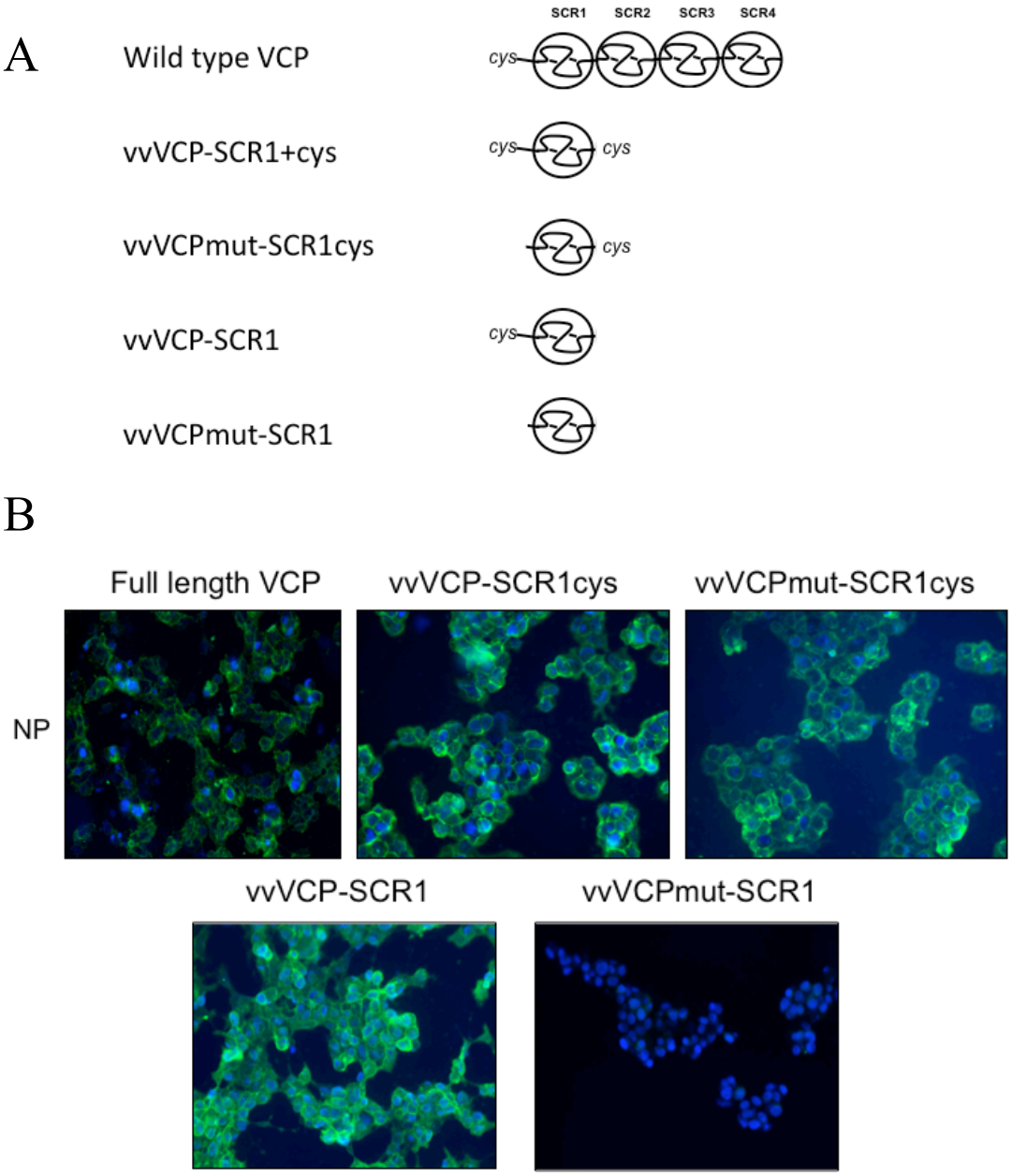

Fig. 3.4: Analysis of truncated VCP proteins' surface expression. (A) Schematic of four different truncated VCP proteins. vvVCP-SCR1+cys has N- and C-terminal cysteines; vvVCPmut-SCR1+cys has the C-terminal unpaired cysteine. vvVCP-SCR1 only contains the original N-terminal free cysteine, while vvVCPmut-SCR1 has none. (B) Immunofluorescence of infected cells. RK-13 cells were infected with the indicated viruses for 24 hours and stained for VCP under non-permeablized (NP) conditions.

Discussion

The vaccinia complement control protein has already been well described as a virulence factor that is secreted from infected cells (50, 57). Here, we have begun to characterize VCP as a protein that is also expressed on the cell surface and also started to uncover the mechanism of surface expression. We have discovered that VCP binds to A56, a vaccinia protein that possesses a transmembrane domain. This relationship has also been shown between A56 and a viral protein before, with K2, also known as serine protease inhibitor 3 (SPI-3). K2 is a soluble viral protein that cannot localize to the cell surface on its own. A56 binds K2 non-covalently (125), forming a complex that prevents syncytia formation and viral superinfection (125, 128, 131, 132). Since complement can act at the cell surface, it is possible that surface-bound VCP has a biological function there that has implications in pathogenesis.

Work presented here and elsewhere has established that the N-terminal free cysteine of VCP is necessary for stable interaction with A56, and therefore cell-surface expression. We also showed that only the first SCR of VCP is necessary for surface expression, meaning that the critical interaction residues are located in that region. Two different hypotheses for this mechanism of the interaction and cell-surface expression can be proposed. The first is that a cysteine-bridged VCP dimer formation is necessary for VCP to bind non-covalently to A56. The second is that an intermolecular disulfide bridge forms between VCP and A56. This is possible because the ectodomain of the A56 protein has three cysteines. As A56 has an Ig domain, two of cysteines are predicted to form an intramolecular disulfide bridge. The third cysteine has no described function, and so may

allow the formation of an intermolecular disulfide bond with VCP. In the next chapter we will describe a series of experiments with A56 mutants and their ability to bind to poxvirus complement control proteins.

Our findings with vvVCP-SCR1 and vvVCPmut-SCR1cys (Fig. 3.4A) are also tantalizing. It suggests that if the first SCR is available to interact with A56, then a free cysteine, not necessarily the N-terminal cysteine, is all that is needed for cell surface expression (Fig. 3.4B). In addition, this piece of data can be used to argue against the hypothesis that a VCP dimer binds to A56 since if vvVCPmut-SCR1cys expresses a protein that forms homodimers, the first SCR would be in a tail-to-tail conformation instead of a head-to-head conformation. It is unlikely that the first SCRs of VCP would form the same binding conformation when they are attached by C-terminal cysteines and when they are bound as a dimer by their N-terminal cysteines. But equally perplexing is that an initial interaction between A56 and VCP can occur with either an N- or C-terminal free cysteine on SCR1. Further work involving mutagenesis of A56 will provide answers whether A56 and VCP form an intermolecular disulfide bond, and in vivo work with mutated and knockout viruses may find a role for surface-bound VCP in vaccinia pathogenesis and virulence.

CHAPTER FOUR:

Poxvirus complement control proteins are expressed on the cell surface through an intermolecular disulfide bridge with the viral A56 protein

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Abstract

The vaccinia virus (VACV) complement control protein (VCP) is an immunomodulatory protein that is both secreted from and expressed on the surface of infected cells. Surface expression of VCP occurs though an interaction with the viral transmembrane protein A56, and is dependent on a free N-terminal cysteine of VCP. Although A56 and VCP have been shown to interact in infected cells, the mechanism remains unclear. To investigate if A56 is sufficient for surface expression, we transiently expressed VCP and A56 in eukaryotic cell lines, and found that they interact on the cell surface in the absence of other viral proteins. Since A56 contains three extracellular cysteines, we hypothesized that one of the cysteines may be unpaired and could therefore form a disulfide bridge with VCP. To test this, we generated a series of A56 mutants in which each cysteine was mutated to a serine, and found that mutation of cysteine 162 abrogated VCP cell surface expression. We also tested the ability of other poxvirus complement control proteins to bind to VACV A56. While the smallpox homolog of VCP is able to bind VACV A56, the ectromelia virus (ECTV) VCP homolog is only able to bind the ECTV homolog of A56, indicating that these proteins may have co-evolved. Surface expression of poxvirus complement control proteins may have important implications in viral pathogenesis, as a virus that does not express cell surface VCP is attenuated in vivo. This suggests that surface expression of VCP may contribute to poxvirus pathogenesis.

Introduction

Poxviruses, including vaccinia virus (VACV), encode large numbers of immunomodulatory proteins that help them establish an infection and combat the host's

immune response (40, 104). One of these is the vaccinia complement control protein (VCP), which is both secreted from and expressed on the surface of infected cells (36, 50, 57, 59). VCP acts against the complement system, a series of soluble proteins that is an important early component of the innate immune system and also shapes adaptive immune responses (55, 134, 135). In response to viral infection, complement can opsonize or inactivate virions and can lyse enveloped virus or infected cells (8, 12, 20, 44). Because of these pressures, a number of viruses, such as HSV, flaviviruses, and poxviruses encode novel or host-derived regulators of complement, while others such has HIV and poxviruses incorporate host complement regulatory proteins into virus particles (20, 42, 101, 130). Many orthopoxviruses encode a complement regulator (21, 68, 80, 98), and the most studied of these is VCP. Structurally, VCP is made up of four short consensus repeats (SCR) that are the basic units of mammalian complement regulators (59, 86) and has been shown to interfere with the complement cascade at multiple steps (9, 57, 68, 69, 76, 86, 97, 98, 100, 107). Additionally, a VCP knockout virus generates smaller lesions in animal models (50, 57). While some host complement control proteins (CCPs) are secreted, many contain transmembrane domains (or a GPI anchor) and are thus expressed on the cell surface (134, 135). Thus, when we found that VCP is also expressed on the infected cell surface and protected infected cells from complement mediated lysis in vitro (36), we believed this to be an important interaction that required further investigation. We previously found that the N-terminal cysteine on VCP was needed for surface expression and that the VACV transmembrane protein, A56, was also required (36). The vaccinia A56 protein is a type 1 transmembrane glycoprotein that is found on the surface of infected cells and on extracellular virus particles (14, 60, 91, 93,

125). It interacts with another viral protein, K2 (62, 127, 147), which lacks a transmembrane domain and binds to A56 non-covalently (125). The A56/K2 complex prevents syncytia formation between infected cells and superinfection by interacting with the vaccinia entry/fusion complex on virions (83, 128, 131, 132). Here we provide evidence that the N-terminal cysteine on VCP forms an intermolecular disulfide bond with cysteine 162 on the ectodomain of A56. We also demonstrate that similar interactions can occur with other poxvirus CCPs, as the smallpox and ectromelia homologs of VCP also exhibit A56-dependent surface expression.

Methods

Cells and viruses

BSC-1, 293T, and RK-13 cells were grown and maintained in Minimum Essential Media (MEM) supplemented with 10% Fetal Bovine Serum. Viruses were grown and titered in BSC-1 in MEM with 2.5% FBS. The generation and isolation of vaccinia virus (vv)-VCPko, -VCPmut, and -VCPwt from the parental stain WR has been described previously (36). The VCPrescue virus was made by reinserting the VCP open reading frame (ORF) under its native promoter into the VCPko virus. After the initial infection of cells with VCPko and transfection with a plasmid containing wild type VCP ORF, the progeny virus was amplified on BSC-1 cells, and the cell lysate was used to infect fresh RK-13 cells. After 24 hours of infection these cells were stained for VCP with the anti-VCP mAb 3F11 and an anti-mouse APC secondary Ab under non-permeablized conditions and positive cells were collected through live cell sorting. The resulting cells were lysed, the virus was plaque purified 3-times on BSC-1 cells, and a stock of virus

was grown and purified. PCR confirmed proper insertion of the gene back into the C3L position and that no mutations occurred during PCR amplification or virus isolation. Expression of VCP in the rescued virus was confirmed by western and FACS analysis.

Cloning of poxvirus genes into expression plasmids

The previously generated plasmids containing the ORF of VCP and VCPmut (36), as well as SPICE, MoPICE and EMICE (68) were used to insert the ORFs into pCAGGS. The VACV A56 ORF was PCR amplified from VACV (strain WR) and initially cloned into Topo 2.1 using the primers 5'-CGG GGT ACC ATG ACA CGA TTA CCA ATA CTT TTG -3' and 5'-CGC GCG GCT ACG CTA GAC TTT GTT CT-3'. This plasmid was then used to generate A56mut1, mut2 and mut3 by site directed mutagenesis using overlapping primers. A56mut1 primers were 5'- GCA ACT CTA TCA TCT AAT CGA AAT AAT ACA AAT G-3' and 5'- CAT TTG TAT TAT TTC GAT TAG ATG ATA GAG TTG C-3'; A56mut2 primers were 5'- GCC GGT ACT TAT GTA TCT GCA TTC TTT ATG ACA TC-3' and 5'- GAT GTC ATA AAG AAT GCA GAT ACA TAA GTA CCG GC-3'; A56mut3 primers were 5'- GAT TAT ATA GAT AAT TCT AAT TCC TCG TCG GTA TTC G-3' and 5'- CGA ATA CCG ACG AGG AAT TAG AAT TAT CTA TAT AAT C-3'. A56mut1+2 was created by site directed mutagenesis of A56mut1 using the primers for mut2. All 4 mutated ORFS were then inserted into pCAGGS. The ectromelia A56 homolog was PCR amplified from the ECTV Moscow strain (primers: 5'- CGG GGT ACC ATG GCA CGA TTG TCA ATA CTT TTG-3' and 5'- CGC GCG GCT AGC CTA GAC TTT GTT CTC TGT TTT G-3') and the monkeypox A56 homolog PCR amplified from MPXV-Zaire and cloned into Topo2.1 prior to insertion

into pCAGGS (forward primer: 5'- CGG GGT ACC ATG ACA CAA TTA CCA ATA CTT TTG-3'; reverse primer is the same as ECTV A56). K2 was cloned from VACV (strain WR) with the addition of a C-terminal HA tag using the primers 5'- CGG GGT ACC ATG ATT GCG TTA TTG ATA CTA TCG-3' and 5'- CGC CGA TCG TTA GGC ATA ATC GGG AAC ATC GTA GGG GTA AGA GCC ACC GCC ACC AGG AGA TTC CAC CTT ACC CAT AAA C-3'. The ORFs in all final plasmids were sequenced to confirm sequences (or site directed point mutations) were correct.

Transfection of poxvirus genes and flow cytometry

Plasmid transfections were performed in 6-well plates of 95% confluent 293T cells using Lipofectamine and serum-free media. Two micrograms of each plasmid was used per well, along with 10 μl of Lipofectamine. Forty-eight hours later, the cells were lifted in FACS buffer (PBS without Ca^{++}/Mg^{++} with 1% FBS and 0.04% sodium azide) and transferred to FACS tubes. Cells were then incubated with anti-VCP Ab (either a mouse monoclonal (mAb) 3F11 (48, 131) or a rabbit polyclonal (68)) and anti-A56 Ab (either mAb LC10 or a previously described peptide-raised rabbit polyclonal antibody (131)) at 1:1000 dilutions. After washing, the cells were incubated with appropriate FITC and APC conjugated anti-mouse or anti-rabbit antibodies at a 1:40 dilution and then fixed using 3% PFA (paraformaldehyde). The cells were then read on a FACScaliber and analyzed using FlowJo. Scatter plots were gated on live cells using forward and side scatter. Histograms were created by gating on cells that were A56 positive. For the K2 FACS, collected cells were incubated with the rabbit anti-A56 Ab and a previously described K2 mAb 4A11-4A3 (15).

Affinity isolation of tagged A56 from infected cells

To determine the how much VCP associates with A56, we infected cells with a previously described recombinant VACV expressing a tagged A56 (131). BSC-1 cells were infected with vA56TAP or a virus expressing an untagged A56 (vv-VCPwt). 48 hours later, cell lysates were harvested in RIPA buffer and incubated with streptavidin sepharose beads for 1 hour at 4° C. After washing and eluting the protein, western blots were performed using a rabbit polyclonal anti-A56 antibody and the mouse monoclonal anti-VCP antibody 3D1 (48).

Infection of cells for FACS or immunofluorescence

For VACV and ECTV infection of cells for cell sorting, RK-13 cells were infected in wells of a 6-well plate for 24 hours with ectromelia, vv-VCPwt, and vv-VCPko. The cells were then collected, transferred to FACS tubes, and stained with a rabbit anti-VCP Ab and the mouse anti-A56 mAb as described above. For immunofluorescent images of MPXV infected cells, BSC-40 cells in eight-well chamber glass slide (Nunc) at ~50% confluency were infected with 1 pfu/cell of either VACV, MPXV-USA, or MPXV-Congo and incubated for 18 hours in 6% CO_2 incubator at 35.5^{o}C. After a PBS wash, the cells were fixed for 30 min with 2% PFA in PBS, followed by washes and blocking with 5% BSA in PBS for 1 hr. 30 min at room temperature (RT). Cells were then stained for 2 hours at RT with anti-VCP mouse monoclonal antibody 2E5 at a 1:40 dilution in 5% BSA. After PBS washes, wells were incubated in the dark for 2 hr. at RT with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) at 1:200 in 5% BSA. Wells were washed,

mounted in VECTASHIELD hard set mounting medium containing DAPI, and images were taken at X400 magnification under oil immersion.

Mice for intranasal and intradermal infections

For all mouse experiments, 6-8 week old female C57BL/6 mice were purchased from the Jackson Laboratory and housed in a specific pathogen-free facility at the University of Pennsylvania. For intranasal infections, mice (N=5 per group) were infected with 10^3, 10^4, or 10^5 pfu of the indicated viruses and weight loss was measured as has been previously described (140). For intradermal infections, groups of mice were anesthetized and inoculated in both ear pinnae with 10 μl of the indicated virus diluted to $2x10^3$ pfu/μl in PBS ($2x10^4$ pfu/ear) using a 29-gauge insulin syringe (BD). Mice were monitored for lesion development and lesions greater than 1 mm in diameter were measured using digital calipers. Experiments were conducted in accordance with the guidelines of the University of Pennsylvania Institutional Animal Care and Use Committee. Statistical analysis was performed using Prism. Unpaired Student's T tests were used to compare differences in weight or lesion size between two groups, and a two-way ANOVA was used to compare differences in lesion size between three groups.

Results

VCP cell surface expression is dependent on A56 and does not require other viral proteins

Previously, our lab showed that expression of VCP on the infected cell surface is mediated by an interaction with the viral A56 protein and requires a free N-terminal cysteine on VCP (36). To investigate if A56 is sufficient for VCP surface expression, we transiently transfected cells with plasmids expressing wild type VCP (VCPwt), a mutated VCP lacking the free N-terminal cysteine (VCPmut), and wild type A56 (A56wt). We found that when VCPwt is transfected into 293T cells, it is not expressed on the cell surface (Fig. 4.1A). However, when VCPwt and A56wt are co-transected, VCP is expressed on the surface of cells that are also A56 positive (Fig. 4.1B). Importantly, cell surface expression is lost when VCPmut is transfected with A56wt (Fig. 4.1C), indicating that the transient transfection system reproduces what we saw in cells that were infected with viruses expressing either VCPwt or VCPmut (36). In transfected cells that are A56 positive, a 2-$\log_{10}$ shift in mean fluorescence intensity (MFI) can be seen between VCPwt and VCPmut (Fig. 4.1D).

In native western blots, A56 is found as a monomer and a higher molecular weight band ((131) and data not shown). The existence of a strong monomer band suggests that a surplus of A56 exists relative to the amount of K2 and VCP on the surface of cells. To begin to assess how much VCP is bound toA56, a recombinant VACV expressing a TAP-tagged version of A56 (131) was used to co-precipitate VCP. We found that when tagged A56 was pulled down with Streptavidin beads, VCP co-precipitated (Fig. 4.1E). However, only a small amount of VCP is present relative to the amount of A56-TAP and to the proteins from the input infected cell lysates. This suggests that a surplus of A56 exists relative to the amount of VCP on the surface of cells.

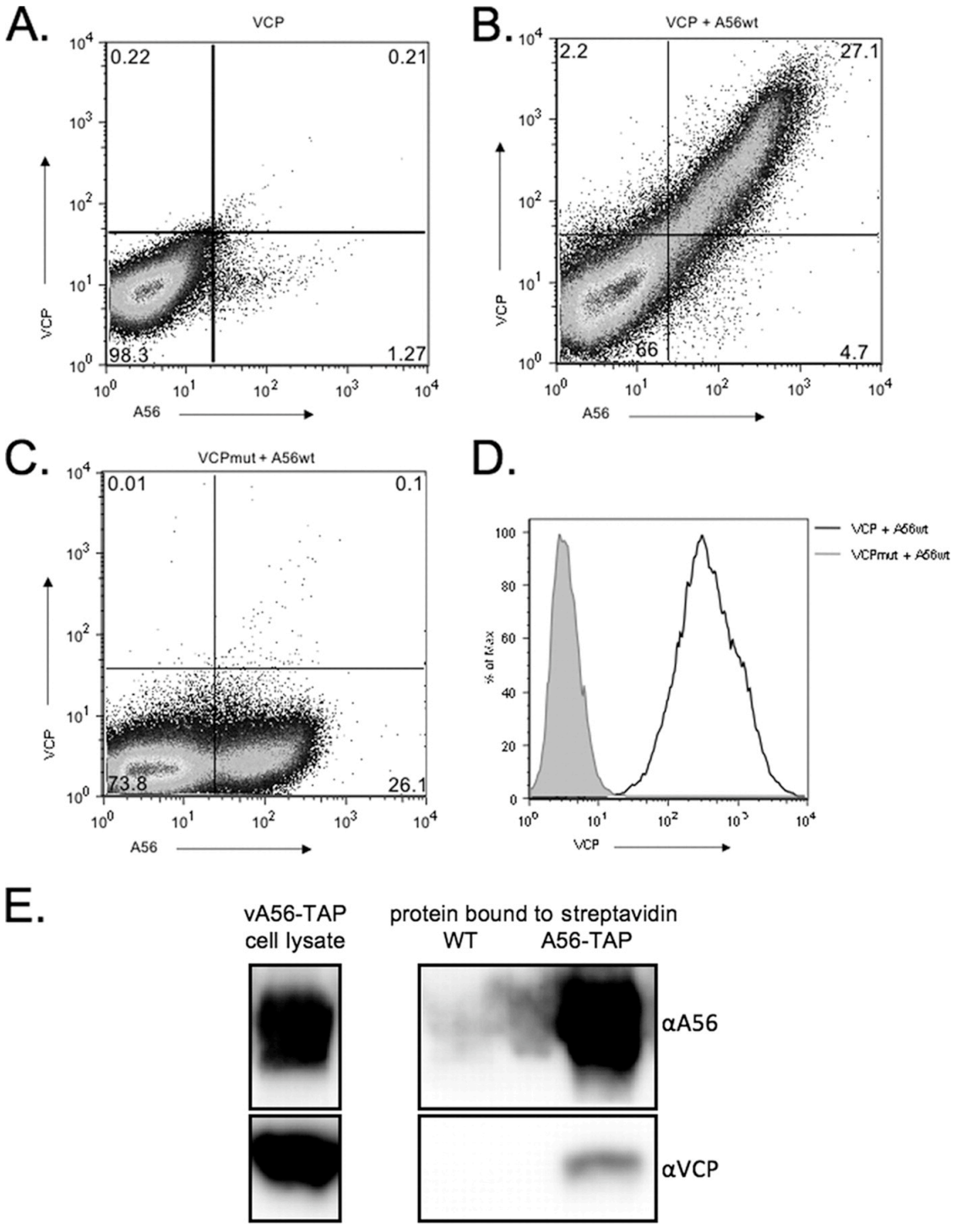
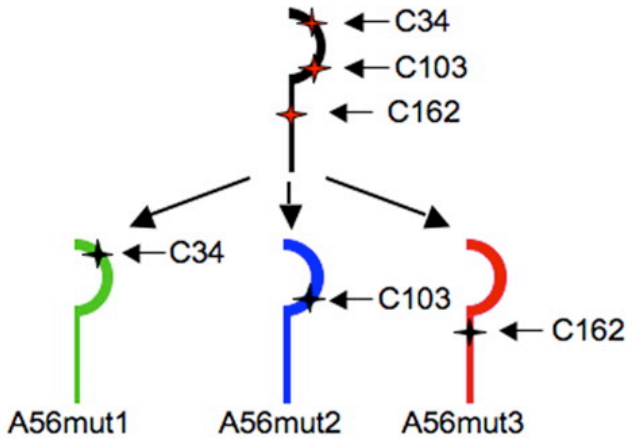

A.
VCP
0.22
0.21
98.3
1.27
VCP
A56
B.
VCP + A56wt
2.2
27.1
66
4.7
VCP
A56
C.
VCPmut + A56wt
0.01
0.1
73.8
26.1
VCP
A56
D.
VCP + A56wt
VCPmut + A56wt
% of Max
VCP
E.
vA56-TAP
cell lysate
protein bound to streptavidin
WT
A56-TAP
αA56
αVCP

Fig. 4.1: VCP and A56 interact on transfected and infected cells. VCP, VCPmut, and A56wt were transfected into 293T cells either alone or in combination. 48 hours later, the cells were collected and stained under nonpermeablized conditions for FACS staining with an anti-VCP polyclonal antibody and anti-56 mAb LC10. A) VCP is not expressed on the cell surface when transfected alone. B, C) VCP, but not VCPmut, is expressed on the cell surface in the presence of A56. D) Levels of VCP expression on A56 positive cells. Black solid line represents cells transfected with VCP and A56wt; gray shaded area is cells transfected with VCPmut and A56wt. E) VCP is present after pulling down A56-TAP with streptavidin from cells infected with vA56TAP. Western blots were probed with anti-A56 and anti-VCP antibodies to show the presence of the indicated proteins. Boxed areas on the left represent blots of the input cell lysate from vA56TAP infected cells. Boxed areas on the right represent blots of the proteins pulled down with streptavidin from cells infected with virus expressing untagged A56 (WT) or from cells infected with virus expressing TAP-tagged A56 (A56-TAP).

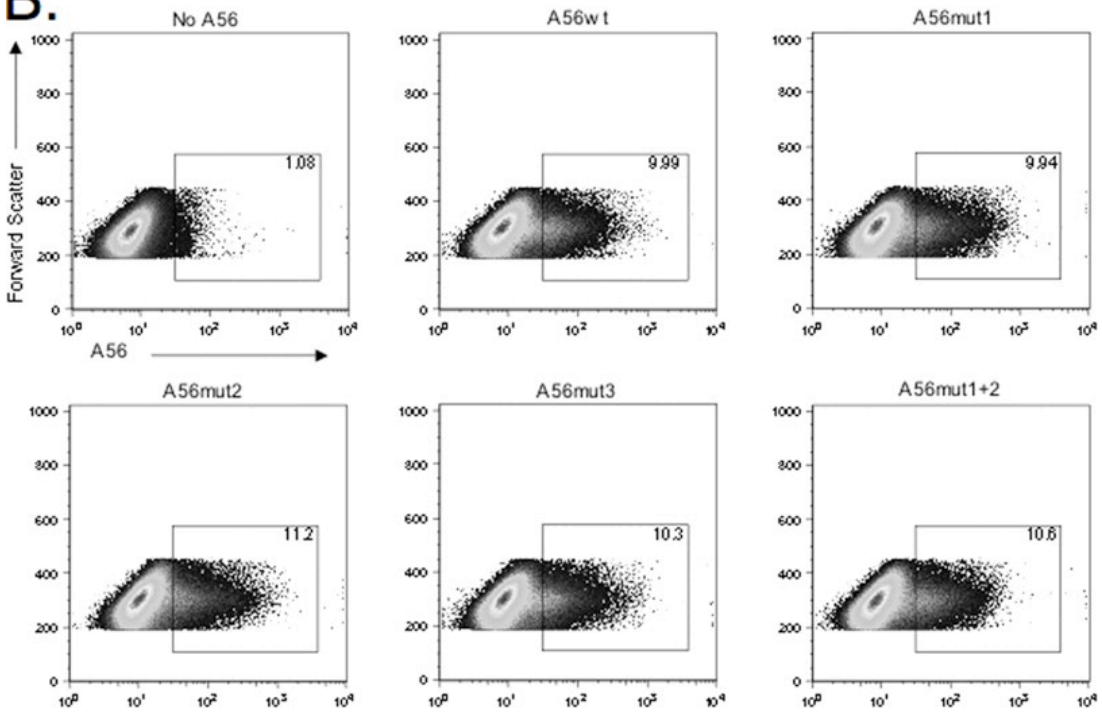
Cysteine 162 of A56 is required for VCP cell surface expression

The fact that VCP required its N-terminal cysteine to bind A56 suggested to us that either a disulfide bonded VCP homodimer (68) interacted with A56 to allow surface expression or the free cysteine on monomeric VCP formed a disulfide bridge with an unpaired cysteine in the A56 ectodomain. To investigate which model best explained the interaction of VCP with A56, we mutated each of the three cysteine residues in the ectodomain of A56. Each cysteine was mutated to a serine residue and the resulting expression plasmids were named A56mut1, A56mut2, and A56mut3, respectively (Fig. 4.2A). If an intermolecular bridge was required for surface expression of VCP, the loss of VCP's cysteine partner on A56 would prevent VCP expression on the cell surface. While the anti-A56 mAb LC10 used in Figure 1 could detect A56mut3, it no longer recognized A56mut1 and A56mut2 (data not shown) indicating that the mAb LC10 recognizes a conformation epitope that is lost when either Cys34 or Cys103 is mutated. However, when we used a rabbit polyclonal Ab raised to a peptide in the A56 ectodomain (131), all 3 mutants were recognized and were expressed at the cell surface at levels similar to A56wt (Fig. 4.2B). This indicates that the conformational epitope lost in A56mut1 and A56mut2 did not result in drastic misfolding and loss of the trafficking of A56muts to the cell surface. Each of the mutants was examined for their ability to interact with VCP and result in surface expression of VCP. When VCP was co-transfected with either A56mut1 or A56mut2, the level of VCP cell surface expression was similar to cells co-transfected with A56wt (Fig. 4.2C). A double mutant

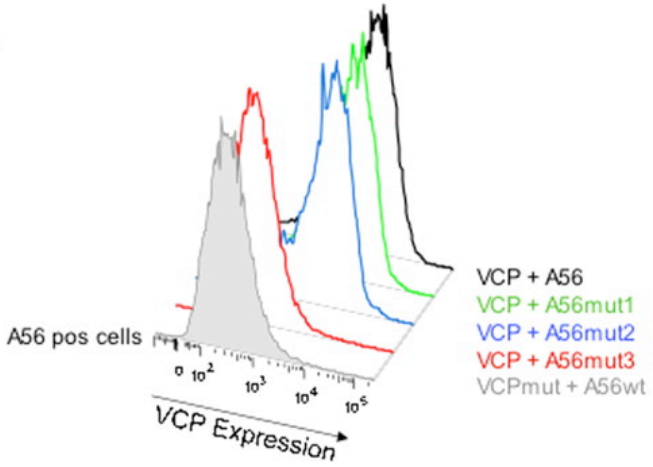
A.



B.



C.



Group	MFI
VCP/A56	4,200
VCP/ A56mut1	3,708
VCP/ A56mut2	3,384
VCP/ A56mut3	428
VCPmut/ A56wt	358

Fig. 4.2: A56 mutagenesis and VCP surface expression. A) Schematic of the cysteine pattern in the ectodomain of A56 and the three cysteine mutant A56 proteins. B) Expression levels of A56wt vs. A56mut1, A56mut2, A56mut3, and A56mut1+2. C) Cells positive for A56 expression were gated and analyzed for VCP expression. Mean fluorescence Intensity (MFI) levels are listed in the table to the right of the figure. VCP was co-transfected with the indicated A56 construct and stained with anti-VCP mAb 3F11 and the anti-A56 rabbit Ab and appropriate secondary Abs, and analyzed.

(A56mut1+2) is also able to interact with VCP and result in surface expression at levels similar to A56wt (data not shown). These results indicate that Cys34 and Cys103 are not required for VCP to interact with A56. However, when cells are co-transfected with A56mut3, VCP is not found on the cell surface, and has an expression profile similar to VCPmut + A56wt (Fig. 2C). This demonstrates that surface expression is lost even when the free N-terminal cysteine on VCP is present and homodimers can form. Taken together, these experimental results support a model in which cell surface expression of VCP is dependent on formation of a disulfide bridge between VCP and A56.

A56mut3 retains the ability to bind K2

It is possible that mutating Cys162 of A56 could cause misfolding of the protein, resulting in the lack of VCP interaction with A56mut3. Therefore, we examined the ability of A56mut3 to interact with the viral K2 protein, which binds to A56 non-covalently. As was previously been shown by the Moyer group (125), transfecting K2 alone does not result in cell-surface expression of K2 (data not shown). Also as previously reported (125, 132), we found that K2 is expressed on the surface of cells in the presence of A56wt (Fig. 4.3). We also found that in the presence of A56mut3, K2 is still expressed on the surface of cells at the same level as with A56wt. These data suggest that mutating cys162 specifically abrogates the A56-VCP interaction without affecting the interaction between A56 and K2.

The variola virus VCP ortholog binds the vaccinia A56 protein

The unpaired N-terminal cysteine in VCP is conserved in many VCP orthologs, including

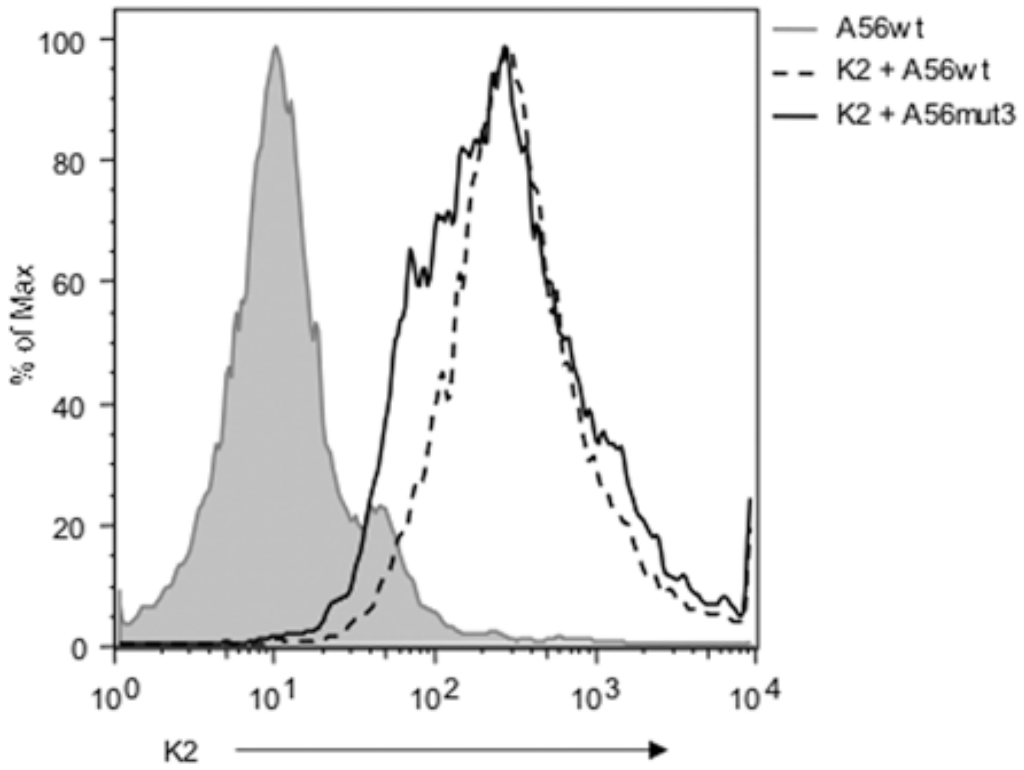


Fig. 4.3: A56mut3 is able to bind K2. 293T cells were transfected with A56wt alone, or with K2 plus A56wt and A56mut3. The cells were then stained with anti-K2 mAb 4A11-4A3 and the rabbit anti-A56Ab. A56 positive cells were gated on and used to create a histogram.

the CCP proteins expressed by variola virus (smallpox inhibitor of complement enzymes (SPICE)) and ectromelia virus (ectromelia inhibitor of complement enzymes (EMICE)). The monkeypox inhibitor of complement enzymes (MoPICE) has lost this N-terminal cysteine, but due to a truncation right after the start of the fourth SCR it has an unpaired cysteine near its C-terminus (19, 68). Additionally, all of these viruses contain a homolog to the vaccinia A56 protein, and interestingly, all of these A56 orthologs retain the 3 extracellular cysteines (Fig. 4.4). Therefore, we hypothesized that the interaction between A56 and poxvirus CCPs may not be limited to VACV. To test this, we first studied the ability of SPICE to interact with VACV A56. We found that SPICE bound to vaccinia A56 (Fig. 4.5A). We also found that SPICE, like VCP, is not expressed on the cell surface when co-transfected with A56mut3 (Fig. 4.5B). This suggests that SPICE and A56 can interact on the cell surface through a disulfide bridge at Cys162 similar to VCP and A56.

EMICE, but not MoPICE, binds to its cognate A56 homolog efficiently

While VCP and SPICE have identical first SCR domains, EMICE has 12 amino acid differences in its first SCR. Co-transfection of EMICE with vaccinia A56 produced weak EMICE surface staining (Fig. 4.5C). However, staining of cells infected with ECTV showed that surface staining of EMICE was clearly evident (Fig. 4.5D) and that EMICE expression was similar to VCP expression on vaccinia infected cells (Fig. 4.5D). This suggested to us that EMICE can interact with its A56 cognate, but not vaccinia A56. To address this possibility, we cloned the ECTV homolog of A56 (ECTV-A56) and co-

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VACV  1    MTRLPILLLLISLVYATPFPQT--SKKIGDDATLSCNRNNTNDYVVMSAWYKEPNSIILLAAKSDVLYFDNYTKDKISYD  78
VARV  1    MTRLSILLLLISLVYSTPYPQIQISKKIGDDATLSCSRNNINDYVVMSAWYKEPNSIILLAAKSDVLYFDNYTKDKISYD  80
MPXV  1    MTQLPILLLLISLVYVTPSPQT--SKKIGDDATISCSRNNTNYYVVMSAWYKEPNSIILLAAKSDVLYFDNYTKDKISYD  78
ECTV  1    MARLSILLLLISLVYATPYPQTQISKKIGDDATISCSRNNTTDYVVMSAWYKEPNSIILLAAKSDVLYFDNYTKDKISYD  80

VACV 79    SPYDDLVTTITIKSLTARDAGTYVCAFFMTSTTNDTDKVDYEEYSTELIVNTDSESTIDIILSGSTHSPETSSKKPDYID  158
VARV 81    SPYDDLVTTITIKSLTAKDAGTYVCAFFMTSTTNDTDKVDYEEYSTELIVNTDSESTIDIILSGSSHSPETSSEKPDYIN  160
MPXV 79    SPYDDLVTTITIKSLTAGDAGTYICAFFMTSTTNDTDKVDYEEYSIELIVNTDSESTIDIILSGST--PETISEKPEDID  156
ECTV 81    SPYDDLVTTITIKSLTAGDAGTYVCAFFMTSTTNDTDKVDYEDYSTELIVNTDSESTIDIILSGSTHSPETSSEKPDYID  160

VACV 159   NSNCSSVFEIATPEPITDNVEDHTDTVT--YTSDSINTVSASSGESTTDETPEPITDK-EDHTVTDTVSYTTVSTSSGIVTT 237
VARV 161   NFNCSLVFEIATPGPITDNVENHTDTVT--YTSDIINTVSTSSGESTTDKTSGPITNK-EDHTVTDTVSYTTVSTSSEIVTT 239
MPXV 157   NSNCSSVFEIATPEPITDNVEDHTDTVT--YTSDSINTVNASSGESTTDETPEPITDKEEDHTVTDTVSYTTVSTSSGIVTT 236
ECTV 161   NSNCSSVFEIATPEPI--------------------------------------TDK-EDHTVTDTVVYTTVSTSS-VVTT 201

VACV 238   KSTTDDADLYDTYNDND--TVPPTTVGGSTTSISNYKTKDFVEIFGITALIILSAVAIFCITYYIYNKRSRKYKTENKV   314
VARV 240   KSTANDA-----HNDNEPSTVSPTTVKNITKSIGKYSTKDYVKVFGIAALIILSAVAIFCITYYICNKRSRKYKTENKV   313
MPXV 237   KSTTDDADLYDTYNDND--TVPPTTVGGSTTSISNYKTKDFVEIFGITTLIILSAVAIFCITYYICNKHPRKYKTENKV   313
ECTV 202   KSTTDDADLYDTYNDNESSTVLPTTVESITKSIGKYSTKDFAEIFGITALIILSAVAVFCITYYICNKRSRKYKTENKV   280
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Fig. 4.4: Comparison of Orthopoxvirus A56 genes. The protein sequences of vaccinia (VACV-WR) A56 and the homologs in variola (VARV-Bangladesh), monkeypox (MPXV-Zaire), and ectromelia (ECTV-Moscow) were downloaded from poxvirus.org and aligned using BLAST. Shaded residues indicate identical amino acids to vaccinia and dashes represent missing amino acids. Conserved cysteines in the ectodomain are indicated in bold and with an arrow. The putative transmembrane domain is marked with a black line above the residues.

transfected it with EMICE. We found that while VACV-A56 and ECTV-A56 were expressed on approximately the same number of cells (19.2% vs. 19.8%), EMICE is expressed to a much higher level at the cell surface when co-transfected with ECTV-A56 (Fig. 4.5E). The peak level of EMICE staining on cells transfected with ECTV-A56 was 2-logs higher than EMICE transfected in cells with VACV-A56 or VACV-A56mut3 (Fig. 4.5F). This shift in staining mirrors the expression levels of VCP when it is transfected with vaccinia A56.

The monkeypox homolog of VCP, MoPICE, differs from these other poxvirus CCPs in several respects. As has been reported elsewhere, MoPICE does not have a free N-terminal cysteine, as this initial residue is a tyrosine (68). Also, MoPICE only contains 3 SCRs due to a point mutation that results in a truncation after the start of the fourth SCR. Interestingly, this truncation results in an unpaired cysteine near the C-terminus through which MoPICE can form homodimers (20). Immunofluorescence of cells infected with MPXV-Congo shows weak cell surface staining of MoPICE when compared to vaccinia-infected cells (Fig. 4.6A). Consistent with the lower level of surface expression compared to VCP expression on vaccinia infected cells, co-transfection of MPXV A56 with MoPICE also results in low level surface expression of MoPICE (Fig. 4.6B, C), with only 2.48% of cells double positive. These data suggest that MoPICE is not expressed at high levels on infected or transfected cells. Taken with the EMICE data, it appears that the first SCR domain with an N-terminal free cysteine is crucial for optimal binding to A56.

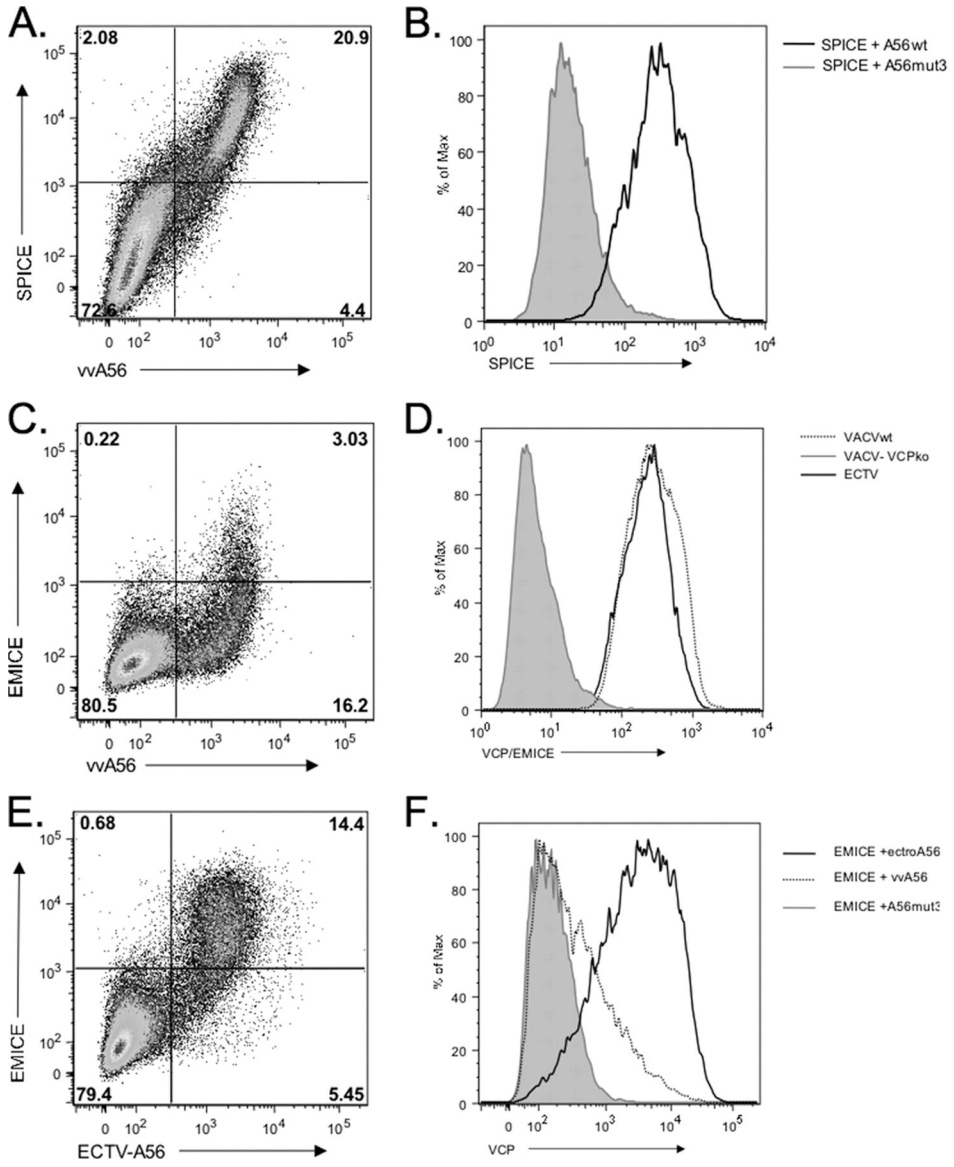

Fig. 4.5: SPICE and EMICE are also expressed on the cell surface in the presence of A56. SPICE is expressed on cells co-transfected with vaccinia A56wt. A) Scatter plot of SPICE co-transfected with A56wt. B) Histogram of SPICE expression on A56 positive cells. The black line is SPICE transfected with vaccinia A56wt, and the shaded grey area is SPICE transfected with A56mut3. C) Scatter plot of EMICE transfected with VACV A56. D) Histogram of CCP expression on virus infected cells. RK-13 cells were infected with ECTV, VACV, or vv-VCPko and then stained with a polyclonal anti-VCP antibody. Shown is a histogram of surface staining due to EMICE (black line), VACV (dotted line) or vv-VCPko (grey shaded area). E) Scatter plot of EMICE co-transfected with ECTV A56. F) Histogram of EMICE expression of cells co-transfected with ECTV A56 (black line), VACV A56 (dotted line), and VACV A56mut3 (grey shaded area).

Vaccinia expressing VCPmut is attenuated in vivo

The N-terminal cysteine residue in VCP is conserved among other PICEs and has been shown to mediate both cell surface expression and dimerization of VCP (36, 68). Both cell surface expression and dimerization of recombinant VCP have been shown to enhance its complement regulatory function in vitro (68). In order to determine if cell surface expression and/or dimerization contribute to VCP's function as a virulence factor, we assessed the virulence of our recombinant VACVs using two mouse models of VACV pathogenesis (Fig. 4.7). We first compared vv-VCPmut to vv-VCPko and vv-VCPrescue after intranasal inoculation. At the lowest dose (10^3 pfu), all of the mice survived, but mice infected with vv-VCPrescue lost slightly more weight (Fig. 4.7A). The difference in weight loss between vv-VCPrescue and the other two viruses was significant at days 7 and 8. At the intermediate dose (10^4 pfu), all of the mice infected with the VCP rescue virus died, while mice infected with the VCPko and VCPmut mice lost approximately the same amount of weight (Fig. 4.7B). At a high dose (10^5 pfu), all of the animals lost weight at a similar rate and died (data not shown). These experiments show that after intranasal inoculation a virus expressing wild type VCP is more pathogenic than viruses with VCP deleted or ones lacking the N-terminal cysteine on VCP (and thus unable to form homodimers and/or be expressed on the cell surface). Interestingly, by this route of infection, vv-VCPko and vv-VCPmut have similar virulence. We also examined pathogenesis of vv-VCPko and vv-VCPmut using an intradermal model of infection (122, 123). We initially showed that vv-VCPko made smaller ear lesions than vv-VCPwt (and vv-VCPrescue; Fig. 4.7C). We then infected mice intradermally with a recombinant VACV encoding VCP lacking the free N-terminal cysteine (vv-VCPmut) and compared

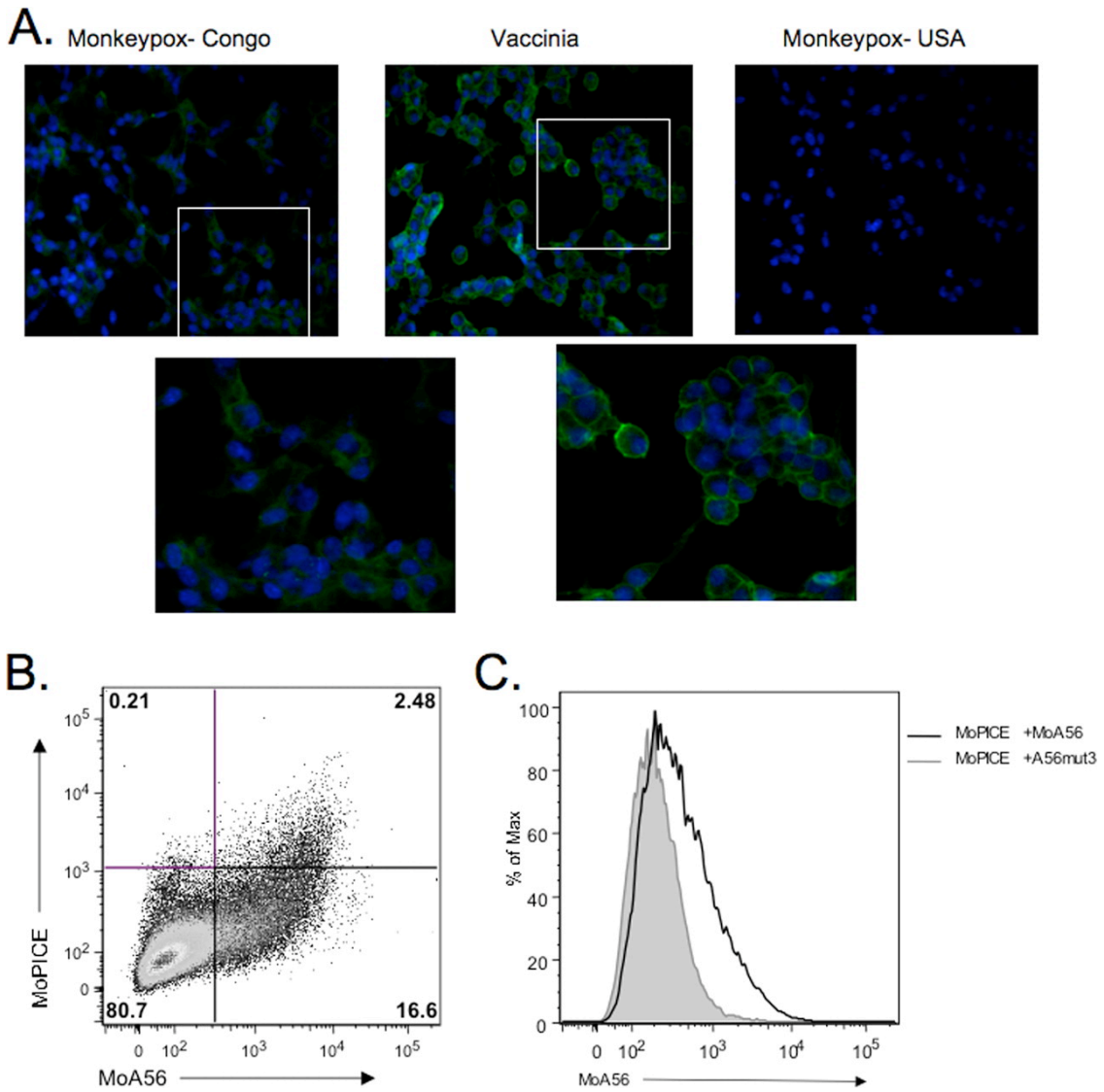

Fig. 4.6: MoPICE is expressed on the cell surface at low levels. A) Immunofluorescence of infected cells. BSC-40 cells were infected for 18 hrs with MPXV-Congo, VACV, or CCP-minus strain of MPXV, MPXV-USA, and fixed and stained using anti-VCP mAb 2E5 and fluorescent secondary Ab. To better visualize the cell distribution of VCP or MoPICE, the white box indicates the area of the image that was enlarged, but unaltered. B) Scatter plot of MoPICE co-transfected with MPXV A56. C) Histogram of MoPICE expression on A56-positive cells. MoPICE was co-transfected with MPXV-A56 (black line) or VACV A56mut3 (grey shaded area) and cells were then stained for FACS with polyclonal rabbit anti-VCP and anti-A56 mAb.

lesion sizes formed by this virus to those formed by a virus encoding wild type VCP (vv-VCPwt) or a virus that lacks VCP (vv-VCPko) (Fig. 4.7D). The lesions formed following infection with vv-VCPmut were larger than those formed by vv-VCPko, but smaller than those formed by vv-VCPwt. These differences in lesion sizes were statistically significant. The finding that vv-VCPko is attenuated following intradermal infection in mice confirms that VCP contributes to pathogenesis and is similar to what was reported in the past in rabbits and guinea pigs (50). Furthermore, as opposed to the intranasal route of inoculation where VCPko and VCPmut were similarly attenuated, by the intradermal route vv-VCPmut formed lesions of intermediate size between vv-VCPko and vv-VCPwt. This suggests that the ability of VCP to bind to the cell surface and/or dimerize contributes to pathogenesis.

Discussion

VCP has been previously characterized as the major protein secreted from VACV infected cells (57, 59) and has only recently found to also be expressed on the infected cell surface (36, 57, 59). The N-terminal cysteine of VCP is necessary for surface expression; however, the precise mechanism by which VCP interacted with A56 was unknown. We have found that the primary method for VCP surface expression is through a disulfide bridge with Cys162 of the viral A56 protein. Mutation of A56 Cys34 and/or Cys103 results in loss of recognition by an anti-A56 monoclonal antibody LC10. However, despite the loss of recognition by this antibody, these mutated proteins can still traffic to the cell surface and interact with VCP. Our work therefore also provides important information about A56. While cysteines 34 and 103 of A56 are unnecessary

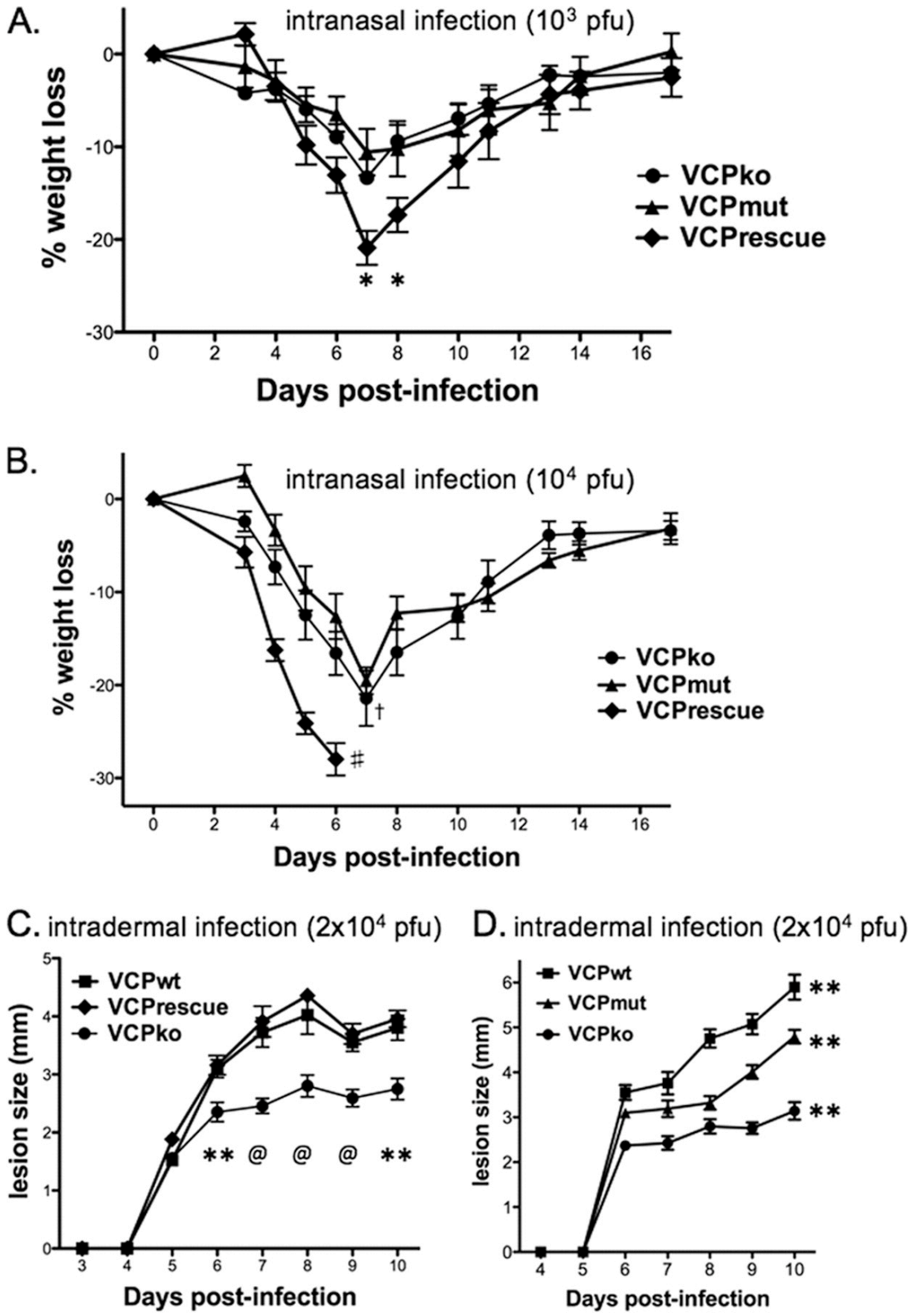
A.
intranasal infection (10³ pfu)
% weight loss
Days post-infection
VCPko
VCPmut
VCPrescue
B.
intranasal infection (10⁴ pfu)
% weight loss
Days post-infection
VCPko
VCPmut
VCPrescue
C. intradermal infection (2x10⁴ pfu)
lesion size (mm)
Days post-infection
VCPwt
VCPrescue
VCPko
D. intradermal infection (2x10⁴ pfu)
lesion size (mm)
Days post-infection
VCPwt
VCPmut
VCPko

Fig. 4.7: Wild type VCP is needed for full virulence in mice. Our panel of recombinant viruses was studied in 6-8 week old female C57BL/6 mice either after intranasal (A & B) or intradermal (C & D) inoculation. In A & B, shown is the average weight loss of 6-week old mice infected with vaccinia encoding VCPrescue, VCPmut, or VCPko at 10^3 pfu (A) or 10^4 pfu (B). N=5 for all groups. # sign indicates that all mice infected with vv-VCPrescue died; † sign indicates a single mouse in the group infected with vv-VCPko died. * indicates $p<0.05$ on the days indicated between VCPrescue and VCPko and between VCPrescue and VCPmut (unpaired Student's T test). In C & D, shown are lesion diameters in 6-8 week old mice inoculated with $2x10^4$ pfu of C) VCPrescue, VCPwt, and VCPko and D) VCPwt, VCPko and VCPmut. For C, data points represent the mean lesion diameter ± SEM of 10 infected ears per group (two ears per mouse). P values for the difference between VCPko and VCPwt and between VCPko and VCPrescue for each day are as indicated: * for $p<0.05$, ** for $p<0.01$, and @ for $p<0.001$ (unpaired Student's T test). For D, data points represent the mean lesion diameter ± SEM of 20 (VCPko), 14 (VCPwt), or 10 (VCPmut) infected ears per group (two ears per mouse). Differences in lesion size between the groups of mice were statistically significant (two-way ANOVA; $p < 0.01$).

to bind VCP, they may play a role in the folding of A56. The N-terminal domain of A56 has homology to the Ig superfamily, which is heavily disulfide bonded. It is possible that the first two cysteines in A56 form an intramolecular bridge that when mutated, alters an epitope recognized by mAb LC10. But the mutated protein still maintains sufficient folding to allow the protein to properly traffic to the cell surface.

VCP forms a disulfide bond with Cys162 of A56 and we have shown that this specific interaction also occurs with other poxviruses proteins, like SPICE. Soluble SPICE is capable of the same catalytic activity as VCP, but with a 100-1000 fold increase in activity against human complement (69, 98, 107). Therefore, it is interesting to speculate that expression of SPICE on the infected cell surface may have contributed to its significant pathogenesis in humans. Another significant finding is the interaction between the ectromelia homolog of VCP and A56. EMICE, in contrast to SPICE, is only able to interact efficiently with its own homolog of A56. This raises the intriguing possibility that co-evolution has occurred in these two proteins, as EMICE and ectromelia A56 are less similar to the vaccinia proteins than their variola counterparts. Conversely, the lack of high surface expression of MoPICE may mean that efficient cell surface expression of MoPICE is not possible without an N-terminal cysteine, even in the presence a C-terminal free cysteine. This is interesting because it has been shown that this free C-terminal cysteine can form homodimers in a similar fashion as the orthologs with a free N-terminal cysteine (68).

We have also shown that a virus that cannot express cell surface VCP is modestly attenuated in vivo. This finding, combined with earlier in vitro data showing that compared to cells infected with a VCP-deletion virus, wild type vaccinia infected cells

are resistant to complement mediated lysis (36), suggests that the ability of VCP to interact with A56 and be expressed on the cell surface may have important implications for poxvirus pathogenesis. After ear pinnae infection, VCPmut formed larger lesions than VCPko. This is not entirely unexpected since the majority of VCP is a secreted monomer. Therefore, when VCP is mutated so that it cannot form homodimers and/or be expressed on the cell surface, VCP still has complement regulatory activity. However, since mutating the N-terminal cysteine also abrogates dimerization of VCP, the contribution of secreted VCP homodimers to the attenuated lesion size we observed is not known. Our biochemical analysis of the interaction between VCP and A56 has identified a mutation in A56 that disrupts the A56-VCP interaction without disrupting the A56-K2 complex. Comparing pathogenesis of vv-VCPmut with a recombinant virus expressing A56mut3 will allow us to define the roles of surface bound VCP and dimeric VCP in poxvirus pathogenesis. As opposed to the intradermal route of infection where we found statistically significant differences between lesions formed by VCPko and VCPmut, these viruses appeared to be similarly attenuated after intranasal inoculation of mice. This may indicate that in a lung pneumonia model, the contribution of cell surface expression and/or the formation of the VCP homodimer is important for VCP's contribution to pathogenesis. Again, work with a recombinant virus expressing A56mut3 will allow us to define the roles of surface bound VCP and dimeric VCP in poxvirus pathogenesis.

CHAPTER FIVE:

Surface expression of the vaccinia virus complement control protein is important for full viral virulence

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Manuscript in preparation

Abstract

The vaccinia virus complement control protein (VCP) is both secreted from and expressed on the surface of infected cells. Surface expression of VCP is due to an intermolecular di-sulfide bond formation with the viral transmembrane glycoprotein called A56. The covalent interaction of these two proteins require a free N-terminal cysteine on VCP and an unpaired cysteine in the ectodomain of A56 at amino acid 162. Here, we generated and characterized a novel recombinant vaccinia virus that contains a cysteine to serine mutation at residue 162 in A56 (vvA56mut3). We show that the mutated A56 surface expression is unaffected on cells infected with vvA56mut3, as well as the ability of A56 interact with K2 and inhibit syncytia formation. However, VCP is no longer expressed on the cell surface. In vivo, when vvA56mut3 is compared to its parental virus (vvA56ko) and the rescue virus (vvA56rescue), vvA56mut3 is attenuated and this attenuation is dependent upon the presence of the host complement system. These results indicate that the ability of A56 to bind VCP and express it on the surface of infected cell is important for full vaccinia virus virulence.

Introduction

Poxviruses are some of the largest and most complex of all viruses. Vaccinia virus (VACV), the most studied and prototypical poxvirus, has a ~190 kb long genome (38). Besides genes necessary for viral replication, poxvirus genomes contain a large number of non-essential genes. Many of these are needed to replicate in certain cell types (i.e., host range genes) or increase pathogenesis in vivo (i.e., virulence genes) (27, 73, 95). One non-essential VACV gene is in the open reading frame (ORF) A56R, which encodes a protein initially called the VACV hemagglutinin (46, 87). This protein is now more

commonly called A56. A56 is a glycosylated transmembrane protein that is expressed on the surface of infected cells as well as on an infectious form of VACV called the extracellular virus (EV) (11, 94, 109). Although it was initially characterized as a viral hemagglutinin due to its ability to bind chicken erythrocytes, the important functions of A56 during viral infection are unrelated to its hemagglutinating activity. A56 binds two viral proteins and allows them to be expressed at the infected cell surface. A56 interacts noncovalently with K2 (125). This A56/K2 complex interacts with the VACV entry/fusion complex to prevent syncytia formation and superinfection of infected cells (128, 131-133). K2, which is also called serine protease inhibitor (SPI)-3, is a soluble protein that is dependent on A56 for surface expression. While the serine protease inhibitor activity is not required for prevention of infected cell synctia formation (125), both A56 and K2 must be present at the infected cell surface to prevent syncytia formation (47, 62, 127, 147) and superinfection (128, 132).

A56 also forms a heterodimer with the vaccinia complement control protein (VCP) (36). While A56 interacts non-convalently with K2, the A56-VCP interaction results in a covalent bond between unpaired cysteines present in both proteins (23). VCP is related to the superfamily of host regulators of complement activation and is able to disrupt the complement cascade at multiple steps (9, 68, 69, 76, 86, 97, 98, 100), and has been shown to be a virulence factor during in vivo infections (23, 50). VCP was initially characterized as the major protein secreted from infected cells (59), and most of its complement regulatory activity has been examined as a purified soluble protein. However, surface bound VCP can protect infected cells from complement attack (36),

and recent evidence suggests that surface expression is necessary for full virulence in vivo (23, 36).

Studies of A56's contribution to VACV virulence have been inconsistent. In some models, A56 knockout viruses are attenuated relative to wild-type viruses. Studies that showed attenuation in mice were with the NYCBH strain and WR strain given intracranially (31, 64). This attenuation with NYCBH strain was also seen after intranasal challenge (64). However, little or no attenuation in mice was seen using VACV strains LC16mO or LO given either by an intracranial or intraperitoneal route (112). In retrospect, this is not surprising given that we now know A56 has multiple functions, which may have varying importance to virulence depending on virus strain and the model of infection used. Here, for the first time, we attempt to separate the functions of A56. We show that a recombinant VACV containing an A56 protein that can bind K2 but not VCP is attenuated in vivo, using both mouse intranasal and intradermal models of infection. Importantly, using mice with the central complement component C3 genetically deleted, we also show that the attenuation of the mutant virus that does not express VCP on the surface of infected cells is complement-dependent.

Methods

Cells and Viruses

Viruses were grown and titered in BSC-1 cells (ATCC® Number CCL-26™) in MEM supplemented with 2.5% FBS and antibiotics/antimycotics at 37°C in a 5% CO_2 atmosphere. Plasmids were first constructed to allow the generation of recombinant vvA56mut3 and vvA56rescue. The plasmid containing the A56mut3 gene was created

using Splicing by Overlap Extension (SOE) PCR with primers that mutate the third cysteine of A56 (ol-205 and ol-206 (23)), paired with ones that anneal to the left and right flanking DNA adjacent to the A56R ORF (ol-156 and ol-159 (36)) These latter primers were then used to generate a single PCR product that contained the mutated A56R ORF along with flanking sequences. This PCR product was cloned into the TOPO 2.1 plasmid to generate pAS279 (pA56mut3). A56rescue was created by first digesting out bases 42-505 of the A56 ORF from pAS279 using the restriction enzymes NruI and BstZ17I and isolating a 463 bp fragment of A56 (bases 42-505 of the ORF). This DNA was used along with primers that annealed to the left and right flanking DNA adjacent to the A56R ORF (ol-156 and ol-159) and WR DNA as a template to generate two fragments. Primers ol-156 and ol-159 were then used to generate a single PCR product that contained the wild type A56R ORF along with flanking sequences. This product was cloned into the TOPO 2.1 plasmid to generate pBD281 (pA56rescue). The plasmids were sequenced to confirm that no unwanted mutations were accidentally generated during the PCR reactions and cloning. The plasmids then transfected into individual flasks containing CV-1 cells (ATCC CCL-70™) that had been infected with a previously described recombinant VACV lacking the A56R ORF (vvA56ko, (36)). vvA56ko had the entire A56R ORF replaced with a beta-glucuronidase (GUS) screening cassette (16). Fourty-eight hours after infection and transfection, the cells were harvested, lysed with freeze-thawing and sonication, and used to infect RK-13 cells (ATCC® Number CCL-37™) for a second round of growth. The lysate from this growth was then used to infect RK-13 cells and 24 hours after infection, infected cells underwent live-cell sorting to isolate cells infected with the recombinant viruses. Cells containing the A56 rescue virus

(vvA56rescue; virus # vBD219e4-1) were isolated by collecting cells with surface expression of VCP using an anti-VCP monoclonal antibody 3F11 (48). Cells containing the mutated A56 (vvA56mut3; virus #vBD217f2-1) were collected based on cell-surface A56 expression using the anti-A56 mAb LC10 (78). Collected cells were lysed and the released virus underwent three rounds of plaque purification in the presence of X-Gluc (16). During plaque purifications, any remaining parental virus (vvA56ko) plaques stained blue. By the third plaque purification, all plaques were white in the presence of X-Gluc indicating that the GUS cassette had been successfully replaced with A56wt or A56mut. Isolated viruses were confirmed by PCR. Virus from plaques were grown and amplified in BSC cells and virus used for animal challenge were pelleted through two sequential 36% sucrose cushions.

Antibodies and flow cytometry

Infected cells were studied by flow cytometry using methods similar to what were previously described (23). Briefly, RK-13 cells were infected in 6-well plates at an MOI of ~1 pfu/cell with the indicated virus. After 24 hours, cells were harvested in FACS buffer (PBS with 1% BSA and 0.04% sodium azide) and stained with primary antibodies at dilutions of 1:1000. Primary antibodies included an anti-K2 mouse mAb 4A11-4A3 (15), an anti-A56 rabbit polyclonal antibody raised to an A56 peptide (131), an anti-VCP mouse mAb 3F11 (48), and a polyclonal anti-B5 rabbit antibody (UP2197). After washing in FACS buffer for 45 minutes, an anti-mouse APC-conjugated secondary Ab and an anti-rabbit FITC-conjugated secondary Ab were added at dilutions of 1:40. After

30 minutes, the cells were washed, fixed in 3% paraformaldehyde, and loaded into a FACScaliber machine. Data analysis was performed via FlowJo.

Western blots

BSC-1 cells in a 6-well plate were infected with the indicated virus at an MOI of 5. After 24 hours, the cells were harvested and prepared in 100 μl of RIPA buffer (150 mM NaCl, 0.1% SDS, 50 mM Tris). 4× reducing/denaturing sample buffer was added to a 20 μl volume of each sample, and loaded onto a 10% Tris-Glycine gel. After transferring to nitrocellulose, the blot was blocked PBS/0.01% Tween with 10% milk fat, and then incubated overnight at 4 ^{o}C with shaking with an anti-A56 rabbit antibody (131) at a 1:5000 dilution. After washing in PBS-0.01% Tween, an anti-rabbit HRP-conjugated secondary Ab at 1:5000 was added for one hour. Chemiluminescence was used to visualize bands.

Animal infections

C57BL/6 mice were purchased from the Jackson Laboratory. Complement component C3 knockout mice (C3-/-) on the C57BL/6 background (originally provided by J. D. Lambris, University of Pennsylvania) were bred at the University of Pennsylvania. Mice were housed in a specific-pathogen-free facility at the University of Pennsylvania. For intranasal infections, mice were anesthesized and infected with ~10^4 PFU of the indicated viruses in 20 μl volume (10 μl/nostril), and weight loss was measured as has been previously described (23). For intradermal infections, groups of mice were anesthetized and inoculated in both ear pinnae with ~$2x10^4$ pfu of the indicated virus using a 29-gauge

insulin syringe (BD). Mice were monitored for lesion development, and when lesions reached a 1 mm in diameter threshold, they were measured using digital calipers. Experiments were conducted with the approval of the University of Pennsylvania Institutional Animal Care and Use Committee.

Results

Characterization of recombinant viruses

Previously, our lab has shown that the N-terminal cysteine (residue 20) on VCP (36) and the third extracellular cysteine of A56 (residue 162) (23) were necessary for surface expression of VCP. The prior data showing the importance of A56 cysteine-162 for VCP surface expression was based on the transfection of plasmids expressing the proteins in uninfected cells. To investigate whether this finding was also true during viral infection, we constructed a recombinant VACV that contained a cysteine to serine mutation at residue 162 (vvA56mut3). This virus was generated from an A56-knockout virus. As a control, we also made a rescue virus producing wild-type A56 (vvA56rescue). A56rescue contains the VCP-binding cysteine residue at position 162, while in A56mut3 this cysteine has been mutated to a serine (Fig. 5.1). As shown in western blots, these two new viruses, but not the parental A56ko virus, produce the A56 protein (Fig. 5.2A). Using flow cytometry of infected non-permeablized cells, both A56rescue and A56mut3 are able to bind K2 and express it on the cell surface at levels comparable to cells infected with a stock of wild type WR virus (Fig. 5.2B).

In contrast to the ability of A56 to bind K2, mutating the third cysteine of A56 abrogates its ability to bind VCP. VCP expression on infected cells is markedly lower

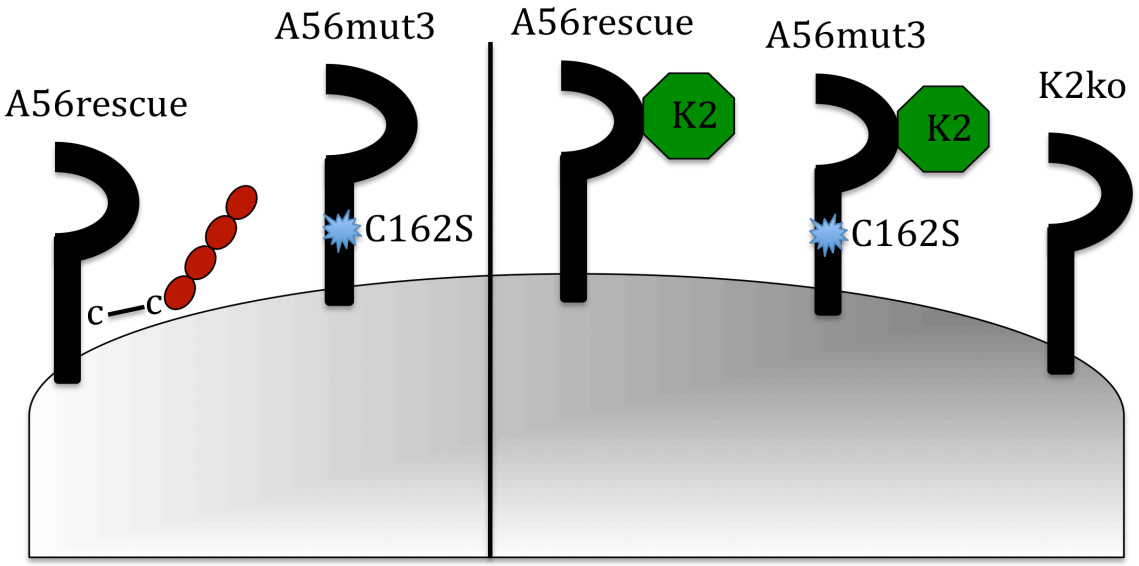


Fig. 5.1: Schematic of A56 proteins. The ectodomain of A56 contains an unpaired cysteine at residue 162. This cysteine can form a disulfide bridge with VCP (23). In the virus vvA56mut3, this cysteine has been mutated to a serine, abrogating surface expression of VCP. A56 and A56mut3 can also bind K2; we use a K2ko virus to remove the A56/K2 complex.

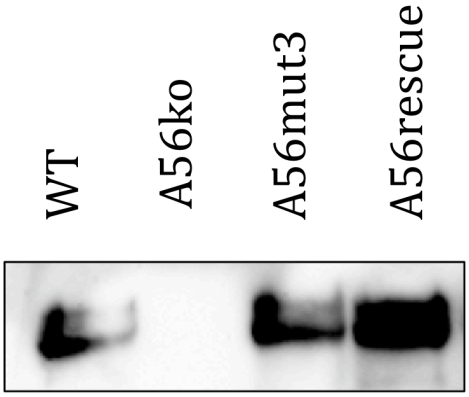
than the rescue virus (Fig. 5.2C). In fact, vvA56mut3 has approximately the same level of surface VCP as vvVCPmut (36) (Fig. 5.2D). In VCPmut, the N-terminal cysteine has been mutated to a threonine residue, so it cannot form dimers with either itself or A56. Interestingly, both vvA56mut3 and vvVCPmut have slightly higher levels of surface VCP expression than vvVCPko (Fig. 5.2D). Thus, in addition to the high level of surface expression due to interactions with A56, some VCP can also bind the RK cell surface via non-covalent interactions as suggested by others (68, 116).

A56mut3, but not A56ko, is attenuated in an intranasal mouse challenge model

We first examined virulence by infecting groups of mice intranasally with each virus. At a challenge dose of ~10^4 pfu per mouse, we found that vvA56rescue is as virulent as the previously characterized wild-type virus vvVCPrescue (23). We also found that in this challenge model, vvA56ko is not attenuated, as mice infected with this virus lose the same amount of weight as the two rescue viruses (Fig. 5.3). Somewhat surprisingly, given this finding with the A56ko virus, vvA56mut3 is attenuated in this model. We also saw a similar amount of attenuation with vvVCPmut in this system (data not shown). Mice infected with this virus lose less weight and this difference is statistically significant at the peak of infection. This suggests that an intranasal model 1), losing the VCP/A56 complex attenuates the virus, and 2) the loss of the A56/K2 complex may actually restore this virulence.

A56mut3 and A56ko are attenuated in an intradermal mouse challenge model

A



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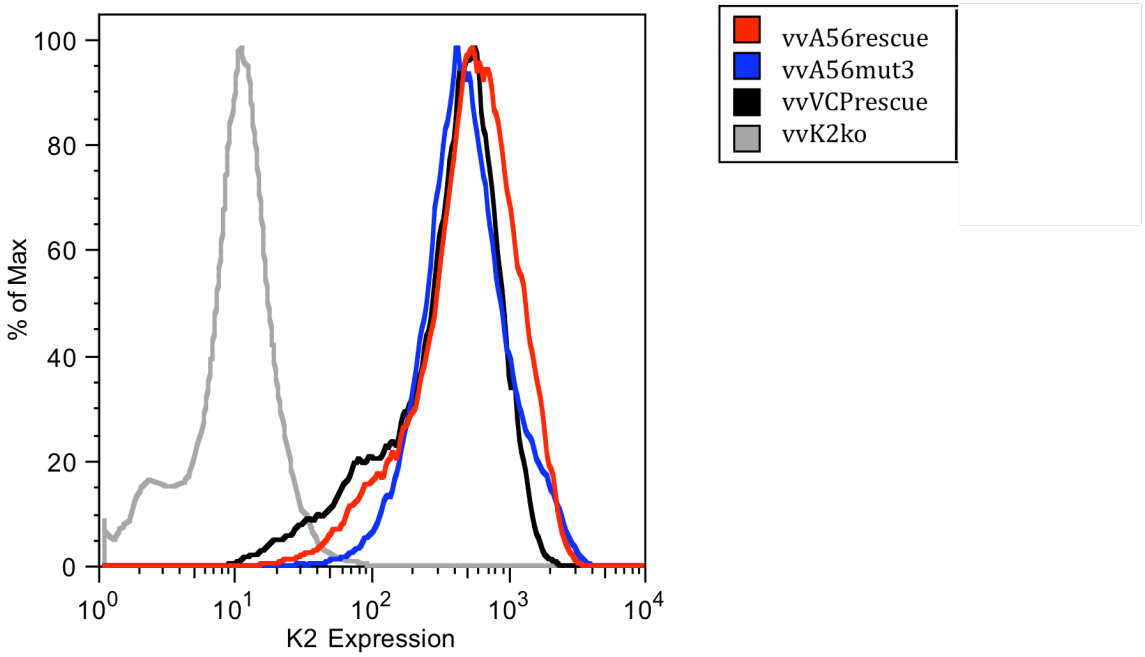


Fig. 5.2: Characterization of recombinant viruses. A) vvA56mut3 and vvA56rescue produce the A56 protein while vvA56ko does not. Western blotting was performed on infected BSC cell lysate using an anti-A56 antibody. A stock of WR virus was used as a positive control. B) Recombinant A56 viruses still express K2 on the cell surface. Infected RK-13 cells were stained with anti-K2 and -A56 antibodies under non-permeablized conditions. Infected (A56 positive) cells were gated on and analyzed for levels of K2 expression.

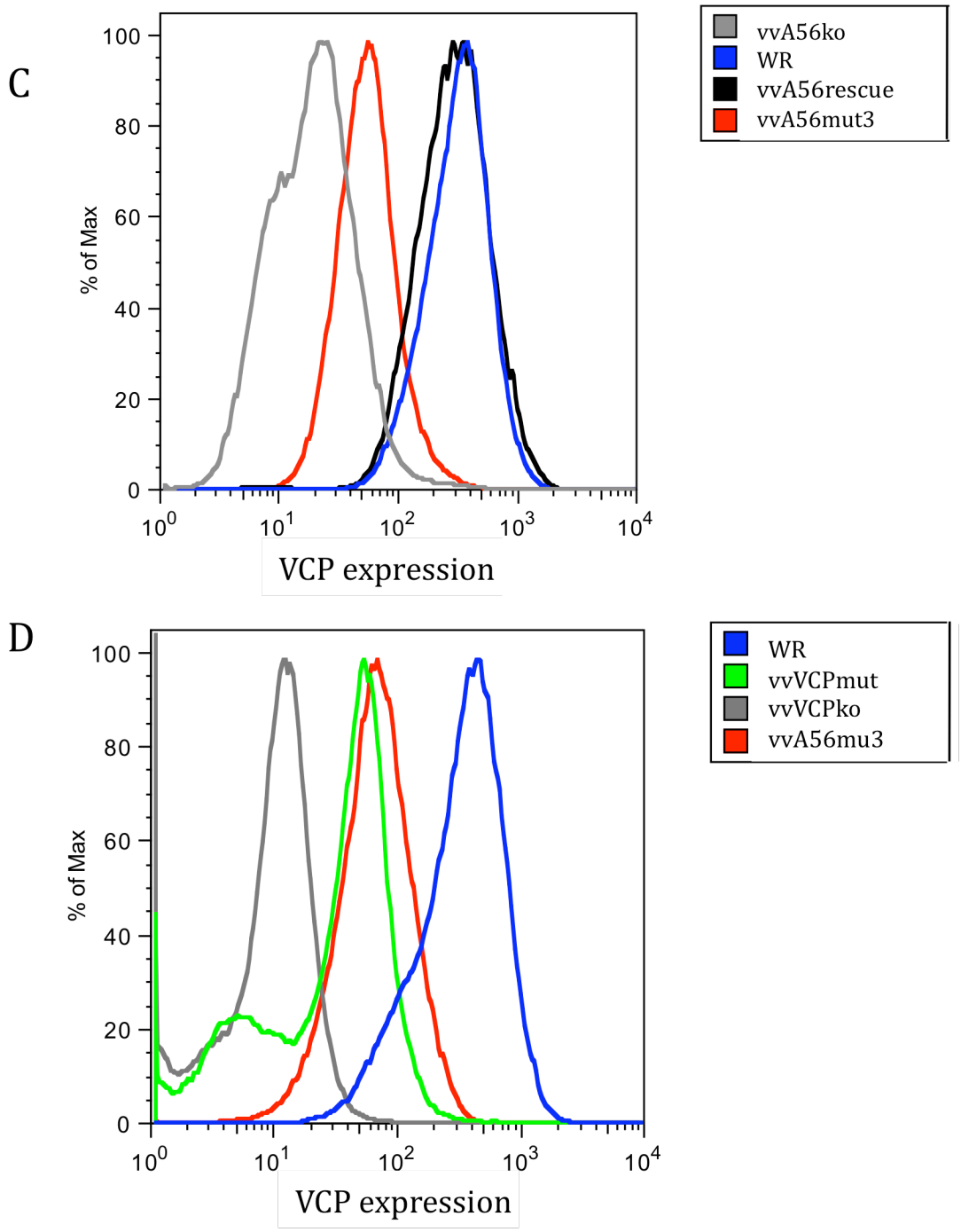


Fig. 5.2, continued: C, D) A56mut3 does not express VCP on the cell surface. Infected RK-13 cells not otherwise gated were stained with an anti-VCP antibody under non-permeablized conditions. Panel C compares the recombinant A56 viruses to a stock of WR virus; panel D compares vvA56mut3 to the previously characterized recombinant VCP viruses vvVCPmut and vvVCPko (36), as well as to a stock of WR virus.

In addition to the intranasal model, we also infected mice intradermally using an ear pinnae model of infection (122). This system has the advantages of mimicking VACV vaccination, and also is able to differentiate small changes in virulence (122). C57BL/6 mice were first infected with vvVCPrescue (23) and vvA56rescue to establish the size of wild type lesion sizes. We found that our A56rescue virus produced the same size lesions as vvVCPrescue, which has previously been shown to be virulent in this model compared to a related panel of mutant viruses (23, 37). Next, we infected groups of mice with vvA56rescue, vvA56ko, or vvA56mut3. In this model of infection, vvA56ko produces smaller lesions than vvA56rescue. These differences are statistically significant at the peak of infection. vvA56mut3 produced lesions that were of similar size to vvA56ko; at day 8 the difference between vvA56mut3 and vvA56rescue is statistically significant. However, at days 9 and 10 these differences fall just short of statistical significance ($p= 0.1$ compared to A56rescue) (Fig. 5.4B). The differences in lesion sizes between A56resue and A56mut3 were seen in repeated experiments. Additionally, lesion sizes between vvA56mut3 and vvVCPmut were of equivalent size (data not shown). This may mean that while the A56/VCP complex is important for virulence in this model, the A56/K2 complex also contributes to maximum pathogenesis.

A56mut3 and A56rescue generate equivalent sized lesions in C3-/- mice

VCP acts by inhibiting the complement cascade and has previously been shown to be important for virulence (50). Critically, in the intradermal model of infection, differences in virulence between a VCPrescue and VCPko virus were not seen in complement deficient (C3-/-) mice (37). If the differences in lesion sizes between vvA56rescue and

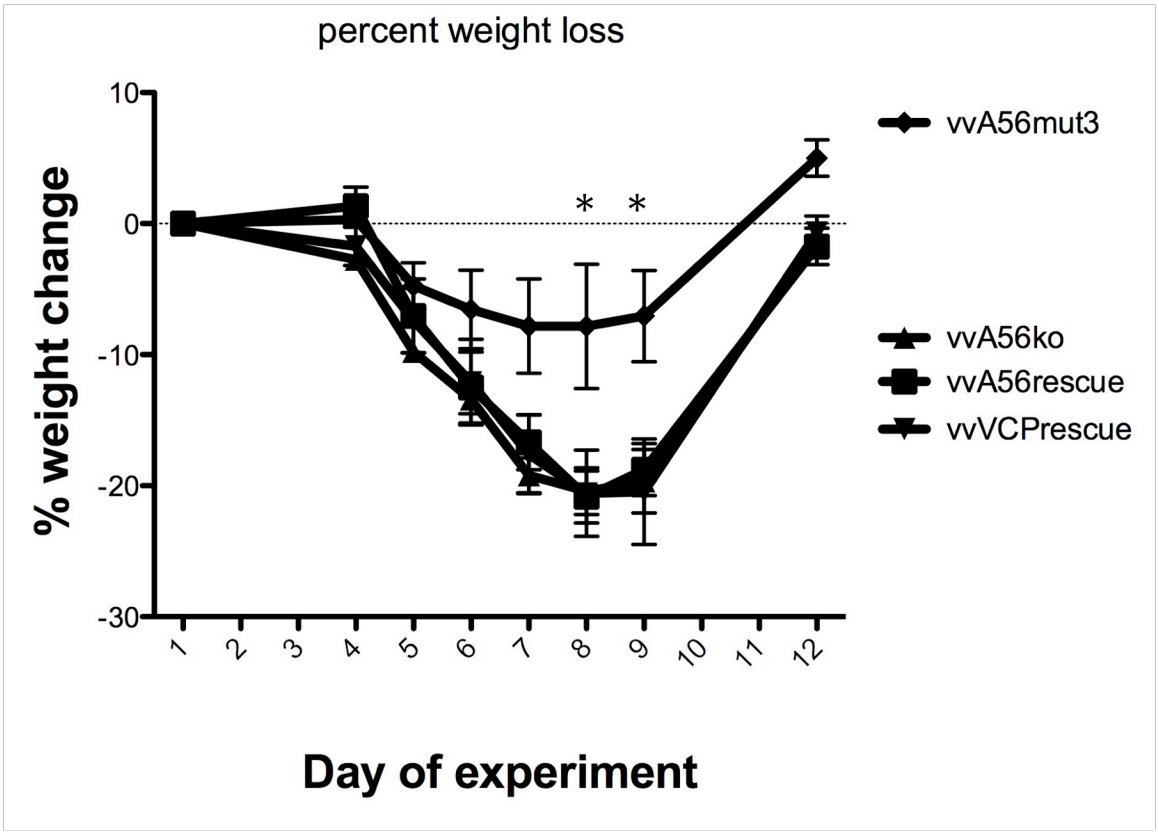


Fig. 5.3: Weight loss after intranasal challenge. 6-week-old female C57BL/6 mice (N=5 per group) were challenged intranasally with $2x10^4$ pfu of virus. Average weight loss was measured as a percentage of the mice's starting weight. * represents $p<0.05$ between vvA56mut3 and vvA56rescue using an unpaired T-test. Error bars represent SEM.

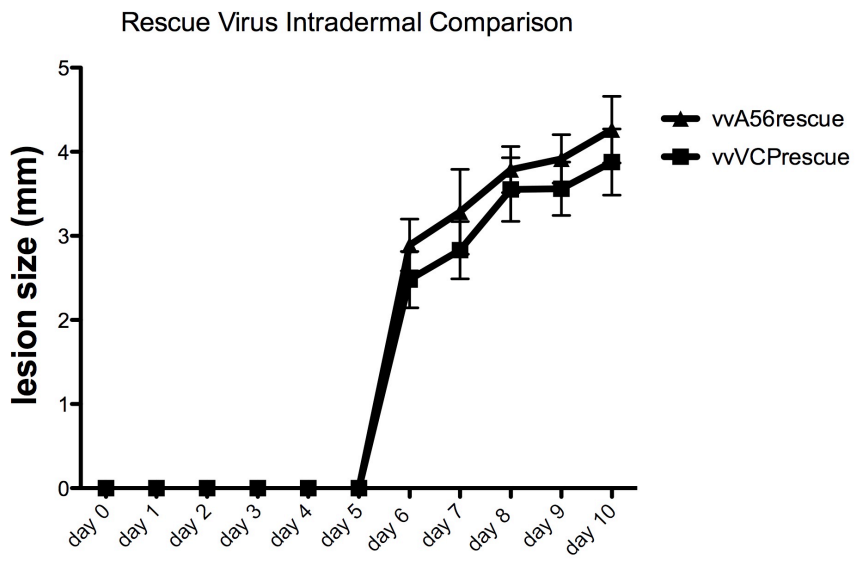
vvA56mut3 are in fact due to the complement-regulatory properties of surface-bound VCP, then these two viruses should form equivalent lesions in C3-/- mice. We therefore infected aged-matched groups of C3-/- mice with either vvA56mut3 or vvA56rescue intradermally and measured their lesions. We found that these two viruses do in fact form equivalently sized lesions on all days measured (Fig. 5.4C). The same results were seen in groups of male mice (data not shown). These findings in C3-/- mice indicates that the consistently smaller lesions formed by vvA56mut3 in C57BL/6 mice is likely dependent on a lack of surface expression of VCP and the presence of host complement.

Discussion

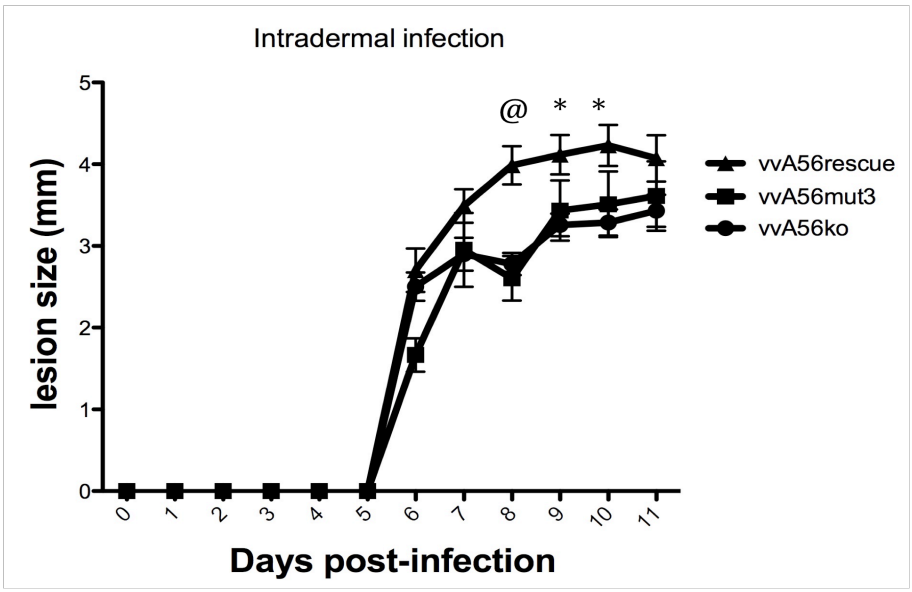
Currently the VACV A56 protein has three known activities. It was first described and identified a hemagglutinin protein in vitro. However, at this point, no relevant in vivo function has been correlated with this activity. More recently, two new properties have been ascribed to A56. It binds K2 and VCP and allows these two proteins to be expressed at the infected cell surface. Testing the contribution of A56 to VACV virulence has been inconclusive, possibly because of these dual (and/or other) functions. Prior in vivo work has focused on gene knockout viruses; however, the two known functions of A56 may have differing levels of importance, or even opposite effects in vivo.

Here, for the first time, we provide in vivo data using a recombinant virus with a site-directed mutation in A56. We show that vvA56mut3 is attenuated relative to wild-

A


Rescue Virus Intradermal Comparison
lesion size (mm)
0
1
2
3
4
5
day 0
day 1
day 2
day 3
day 4
day 5
day 6
day 7
day 8
day 9
day 10
vvA56rescue
vvVCPrescue

B


Intradermal infection
lesion size (mm)
0
1
2
3
4
5
@
*
*
0
1
2
3
4
5
6
7
8
9
10
11
Days post-infection
vvA56rescue
vvA56mut3
vvA56ko

C

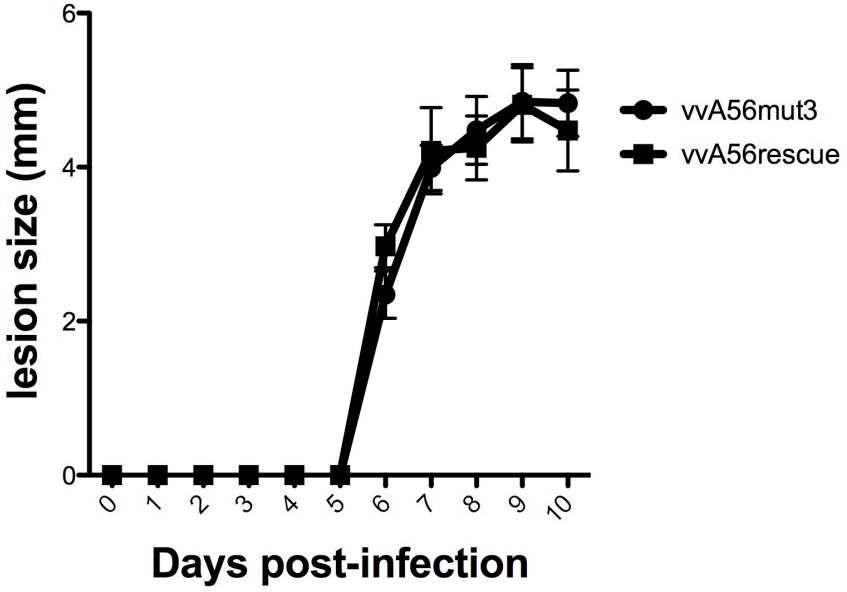


Fig. 5.4: Intradermal infection in C57BL/6 and C3-/- mice. A,B) 6-week old female C57BL/6 mice (n=5) were challenged intradermally with $1x10^4$ pfu of virus in each ear. Lesions greater than 1 mm were measured and averaged each day until peak lesion size. A) Comparison of rescue viruses. B) Comparison of vvA56ko, vvA56mut3, and vvA56rescue. C) Comparison of vvA56mut3 and vvA56rescue in 6-8 week old female C3(-/-) mice. * represents $p<0.05$ between vvA56ko and vvA56rescue using an unpaired T-test; @ represents $p<0.05$ for both vvA56ko and vvA56mut3 vs. vvA56rescue. Error bars represent SEM.

type A56 in two different models of VACV pathogenesis. From this work, it appears that the A56/VCP interaction is responsible for most of A56's contributions to VACV virulence. In an intradermal model, both A56ko and A56mut3 have similar levels of reduced virulence compared with vvA56rescue. Importantly, vvA56mut3 and A56rescue do not have these differences in C3-/- mice. This implies that the differences in C57BL/6 mice are complement dependent, and most likely related to A56mut3's inability to bind VCP. Previous in vivo work focused on mutating the N-terminal cysteine of VCP to abrogate cell-surface expression (23). However, this cysteine also mediates the formation of a VCP homodimer. It is not known whether this dimer contributes to pathogenesis, but in purified form the dimer does possess a higher level of complement regulatory activity than the monomer (68). The recombinant vvVCPmut virus (which has the N-terminal cysteine mutated and thus does not form homodimers and is not expressed on the cell surface) and vvA56mut3 come from different lineages of parental viruses, so direct comparisons may not be valid. However, vvVCPmut seems to be slightly more attenuated than vvA56mut3 compared with their rescue viruses. This may mean that the additional loss of VCP dimers seen in vvVCPmut also has an effect on virulence. Regardless, our data with A56mut3 now allows us to conclude that the VCP/A56 disulfide bridge is important virulence in VACV. However viruses from the same parental line will need to be constructed to make definitive conclusions.

It is interesting to note that vvA56mut3 is attenuated in the intranasal model of infection, while vvA56ko is not. This may mean that the lack of the A56/K2 complex actually may increase virulence in this model of infection. Perhaps in a lung pneumonia model, the potential ability of the virus-infected cells to form syncytia enhances

pathogenesis. However, more research is needed in this area. It is also possible that in other models of infection that the A56/K2 complex has a larger contribution to virulence in VACV. Ideally, a companion mutant A56 virus would be created that cannot bind K2; however, little is known about the interaction between these two proteins. Currently, it is only known that the Ig domain in A56 is needed to bind K2 (105). The first two cysteines in A56 have been hypothesized to form a disulfide bridge. One A56 mutant has been described that has hemagluttinating activity but also form syncytia; in this A56 protein, the 2nd cysteine in the A56 ectodomain is mutated. This mirrors findings from our lab, where tranfected proteins A56mut1 (C34S) or A56mut2 (C103S) appear to have reduced ability to bind K2 (unpublished). Unfortunately, our attempts to isolate recombinant viruses producing these proteins were unsuccessful. In conclusion, the generation of a recombinant viaccinia virus with a cysteine-to-serine change at residue 162 in A56, which can no longer result in surface expression of A56, we have shown that the A56/VCP complex is important for vaccinia virulence.

Chapter 6- Concluding remarks

VACV replication is complex, as it involves both producing new infectious particles as well as a myriad of proteins dedicated to combating the host's immune response. Although these proteins are often called "non-essential", they are frequently needed for the virus to establish an infection and spread to other hosts. This thesis focuses on two non-essential proteins: VCP and A56. VCP, the vaccinia complement control protein, which had previously been characterized as the major secreted protein from VACV infected cells, has already been established as a viral virulence factor (50). In chapter three, we established that VCP interacts with A56 and that this complex is necessary for VCP cell-surface expression. We also showed that the N-terminal cysteine of VCP is a critical residue for this interaction. In chapter four, we located the residue on A56 that allows it to bind VCP. We also proved that the interaction between these two proteins occurs through a disulfide bridge. Finally, we began to establish that viruses that cannot form the A56/VCP complex are attenuated in vivo, using a virus that produces mutated VCP. In chapter five, this in vivo work was extended using recombinant A56-producing viruses in intradermal and intranasal mouse challenge models. A virus producing an A56 protein that cannot bind VCP was attenuated in both of these infection models. In this section, I will review the major conclusions from chapters three, four, and five; I will also discuss possible future directions.

Forming the A56/VCP complex

The observation that VCP was expressed at high levels on the surface of infected cells was initially puzzling. VCP has been well characterized as a secreted protein and while

others had shown that purified recombinant expressed protein could bind to the cell surface through interactions with heparan sulfate, we found surface expression could still occur in heparan sulfate deficient cell lines (36). We also discovered that a virus with the A56 gene deleted (A56ko) could not express VCP at the cell surface (36). This was noteworthy, as A56 is expressed on the surface of cells and could serve as a binding partner for VCP. Work in chapter three focused on proving that these proteins interacted, and began to elucidate the mechanism of this interaction. Using non-reducing, non-denaturing western blots, we found that in addition to formation of a VCP monomer and dimer, a high molecular weight complex forms in wild-type VACV infection. This complex is lost in cells infected with a A56ko virus, and also when the unpaired N-terminal cysteine of VCP is mutated to a threonine residue (VCPmut). In order to show that these two proteins physically interact, we incubated cell lysates from cells infected with 6x-histidine tagged VCP-producing viruses with nickel agarose. We found that A56 co-purified with his-tagged wild-type VCP, but not with his-tagged VCPmut. This provided evidence for an A56/VCP complex, and pointed to the N-terminal cysteine of VCP as critical in forming this complex. However, it was not possible to determine the exact nature of the interaction between these two proteins based on this data. The importance of the N-terminal cysteine could mean that a disulfide linkage occurs between A56 and VCP. However, VCP also forms a homodimer via this cysteine (68) and thus the importance of the N-terminal cysteine in formation of the A56/VCP complex could be the result of a non-covalent interaction between the VCP dimer and A56. While not conclusive, additional work we did with viruses that produced truncated VCP proteins supported the first hypothesis. We found that only the first SCR domain, a portion that

represents only ~25% of the full-length protein, was expressed on the cell surface as long as a free cysteine was present. This first SCR domain could be expressed on the cell surface even if the unpaired cysteine was located at the C-terminus of the protein. Since the homodimer of such a protein would form a "tail-to-tail" configuration, this suggested to us that a free cysteine residue, rather than formation of a VCP homodimer is what drives the stable interaction of VCP with A56.

In chapter four, we show that VCP and A56 form a covalent disulfide bridge. The ectodomain of A56 contains three cysteines. The first two are in an Ig-like domain and have been hypothesized to form an intramolecular disulfide bond (51). Thus the 3rd cysteine is unpaired and we hypothesized that it was this cysteine that formed a disulfide bond with VCP's free N-terminal cysteine. We constructed expression plasmids for transient transfection of uninfected cells. We made plasmids that expressed VCPwt, VCPmut, A56wt and a series of A56 mutants, where each mutant had one of the three ectodomain cysteines mutated to a serine residue. We found that VCP can only be expressed on the cell surface when it was cotransfected with the A56 plasmid. Mutating the first two cysteines of A56 (alone or together) did not affect surface expression of VCP. However, when the third cysteine at position 162 was mutated, VCP surface expression was lost. This loss of expression closely mirrors what was seen when wild-type A56 was transfected with VCPmut. Taken together, these mutations allowed us to conclude that the A56/VCP complex is formed by a disulfide bridge between the N-terminal cysteine (C20) of VCP and third cysteine (C162) of A56. Surface expression of VCP on infected cells can result in additional functions. As a secreted protein, VCP had been thought to act by decreasing complement activation in the extracellular milieu

around an infection. Since VCP is also expressed at high levels on the cell surface, this meant that the protein could be directly protecting infected cells from the host complement attack. Work done in our lab has supported this hypothesis, as cells infected with a VCPmut virus are more susceptible to complement lysis than those infected with wild-type virus (36).

Extensions of surface expression of viral complement control proteins to other poxviruses

Many other poxviruses besides VACV encode complement control proteins. Of these, the best characterized are SPICE from smallpox, MoPICE from monkeypox, and EMICE from ectromelia (mousepox) (67-69, 85, 98, 114). They all share significant homology to VCP and are made up of the same SCR domain repeats. SPICE and EMICE both contain four SCR domains, while MoPICE, due to a point mutation that results in an early stop codon, contains three SCR domains and a few residues of the 4th SCR domain. EMICE and SPICE contains the same free N-terminal cysteine as VCP; while MoPICE does not have this cysteine, there is an unpaired C-terminal cysteine. In chapter four, we found that SPICE is expressed on the surface of cells only when VACV A56 is contransfected. Like VCP, SPICE surface expression is lost when A56mut3 is co-transfected. This finding was somewhat expected, as VCP and SPICE have identical first SCR domains and their A56 proteins are highly homologous. These findings suggest that SPICE could also protect infected cells from complement attack during a smallpox infection. Our data with EMICE was also interesting. Both EMICE and ectromelia virus A56 are less similar to VACV than the smallpox homologs. We found that there was only minimal surface

expression of EMICE when co-transfected with VACV A56. However, data from infected cells showed a high level of EMICE surface expression. We found that while surface expression of EMICE in the presence of VACV A56 was low, when EMICE was co-transfected with the plasmid expression ectromelia A56, high EMICE surface expression was seen. Excitingly, this means that not only is the complement control protein/A56 interaction preserved in different poxviruses, but also that co-evolution of these two proteins may be occurring.

In contrast to the work with VCP, SPICE, and EMICE, we found that surface expression of MoPICE is minimal in cells co-transfected with VACV A56 or it's own cognate A56 protein. Based on work with the truncated VCP proteins, where a C-terminal cysteine on the first SCR could result in surface expression, We took this finding with MoPICE, which has a free C-terminal cysteine after the 3rd SCR domain, to mean that the free cysteine on a complement control protein must be on the first SCR domain to efficiently interact with A56. This may provide some insight into the residues needed for the initial interaction of an SCR with A56 to allow the disulfide bond form. The low level of surface expression of MoPICE on transfected cells was also seen with immunofluorescence staining of monkeypox virus infected cells. MoPICE stains faintly on the infected cell surface, but at much lower levels than VCP on VACV-infected cells.

The importance of the A56/VCP complex to in vivo pathogenesis

The in vitro activity of VCP in regulating complement activation has been well characterized (50, 57, 68, 100). Girgis et al (36) provided the first evidence of an activity of surface expressed VCP in vitro by studying its role in inhibiting complement-mediated lysis of infected cells. There have been fewer in vivo studies that focus on the role of

VCP during a VACV infection. In addition, previous in vivo studies used gene knockout viruses (50). This thesis provides the first in vivo studies that specifically study the contribution of cell-surface VCP to virulence. In chapter four, we studied viruses that produced VCPmut in both intradermal and intranasal models of mouse infections. We found that in intranasal infections, a VCPmut-producing virus was as attenuated as a VCPko virus. Intradermally, vvVCPko was attenuated relative to vvVCPwt, while vvVCPmut had an intermediate phenotype. This suggested that surface expression of VCP contributes to virulence; this is in addition to the effect of soluble VCP. However, because VCPmut cannot form dimers, we could not say with 100% certainty that the attenuation we saw was due to the absence of the A56/VCP complex on the surface of infected cells.

In chapter four we generated mutant A56 proteins and identified one that could not bind VCP, but was unaffected in its other function (i.e., binding K2, which results in a complex that can inhibit syncytia formation and superinfection). In chapter five, we constructed a recombinant virus expressing this mutated A56 protein, and tested it alongside its parental A56 knockout virus and the A56 rescue virus. We found that vvA56mut3 was attenuated in an intranasal model of infection, even though vvA56ko was not. We interpreted this to mean that the A56/VCP complex contributes significantly to virulence in this model. In an intradermal model of infection, vvA56ko is significantly attenuated compared to vvA56rescue. We took this to mean that either the A56/VCP or A56/K2 complex is important for pathogenesis in this model; it is also possible that both complexes contribute. Like A56ko, vvA56mut3 is also attenuated after intradermal infection. We concluded that the A56/VCP complex is important for full virulence in this

model; the slight differences between vvA56ko and vvA56mut3 may mean that the A56/K2 complex also plays a role. vvA56mut3 forms the same size lesions as VCPmut; this supports the conclusion that the lack of the A56/VCP complex is what attenuates these viruses. Importantly, no differences were seen between vvA56mut3 and vvA56rescue in C3-/- mice. This means that the differences we saw in the other models were likely due a fully functional complement system and the absence of surface expressed VCP.

Future directions

There are other sets of experiments that could be done to further this work and our understanding of the contribution to surface expression of VCP during a viral infection. In chapter four, we studied SPICE, but only in a transfection system and only with vaccinia A56. It may be possible to observe SPICE in the context of a variola virus infection at the CDC. From a biochemical standpoint, further mutagenesis of A56 around the third cysteine and the first SCR domain could be performed to see what residues are important for the initial interaction between A56 and VCP. Recent proteomic work showed that VCP is present on some EV particles, presumably bound to A56. However, experiments have not yet been done to determine whether virion-bound VCP is protective against complement. Our work with a panel of viruses with mutations in VCP and a panel of viruses with mutations in A56 makes direct comparisons between these sets of viruses difficult because the starting parental viruses were different. Thus creating a series of virus mutants (K2ko, VCPmut and VCPko, A56ko, and A56mut3) from the same parental virus with rescues would allow for more direct comparisons of each

mutant's contribution to virulence. Such a panel of viruses would allow more definitive conclusions about the contribution of the VCP homodimer vs. VCP surface expression to VACV pathogenesis.

In conclusion, this thesis has provided evidence that VCP and A56 form a covalently bonded complex at the surface of infected cells, and this complex is necessary for full viral pathogenesis in two different models of infection.

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