ABSTRACT

Title of Document: REGULATION OF INFECTED CELL FUSION BY THE VACCINIA VIRUS A56 AND K2 PROTEINS.

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Poxviruses are a group of large double-stranded DNA virus that replicate in the cytoplasm of the cell. The *Orthopoxvirus* genus includes variola virus, the etiological agent of smallpox, and vaccinia virus (VACV), the prototypical member of the genus. Cells infected with VACV display very little cell-cell fusion, however VACV mutants deleted for either the A56R or K2L gene display extensive cell-cell fusion. A56 and K2 interact with one another (A56/K2) and expression of both proteins is important for preventing cell fusion. VACV entry and fusion requires a multiprotein entry fusion complex (EFC) composed of at least eight proteins. In the absence of a functional EFC infected cell fusion does not occur even when the viruses lack either A56 or K2. A panel of recombinant VACVs was used to define protein interaction important for regulation of cell fusion. Affinity purification of A56, K2 and the EFC revealed an interaction between A56/K2 and the EFC. This interaction

required expression of both A56 and K2 as A56 did not bind the EFC in the absence of K2 and vice versa. Interestingly, the ability to bind the EFC correlated with the inhibition of infected cell fusion by A56 and K2. Although the EFC contains eight proteins, only two entry proteins, A16 and G9, were important for binding A56/K2. Individually, A16 and G9 did not bind A56/K2; instead both A16 and G9 were needed for efficient interaction with A56/K2. A16 and G9 copurified with one another when expressed by transfection in uninfected cells, confirming that the two proteins bind to one another suggesting they directly interact within the EFC. To support a biological role for A56/K2 binding the EFC, cells expressing A56 and K2 were tested for infectivity as well as their ability to undergo cell-cell fusion. In both cases, cells expressing A56 and K2, but not individual expression of A56 or K2, showed reduced cell-cell fusion and virus entry. Collectively, these data support a model by which A56/K2 regulate infected cell fusion through an interaction with the viral EFC.

REGULATION OF INFECTED CELL FUSION BY THE VACCINIA VIRUS A56 AND K2 PROTEINS.

By

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Dedication

I would like to dedicate this work to my parents Fred and Diane Wagenaar. Without their support and emphasis on the importance of a good education I would never have reached this point. This work is a tribute to the sacrifices they have made over the years.

Acknowledgements

There are a number of people that have been instrumental in completion of this work. My mentor Dr. Bernard Moss, whose passion for science is infectious and from his helpful suggestions I have learned so much. Dr. Charles Grose, who first introduced me into the wonderful world of viruses and whose lab was a playground for a naïve, inexperienced undergraduate. Finally, Kristie Grebe, who during this process learned more about vaccinia virus then she probably cared to, but whose help and support was critical to my completion.

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List of Abbreviations

A	Adenine
bps	Base pairs
C	Cytosine
^{o}C	Degrees Celsius
CBB	Calmodulin binding buffer
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
DsRed	*Discosoma sp.* red fluorescent protein
E. coli	*Escherichia coli*
EFC	Entry fusion complex
EGFP	Enhanced green fluorescent protein
EGTA	2-[2-[2-[2-[bis(carboxymethyl)amino]ethoxy]ethoxy]ethyl-(carboxymethyl)amino]acetic acid
EM	Electron microscopy
EMEM	Earle's modified Eagle medium
EV	Extracellular virus
FBS	Fetal bovine serum
g	Gravity
G	Guanine
GAGs	Glycosaminoglycans
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescence protein
HcRed	*Heteractis crispa* red fluorescent protein
h	Hours
IPTG	Isopropyl-ß-D-thiogalactopyranoside
kbps	Kilobase pairs
kDa	Kilodaltons

LDS	Lithium dodecyl-sulfate
Luc	Luciferase
mins	Minutes
ml	Milliliters
mM	Millimolar
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MV	Mature virus
nm	Nanometers
ORF	Open reading frame
PCR	Polymerase chain reaction
PFU	Plaque forming unit
RFP	Red fluorescent protein
SBB	Streptavidin binding buffer
SERPIN	Serine protease inhibitor
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
T	Thymidine
TAP	Tandem affinity purification
TBST	Tris-buffered saline with Tween-20
ts	Temperature sensitive
µl	Microliter
µM	Micromolar
UTR	Untranslated region
WR	Western Reserve
WV	Wrapped virus
VACV	Vaccinia virus
VARV	Variola virus

Chapter 1: Review of literature

1.1 The Poxviridae

1.1.1 Classification.

Poxviruses are among the largest animal viruses. They have a characteristic brick-shaped virus particle that contains the virus genome along with all of the viral enzymes and factors required for early RNA synthesis. Poxviruses are unusual among DNA viruses, replicating entirely within the cytoplasm. The family *Poxviridae* is divided into two subfamilies: *Chordopoxvirinae* and the *Entomopoxvirinae* based on their respective vertebrate and insect host range. The chordopoxviruses have been more intensely studied than entomopoxvirues due in part to their ability to infect humans and domesticated animals. There are eight genera within the *Chorodopoxvirinae*: *Orthopoxvirus*, *Parapoxvirus*, *Avipoxvirus*, *Capripoxvirus*, *Leporipoxvirus*, *Suipoxvirus*, *Molluscipoxvirus*, and *Yatapoxvirus*. Viruses within the *Orthopoxvirus* genus are primarily responsible for infection of humans, with the most notable members of the genus including variola virus (VARV), the etiological agent of smallpox, and vaccinia virus (VACV), which was utilized successfully as a vaccine in the eradication of smallpox. VACV is the prototype of the *Orthopoxvirus* genus and has been intensively studied in the laboratory. Many of the poxviruses within the *Chordopoxvirinae* exhibit a narrow host range and infect only a single species, although occasionally the viruses will infect humans by zoonosis. VARV and molluscum contagiosum virus

(*Molluscipoxvirus* genus) are obligate human pathogens with no known animal reservoir.

1.1.2 Smallpox, Edward Jenner and Vaccinia virus.

Smallpox is estimated to have evolved around 10000 BCE, about the same time as the first agriculture settlement [1]. The disease ravaged the human population and spared no class from its effects as evident from the skin lesions found on mummified bodies of Egyptian pharaohs.

Prior to the advent of vaccination, the practice of "variolation" was introduced in India and China around 1000 years ago as a strategy to prevent infection with the smallpox virus [1]. Variolation involved the use of a small amount of infectious material obtained from the smallpox lesion or scab. This infectious material was used to inoculate an individual in the hope of developing a milder form of the disease. Variolation was born from the observation that once individuals infected with smallpox had recovered they were protected from subsequent infection with the virus. Variolation, possessed a significant risk to the individual by modern standards, associated with a low mortality rate which may have in part been due to an inability to control the inoculating dose.

It was the English physician Edward Jenner in the late 18 th century who introduced the concept of vaccination [2]. Edward Jenner observed that local milkmaids infected with cowpox virus failed to be infected by VARV. Jenner's observation led him to inoculate James Phipps with cowpox virus and later challenge the boy with VARV. The prior infection with cowpox virus effectively prevented the

child from developing smallpox and changed the course of modern medicine leading to the eventual eradication of the disease nearly 200 years later.

1.1.3 Genome organization.

The poxvirus family is divided into two subfamilies: viruses that infect vertebrate hosts (*Chordopoxvirinae*) and viruses that infect insect hosts (*Entomopoxvirinae*) [3]. The chordopoxviruses have a dsDNA genome of variable length that ranges from 260 kbps for fowlpoxvirus to 140 kbps for Orf virus. The nucleotide composition of the chordopoxvirus genomes is quite diverse and ranges from 64% G/C in Parapoxvirus to 33% G/C for Yatapoxvirus [3]. Despite the variation in size and nucleotide composition, poxviruses share a common genome organization. The central region of the genome is conserved with respect to gene content and arrangement. Many of the genes located in the central region are essential for virus replication in cell culture and have important roles in virus transcription, replication and assembly of nascent particles. The genome termini are more divergent and the genes located in this region are generally not required for virus replication in cell culture but instead function in host range and immune evasion. There are 90 genes conserved among the *Chordopoxvirinae* with most of the conserved genes being located in the central portion of the virus genome. Inclusion of the entomopoxviruses reduces the number of conserved gene to 49 [4].

Vaccinia is the prototypical member of the poxvirus family. Many of the conserved poxvirus genes have been investigated in the laboratory by examination of VACV. VACV has a linear dsDNA genome of 190 kbps with covalently closed hairpin termini [5]. Prior to sequencing of the genome of VACV it was characterized

by digestion with restriction endonuclease HindIII. The common VACV laboratory strain Western Reserve (WR) contains 15 HindIII fragments designated A to O based on their mobility in an agarose gel, with the A fragment being the largest and O the smallest [6, 7]. VACV genes are named according to their location, position, and direction of transcription within the HindIII fragments. For example, A56R is the 56^{th} gene within the A HindIII fragment and is transcribed to the right as indicated by the R. Meanwhile, K2L is the second gene of the K HindIII fragment and transcribed to the left. It is common to refer to the gene with the L and R designation, while the L and R are omitted when referring to the protein (Figure 1-1).

The ends of the VACV genome are located within the B and C HindIII fragments and consist of 10 kbps of inverted repeat DNA [8, 9]. The sequence of the inverted repeats consists of a total of 30 DNA repeats, with each repeat being 70 bps in length [10]. There is significant diversity in the number of repeats between strains of VACV. The virus genome terminates with a conserved A/T rich region of 104 bps to form a covalently closed hairpin [11]. The terminal portion of the virus genome, encompassing the viral hairpin, which have been suggested to have several important functions including initiation of DNA replication and resolution of the viral genome concatemers [12-15].

1.1.4 Virion Morphology and Infectious particles.

Cells infected with VACV produce three morphologically distinct virus particles: mature virus (MV), wrapped virus (WV) and extracellular virus (EV). The MV particle is the simplest and most abundant. Most MV particles remain

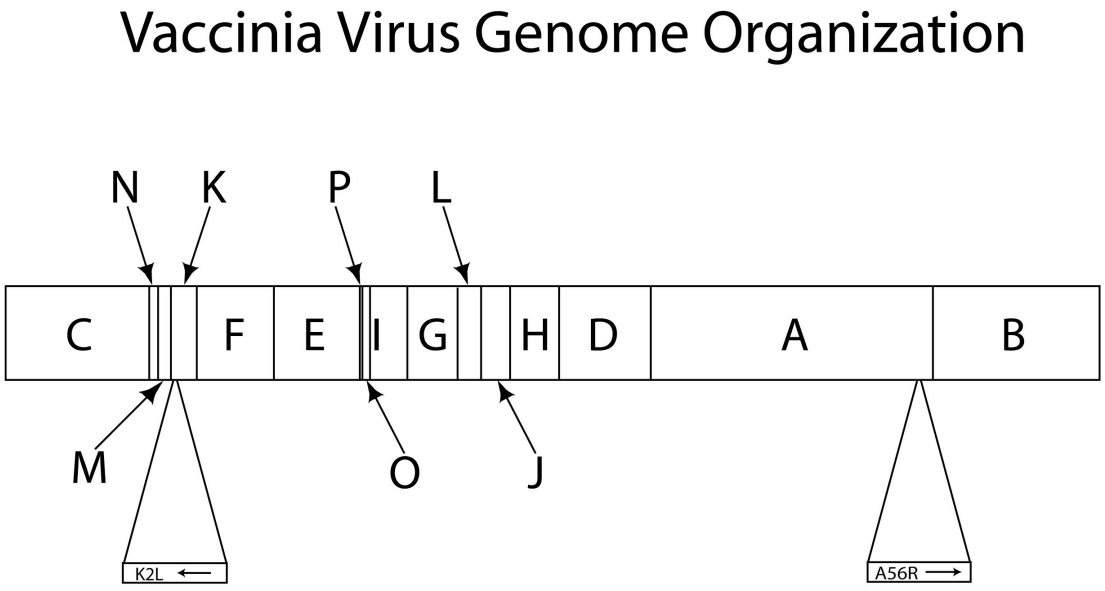

Figure 1-1: Vaccinia virus genome organization. Diagram representing the DNA fragments generated by digestion of the VACV genome with the HindIII restriction endonuclease. The DNA fragments are lettered according to their size; A is the largest fragment, while P is the smallest. Viral genes are named according to their location and position within the HindIII fragment with L or R indicates the direction of transcription. Shown are two examples: K2 is the second gene of the K fragment and transcribed to the left. A56 is the 56th gene of the A fragment and transcribed to the right.

intracellular until cell lysis but a portion acquires two additional membranes of the trans-Golgi or endosomal origin to form WV [16]. The WV is transported to the cell periphery on microtubles and is released from the cell by exocytosis [17]. During the release of WV the outer of the two wrapping membranes is lost to form the EV. The EV particle is surrounded by one additional membrane with respect to MV. EV is important for virus spread in cell culture and is thought to mediate dissemination of the virus *in vivo*. The different stages of VACV lifecycle are indicated in figure 1-2.

The MV particle has been visualized by a combination of cryo-electron tomography and atomic force microscopy [18-20]. These studies define the MV particle as a brick shaped structure with an average dimension of 360nm X 270nm X 250 nm. Transmission electron microscopy of thin-sectioned MV reveal the virus is surrounded by a single lipid membrane [21, 22], although others interpret the images differently, suggesting the MV particle is enveloped by two tightly apposed membranes [23-25]. The virus particle contains a dumbbell shaped electron dense core housing the viral genome with two protein lateral bodies parallel to the biconcave core.

1.1.5 Viral Proteins.

The 190 kbps genome of VACV is predicted to encode nearly 200 proteins. Many of these proteins are incorporated into the virus particle. Early analysis of the MV virus particle by 2D electrophoresis suggested the particle was comprised of nearly 100 proteins [26, 27]. More recently three groups have reevaluated the protein composition of the MV particle by mass spectrometry. The number of viral proteins

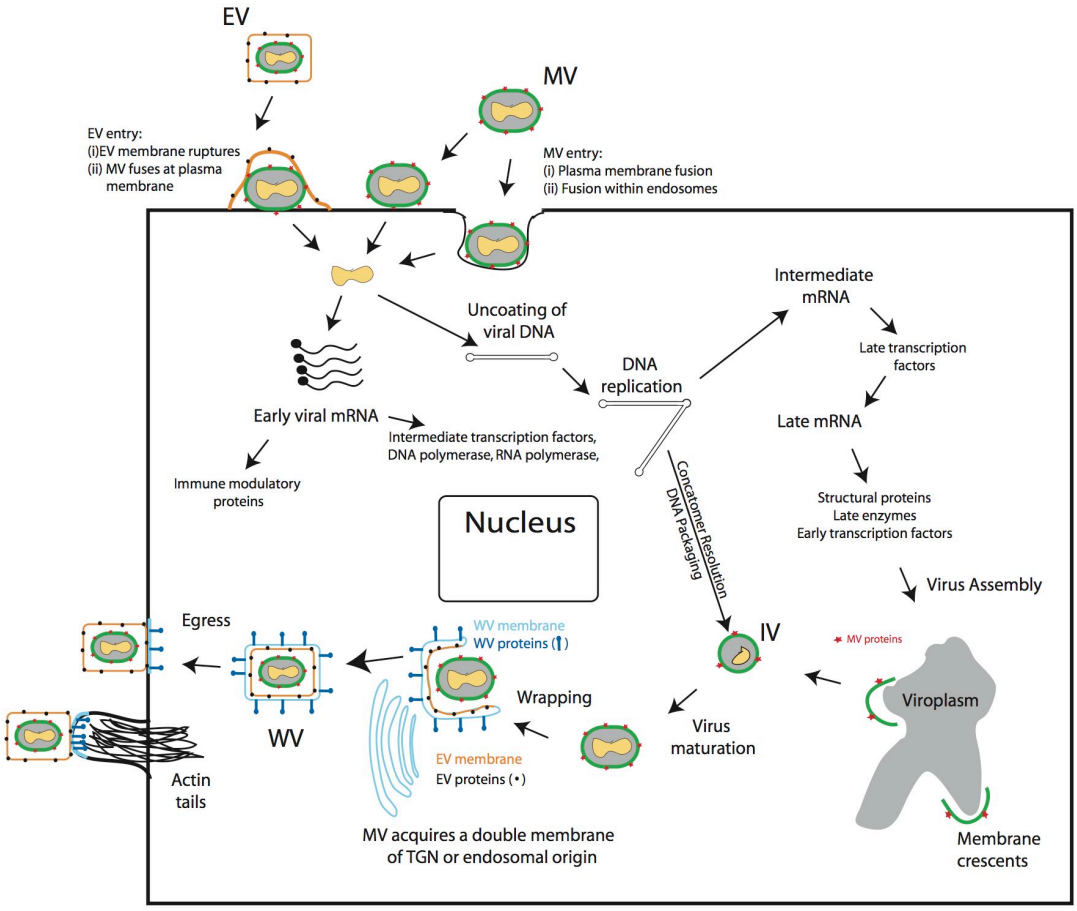

Figure 1-2: Lifecycle of Vaccinia virus.
Virus particles (extracellular virus (**EV**) or mature virus (**MV**) enter the cell by direct fusion at the plasma membrane or an endocytotic pathway to release the virus core into the cytoplasm. The virus core contains the DNA template, viral enzymes and transcription factors required for transcription of **early viral mRNA.** The early gene products encode immunomodulatory proteins, transcription factors and enzymes for viral DNA replication. Disassembly of virus core occurs during early gene expression and leads to **uncoating of viral DNA** and **DNA replication**. **Intermediate mRNA** is transcribed after DNA replication and includes transcription factors for late gene expression. **Late mRNA** encodes many of the structural proteins for nascent particle formation as well as the early transcription factors and enzymes to be packaged into the virus core. **Virus assembly** begins with the formation of **membrane crescents**. The virus crescents are filled with the **viroplasm**, a mixture of viral proteins, which evolves into spherical immature virions (**IV**). **Genome concatemers** are resolved during late gene expression and the unit length genome is packaged into the IV. **Virus maturation** is accompanied by proteolytic processing of viral membrane and core proteins. A double membrane of trans-Golgi or endosomal origin leads to **wrapping** of a portion of the MV to form wrapped virus (**WV**). **Egress** of WV occurs following transport of the WV on microtubles to the plasma membrane and the outer membrane fuses with the plasma membrane to release extracellular virus (**EV**). Viral proteins present in the WV and EV membranes are important for recruitment of cellular proteins to form virus tipped **actin tails** that aid in virus spread.

within the MV particle varied between the studies and ranged from 63 to 80 viral proteins [28-30]. A consensus of 73 viral proteins was identified within two of the three studies [30]. Nearly 50% of the viral proteins within the MV particle have a role in forming the structure or assembling the MV, while the remaining proteins are enzymes and factors important for synthesis of viral RNA. Most of the viral proteins packaged into the MV particle are encoded by genes located in the central conserved region of the genome with few proteins from the more divergent genome termini [30].

Poxvirus replication occurs entirely in the cytoplasm and very few host proteins are required for virus gene expression. Consequentially, poxviruses have evolved an array of proteins for synthesis, regulation and processing of its mRNA [31]. The virus encodes a multisubunit DNA dependent RNA polymerase [32, 33], a heterodimeric capping enzyme [34-36], and a poly (A) polymerase [37]. Viral gene expression is coordinated by early [38, 39], intermediate [40-43] and late [44] transcription factors, while two viral decapping enzymes: D9 [45] and D10 [46, 47] are thought to regulate turnover of host and viral mRNA. Formation of protein disulfide bonds typically occurs within the oxidizing environment of the host ER, as the reducing environment of the cytoplasm is generally thought to prevent their formation. Interestingly, multiple viral membrane proteins (L1, F9, A28, H2, A16, G9 and A21) have been identified to contain intramolecular disulfide bonds. Three viral proteins: E10 [48], A2.5 [49] and G4 [50, 51] are required for the formation of these intramolecular disulfide bonds. The E10 viral protein contains a domain with a conserved thiol active site motif C-X-X-C common to the ERV1 (Essential for Respiration and Vegetative Growth)/ARL (Augmenter of Liver Regeneration) family

of cellular thiol oxidoreductases [52]. Collectively, E10, A2.5 and G4 form a cytoplasmic pathway essential for the catalysis of intramolecular disulfide bonds [53].

VACV encodes numerous immunomodulatory proteins that help the virus evade both the innate and adaptive immune response of the host [54, 55]. During viral gene expression dsRNA is produced, a potent trigger of the innate immune response. However, the virus encodes a dsRNA binding protein, E3, which is important for the inhibition of dsRNA dependent protein kinase R [56, 57]. The VACV C3L gene encodes a soluble complement control protein that inhibits both the classical and alternative pathways of complement by binding C4B and C3B [58, 59]. Poxviruses also express a number of proteins that function as defective receptors for cytokines and chemokines [60]. The terms virokines and viroceptor was coined to describe these decoy molecules, which are important virulence factors as viruses lacking these proteins are attenuated *in vivo*.

1.1.6 Recombinant techniques for the investigation of VACV.

The roles of many VACV genes have been elucidated through targeted deletion of viral genes and examination of the resulting phenotype. Targeted deletion is useful for the study of virus genes that are not essential for the virus life cycle, however alternative strategies are used to investigate the function of essential virus genes. Early investigation of essential viral genes relied on random mutagenesis to generation conditional lethal viruses in which virus growth was temperature sensitive (ts). VACV is normally grown at 37 ^{O}C, however the permissive temperature for ts viruses is typically 31 ^{O}C with viruses containing a ts legion being unable to grow at an elevated temperature of 40 ^{O}C. Temperature sensitive viruses were characterized

biochemically as well as by electron microscopy and placed into complementation groups. Ultimately, characterization of a temperature sensitive virus relied on identification of the defective virus gene through complementation [61]. A more targeted approach for the development of ts viruses employs alteration of charged amino acids to the nonpolar amino acid alanine [62, 63].

A second approach developed to characterize genes essential for the virus lifecycle utilizes components of the *E. coli lac [64]* and *tet* [65] operons. Control of viral gene expression relies on integration of the *lac* or *tet* repressor into the virus genome and constitutive expression of the repressor protein throughout the virus lifecycle. Virus gene expression is controlled at the level of transcription by inserting the DNA sequence of the *lac* or *tet* operator between the virus promoter and the initiating methionine of the protein. An inducer, isopropyl-ß-D-thiogalactopyranoside (IPTG) for the *lac* operon or Doxycycline for *tet* operon, is added to the medium to allow gene expression. The inducer prevents the repressor protein from binding to its cognate operator thereby allowing viral gene expression. In the absence of the inducer the repressor protein binds to the operator and stringently repress transcription, most likely through steric hindrance. Basically, in the presence of the inducer, the gene is expressed, allowing the virus to grow, while in the absence of the inducer gene expression is repressed and the virus is unable to grow. An alternative approach for control of virus gene expression utilizes the phage T7 RNA polymerase along with components of the *lac* operon. Instead of relying of the virus promoter for gene expression the gene is expressed from a T7 promoter. Expression of T7 polymerase is dependent on the addition of IPTG [66] (See Figure 1-3).

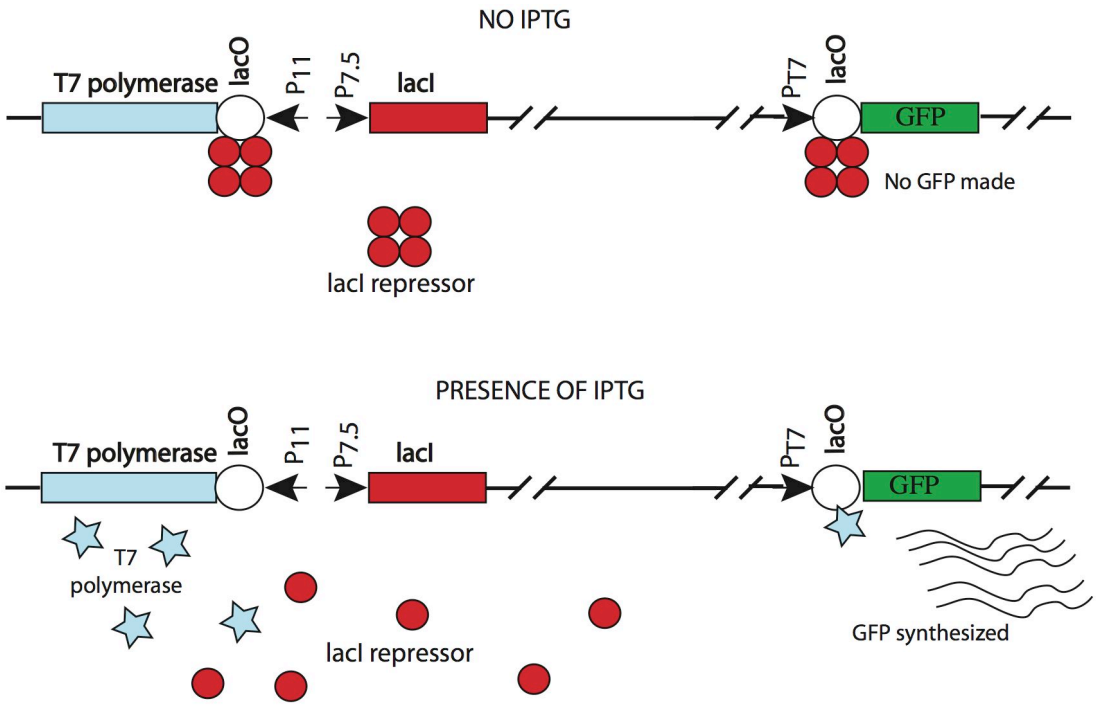

Figure 1-3: Diagram of inducible gene expression in vaccinia virus.
Gene expression is controlled at the level of transcription. The lac repressor (lacI) is expressed constitutively from a viral early/late promoter (P7.5). Expression of the phage T7 polymerase is regulated by the lac operator (lacO) located between the late viral promoter (P11) and the initiating codon of the protein. GFP is expressed from the T7 promoter (PT7), with the lac operator located between the T7 promoter and initiating codon of GFP. In the absence of IPTG the lac repressor binds to the operator preventing expression of the T7 polymerase. A second lac operator downstream of the T7 promoter further represses transcription. In the presence of IPTG the lac repressor does not bind the lac operators, allow expression of T7 polymerase, which catalyzes transcription of the GFP gene.

1.2 Virus Entry and Replication

1.2.1 Virus Attachment.

Entry and fusion of poxviruses is complicated by the existence of multiple forms of the virus. Most of the studies of VACV entry have focused on the MV particle, since it is stable and easily purified. VACV has a broad tropism in cell culture and is able to infect many different types of cells making isolation of a cellular receptor difficult. VACV fails to infect human resting T cells, but does infect activated T cells, suggesting naïve T cells lack specific cellular factors required for virus attachment or entry that are expressed upon activation [67]. A monoclonal antibody reactive to a cell surface antigen was isolated and shown to prevent virus attachment, although the specificity of the antibody remains to be identified [68].

Initial attachment of the MV particle to the cell is facilitated by interactions between the virus and cell surface glycosaminoglycans (GAGs). Three VACV proteins have been reported to mediate virus attachment: D8, H3 and A27. Individually, D8 [69], H3 [70] and A27 [71, 72] are not required for virus growth in cell culture. This may not be all that surprising as A27, H3 and D8 proteins may have slightly redundant function with respect to virus attachment, which would be consistent with individual deletions of the proteins not having a more severe effect on virus growth [73-75]. However, virus attachment is not solely dependent on GAGs as VACV still binds and infects Sog9 cells, which lack the GAGs heparin sulfate and chondroitin sulfate [76]. A recent report suggested that cellular laminin is an important cellular attachment factor since soluble inhibited virus attachment in a dose dependant manner [76]. Cholesterol within the cell membrane has also been

demonstrated to be important for virus entry. Depletion of cholesterol from the cell membrane by treatment with methyl-β-cyclodextrin reduced cell infectivity by 90%. Infectivity was restored by addition of exogenous cholesterol, indicating an important role for cholesterol in VAV attachment/entry [77]. The cellular factors important for virus attachment and entry remain to be fully characterized and further investigations will likely reveal other cellular factors that play a role in this process.

1.2.2 Virus Entry.

Entry and fusion of VACV requires a conserved multiprotein complex of at least eight proteins: A16 [78], A21 [79], A28 [80], L5 [81], G3 [82], J5, G9 [83], and H2 [71]. The proteins of the EFC are integral membrane proteins located within the MV membrane and critical for infectivity of the virus, although it remains to be determined if the EFC directly mediates virus fusion. All of the entry proteins contain a single transmembrane anchor and the proteins fall into two groups based on their predicted topology within the virus particle. A28, A21, H2, G3 and L5 possess an N terminal transmembrane domain and have 0-2 intramolecular disulfide bonds, while A16, G9 and J5 have a C terminal transmembrane domain with 4-10 intramolecular disulfide bonds. The cysteine rich domains of A16, G9 and J5 are homologous, suggesting the proteins arose through gene duplication and diverged to have independent roles in virus entry. There is no detectable homology between A28, A21, H2, G3 and L5. The entry proteins have no role in assembly of the MV, WV or EV particle. Viruses that lack a single entry protein have been isolated and bind normally to cells, but fail to enter indicating a block in virus entry or fusion.

The EFC is critical for virus entry and all proteins of the complex must be expressed for the virus to enter. The absence of a single entry protein is sufficient to prevent the complex from assembling, although if the complex does not form the remaining entry proteins are still incorporated into the MV membrane [84]. This potentially indicates the entry proteins localize to the virus membrane prior to forming the EFC. In fact, the EFC does not form in the absence of the virus membrane, which would be consistent with the entry proteins localizing to the viral membrane prior to assembling into the complex.

Intramolecular disulfide bonds have been shown to form in the cytoplasmic domains of several of the entry proteins: A16 [78], G9 [83], A21 [79], L5 [81] and A28 [85]. Interestingly, repressing the E10 protein, an essential component of the VACV cytoplasmic disulfide bond pathway, had no effect on assembly of the EFC. Therefore disulfide bonds do not appear to be important for assembly of the complex, however because E10 is essential for virus morphogenesis the role of disulfides bonds in the entry of the virus has not been assessed. With eight proteins forming the EFC it is likely that a number of protein interaction are required for formation of the entry complex, however these interactions remained to be defined. In the absence of A16, the H2 and A28 entry proteins were observe to remain associated, suggesting the two proteins may interact within the EFC [84].

In addition to the proteins of the EFC, several other viral proteins have been suggested to be important for MV entry. The ability to isolate neutralizing antibodies to the A27 and L1 proteins suggested that these two proteins might be important for virus entry [86-88]. Analysis of the A27 protein indicated a role in the wrapping of

MV to form WV. Viruses lacking A27 remained infectious, indicating the A27 antibody may inhibit virus entry through steric hindrance [71, 72]. The role of the L1 protein in virus entry has not been investigated because the protein is required for virus morphogenesis preventing isolation of virus particle deficient in L1 [89].

The L1 protein shares 20% amino acid identity with F9, suggesting these two proteins may have had a common gene ancestor (i.e. one of the proteins arose from gene duplication of the other). Unlike L1, there is no block in morphogenesis in the absence of F9 and all of the forms of the virus are produced. Virus particles lacking F9 bind to the cell, however their cores do not penetrate into the cytoplasm [90]. This phenotype is identical to that observed for the proteins of the EFC. The F9 protein was shown to interact with proteins of the EFC, although the interaction was difficult to detect suggested the protein may only associate with a portion of the entry complexes. The functional role of F9 is consistent with its interaction with the EFC, although the significance of the interaction remains to be determined.

The MV particle of VACV has been shown by EM to fuse at the cell surface [91-93]. Virus particles are also observed in intracellular vesicles suggesting entry through an endocytic route [94, 95]. Several recent studies investigating MV entry have used a recombinant VACV that expresses the firefly luciferase under the control of an early viral promoter [94]. Luciferase (Luc) expression, which can be detected as early as 30 minutes postinfection, is measured and used to quantify virus entry. MV bound to the cell surface and exposed to low pH display a 10-fold increase in Luc activity compared to an identical sample treated with neutral pH [94]. The ability of low pH to influence virus entry is consistent with entry through an

endocytic route, with low pH treatment mimicking the pH reduction that occurs within the endosome. Inhibitors of endosomal acidification decrease entry of MV particles by 60-80% depending upon the cell type [94]. The effect of inhibiting endosomal acidification can be particularly rescued by treating the virus bound to the cell surface with low pH. The low pH presumably triggers fusion of the particle with the cell surface thereby bypassing the requirement of the low pH within the endosome.

While most of the studies on the entry of VACV have focused on the MV particle, few studies have looked at EV. Entry of the EV, which contains one additional membrane with respect to the MV particle, is difficult as the EV membrane is fragile and prone to damage during purification. EV was proposed to enter by endocytosis, with the low pH environment of the endosome disrupting the EV membrane [96]. Recently EV has been shown to enter the cell by directly fusion with the plasma membrane. The EV membrane is disrupted by soluble GAGs, to release the MV particle [97]. The EV membrane serves as an accessory during entry of EV with virus-cell fusion depending on the EFC in the MV membrane.

1.2.3 Virus gene expression.

Virus entry is followed by release of the virus core into the cytoplasm. The core is transported on microtubules to a perinuclear site within the cytoplasm [98]. Disassembly of the core, which releases the virus genome into the cytoplasm, requires early gene products and is prevented by protein synthesis inhibitors [99]. The virus core contains all of the enzymes and factors required for viral transcription and early viral mRNA synthesis occurs independent of *de novo* protein synthesis. The

transcriptional apparatus of VACV has been extensively characterized by isolating the enzymes present within the virus core. An extract from the virus core is able to synthesize RNA *in vitro* and the resulting mRNA is capped at its 5' end and contains a 3' poly(A) tail [100].

Virus genes are divided into prereplicative and postreplicative classes depending on whether they are expressed before or after viral DNA replication. Early viral gene expression occurs prior to DNA replication, while intermediate and late gene expression occur after viral DNA replication. Class specific promoters and transcription factors coordinate expression from each class. Early transcription factors are the products of late viral genes that are packaged into the virus particle during the previous infectious cycle. Early viral gene expression occurs shortly after the virus enters into the cytoplasm with RNA/DNA hybridization studies suggesting nearly 50% of the genome is transcribed during this early phase [101, 102].

Intermediate transcription factors are the products of early genes, however intermediate transcription is delayed until after viral DNA replication. Inhibitors of viral DNA replication block intermediate transcription [44]. Interestingly, an intermediate promoter transfected into virally infected cells is transcribed even under conditions in which viral DNA replication is inhibited. This suggests the template for intermediate gene expression is not accessible until after DNA replication, likely indicating the newly replicated DNA serves as the template [44]. Intermediate transcription *in vitro* requires three viral factors: viral intermediate transcription factor (VITF) VITF-1, VITF-2 [41], and VITF-3. The E4L gene encodes VITF-1 [40], while VITF-3 is a heterodimer of A8R and A23R [43]. In contrast, VITF-2 is a

heterodimer of two cellular proteins: Ras-GTPase-activating protein SH3 domain-binding protein (G3BP) and p137 [103]. Both G3BP and p137 are found in distinct regions of the viral factory along with the intermediate transcription factor A23R. G3BP, p137 and A23R localized to similar regions of the viral factories as viral mRNA and two translation initiation factors: eIF4E and eIF4G. These findings suggest virus transcription and translation is coordinated within the virus factory [104].

The viral late transcription factors are the products of intermediate genes. Three viral proteins are required for the transition from intermediate to late gene expression: A1 [105], A2 [106] and G8 [107]. *In vitro,* the H5 protein has been shown to enhance late viral gene transcription [108]. Late gene expression continues until the cellular resources are depleted or cell lysis occurs.

VACV early, intermediate and late gene promoters are located immediately upstream of viral genes. The poxvirus promoters are conserved, with the promoter from one type of poxvirus functioning within cells infected with a different poxvirus. The features of both early and late VACV promoters have been characterized by single nucleotide substitution. Early promoters contain an A+T rich core sequence between –13 and –28, followed by a spacer region of 12 nucleotides and initiation of transcription starting with an A or G nucleotide (Note: +1 is the transcription start site) [109]. Late promoters contain a highly conserved TAAAT sequence with transcription initiating within the A triplet of TAAAT [110]. The TAAAT is commonly followed by a G nucleotide to form TAAATG, with ATG serving as the initiating codon for protein synthesis. Intermediate promoters contain an important

core region from –26 to –13 and have a conserved sequence TAAA, with transcription initiating within the A triplet [111]. The 5' of intermediate and late mRNA contains a poly(A) leader of 30-40 bps [112, 113]. The poly(A) leader may arise as a consequence of the polymerase stuttering within the AAA found in both intermediate (TAAA) and late promoters (TAAAT). Although the poly(A) leader is generally absent from early mRNA, several early promoters contain a TAAAT sequence and analysis of the 5' end revealed a poly A tract, although the length of the leader was reduced to only 15-20 bps [114]. The significance of the 5' poly(A) leader is unknown, but it may have a role in enhancing translation of the mRNA.

The length of the mRNA transcript varies depending on the transcription class. Early transcripts have a defined length resulting from transcription termination downstream of the sequence TTTTTNT, where N is any nucleotide including T [115]. The termination sequence is recognized within the nascent mRNA transcript [116] and transcription ends 20-50 bps after the sequence. Intermediate and late mRNA lack defined termination signals resulting in mRNA of heterogeneous length [117, 118]. Several late viral mRNAs undergo cleavage at their 3' UTR [119]. The activity responsible for *in vitro* cleavage of the 3' UTR copurified with the H5 protein. However, a recombinant H5 protein expressed in *E. coli* was unable to cleave the 3'UTR suggesting there may be additional factors important for cleavage *[120]*.

1.2.4 Genome replication.

VACV encodes most, if not all, of the proteins required for replication of its genome. Enucleated cells support replication of the virus, albeit at a reduced rate [121, 122]. VACV infected cells form discrete cytoplasmic bodies known as "viral

factories" that localize adjacent to the nucleus and stain densely with fluorescent DNA dyes. The viral proteins important for DNA replication are synthesized from early genes and viral replication begins within 1 to 2 h postinfection. The viral proteins important for DNA replication include, but are not limited to, the DNA polymerase (E9) [123], uracil DNA glycosylase (D4) [124], the protein kinase (B1) [125, 126], nucleic acid-independent nucleoside triphosphatase (D5) [127, 128], and a viral DNA processivity factor (A20) [62, 129]. The B1 kinase phosphorylates the cellular protein barrier to auto integration factor (BAF) [130]. BAF is suggested to bind to the viral DNA and inhibit its replication. B1 phosphorylation of BAF is thought to prevent the protein from binding the viral DNA, although the exact mechanism by which BAF inhibits DNA replication is unknown [131]. The viral D5 protein shares limited homology with the archaeoeukaryotic primase superfamily [132] and purified recombinant D5 protein was shown to catalyze oligoribonucleotide synthesis consistent with a role as a primase [133]. The D4 and A20 proteins interact and are important for processivity of the viral DNA polymerase *in vitro* [134].

The mechanism of VACV DNA replication is poorly characterized. Attempts to identify a viral origin of replication were unsuccessful and instead showed the virus is able to replicate plasmid DNA devoid of any viral genomic DNA [135]. Plasmid DNA is thought to replicate by a rolling circle mechanism within the viral factory and was shown to depend on the same viral proteins essential for genome replication [136]. The current model of VACV DNA replication hypothesizes that the viral DNA replicates by a rolling hairpin mechanism, with nicking of the virus genome within hairpin termini serving as a primer for DNA replication. The

replication of both the viral genome and plasmid DNA results in long DNA concatemers (i.e. direct repeats of the genomic DNA). An A/T rich DNA sequence located near the hairpin termini of the viral genome is important for resolution of the plasmid DNA concatemers [137]. This sequence is conserved amongst poxvirus, suggesting an important role in resolution of viral genome concatemers. Resolution of the viral concatemers requires the action of a late viral gene product encoding a holiday junction resolvase (A22) [138]. The purified A22 protein resolves synthetic holiday junctions *in vitro* and repression of the A22 gene prevents resolution of genome concatemers [139, 140]. Following DNA replication, the virus transition to intermediate and late gene expression in which the replicate viral genome is packaged into the nascent virus particle.

1.2.5 MV morphogenesis.

Assembly of VACV occurs within cytoplasmic viral factories. The various stages of MV morphogenesis have been defined by electron microscopy. The first structures of virus morphogenesis observed by electron microscopy are membrane crescents, although the cellular organelle from which the membrane crescents are formed remains controversial. Some studies propose the membrane is synthesized de novo [22] or originates from the ER/Golgi intermediate compartment [25], while more recent evidence favors the idea that the membrane crescents are derived from the ER membrane [141]. Formation of membrane crescents does not occur in the absence of the viral proteins F10 [142], A11 [143], H5 [144] or G5 [63]. Membrane crescents associate with an electron dense granular viroplasm containing viral proteins destined for the viral core. Association of membrane crescents with the

viroplasm requires a complex of seven proteins: F10, A30, G7, J1, D2, D3 and A15 [145].

Membrane crescents evolve into spherical immature virus (IV) particles. The transition from membrane crescent to IV is inhibited by the antibiotic rifampicin. Viral mutants resistant to rifampicin have been isolated and the mutations map to the D13L gene [146]. D13 forms a honeycomb lattice around the IV, but is not packaged into the MV particle. Instead, D13 may serve as a scaffold for the assembly of IV [147]. The viral genome is packaged into the virus core at the IV stage. Two viral proteins, A32 [148] and I6 [149], have been shown to be important for encapsidation of the viral genome.

The transition from IV to MV is poorly defined, but is associated with the proteolytic cleavage of viral membrane and core proteins. VACV has two predicted proteases encoded by G1L and I7L. G1L is a predicted metalloprotease with an essential role in the transition from IV to infectious MV [150], however G1 is not required for cleavage of the viral core proteins. The I7 protein shares homology with other known cysteine proteases and is responsible for cleavage of the viral core proteins A3, A10 and L4 as well as the viral membrane protein A17 [151]. There are several other proteins, including A12 and G7, which also undergo proteolysis. The I7 protease recognizes a consensus AG/X motif [(/) denotes site of cleavage], however other undefined factors influence substrate specificity. For example, the F10 protein contains an AG/X motif, yet does not appear to be cleaved. When synthesis of I7 is repressed, cleavage of the viral core and membrane proteins does not occur and noninfectious irregular virus particles containing an aberrant core structure are formed.

1.2.6 Formation of Wrapped and Extracellular virus.

There are at least eight viral proteins that localize to either the EV or the WV membrane: A33, B5, A34, A56, K2, and F13 are found in the EV membrane, while A36 and F12 specifically localize to the WV membrane. Wrapping of MV is severely compromised in the absence of the EV proteins F13 [152] and B5 [153, 154] as well as the MV protein A27 [155]. WV are transported to the periphery of the cell on microtubles [156]. In the absence of A36, WV transport is impaired, suggesting a role for this protein in WV transport [157]. This was supported by yeast two hybrid screening which identified an interaction between A36 and the light chain of the microtubule motor protein kinesin [158]. A more severe defect in WV transport was noted in the absence of F12, indicating A36 is not solely responsible for transport of WV to the cell surface [159]. Upon reaching the cell surface the WV particle undergoes exocytosis and loses one of the two wrapping membranes to form EV.

The virus recruits cellular machinery to form virus tipped actin tails important for spread of the virus [160]. In the absence of actin tails, the virus forms small plaques, indicating an important role for actin tails in virus-cell spread. Actin tail formation requires multiple WV and EV proteins including A36 [161], A33 [162] and A34 [163]. Phosphorylation of two tyrosine residues within A36 is critical for the recruitment of the cellular proteins required for actin tail formation [164].

1.3 Virus Fusion and Regulation of Infected Cell Fusion

1.3.1 Low pH induced cell-cell fusion.

During a typical VACV infection there is very little fusion between infected cells, however a brief exposure to a pH below 6 induces extensive cell-cell fusion. There are two forms of low pH cell fusion: i) fusion from without and ii) fusion from within. Fusion from without requires adsorption of a large amounts of purified virus (MOI 300-500) to the cell surface [165, 166]. Fusion from within occurs at late times during infection and requires cell surface EV [72, 152]. Following exposure to low pH, cell fusion develops over several hours, while a similar treatment with neutral pH fails to trigger cell fusion. The ability of VACV to induce cell fusion following low pH is characteristic of viruses that enter through a low pH endocytic route, with low pH mimicking the pH drop in the endosome and stimulating virus fusion at the plasma membrane instead of the endosome. It is thought that low pH activates the virus entry machinery and triggers fusion of the virus membrane with the plasma membrane. Consequentially, the viral proteins formally in the viral membrane are relocated to the cell membrane positioning the EFC to mediate fusion with adjacent cells, eventually forming large multinucleated syncytia (Figure 1-4). Neutralizing antibodies are able to inhibit the development of low pH cell-cell fusion consistent with the process of cell-cell fusion being closely related to virus cell fusion [86-88]. More recently VACV entry has been shown to require a multiprotein EFC, although it is uncertain

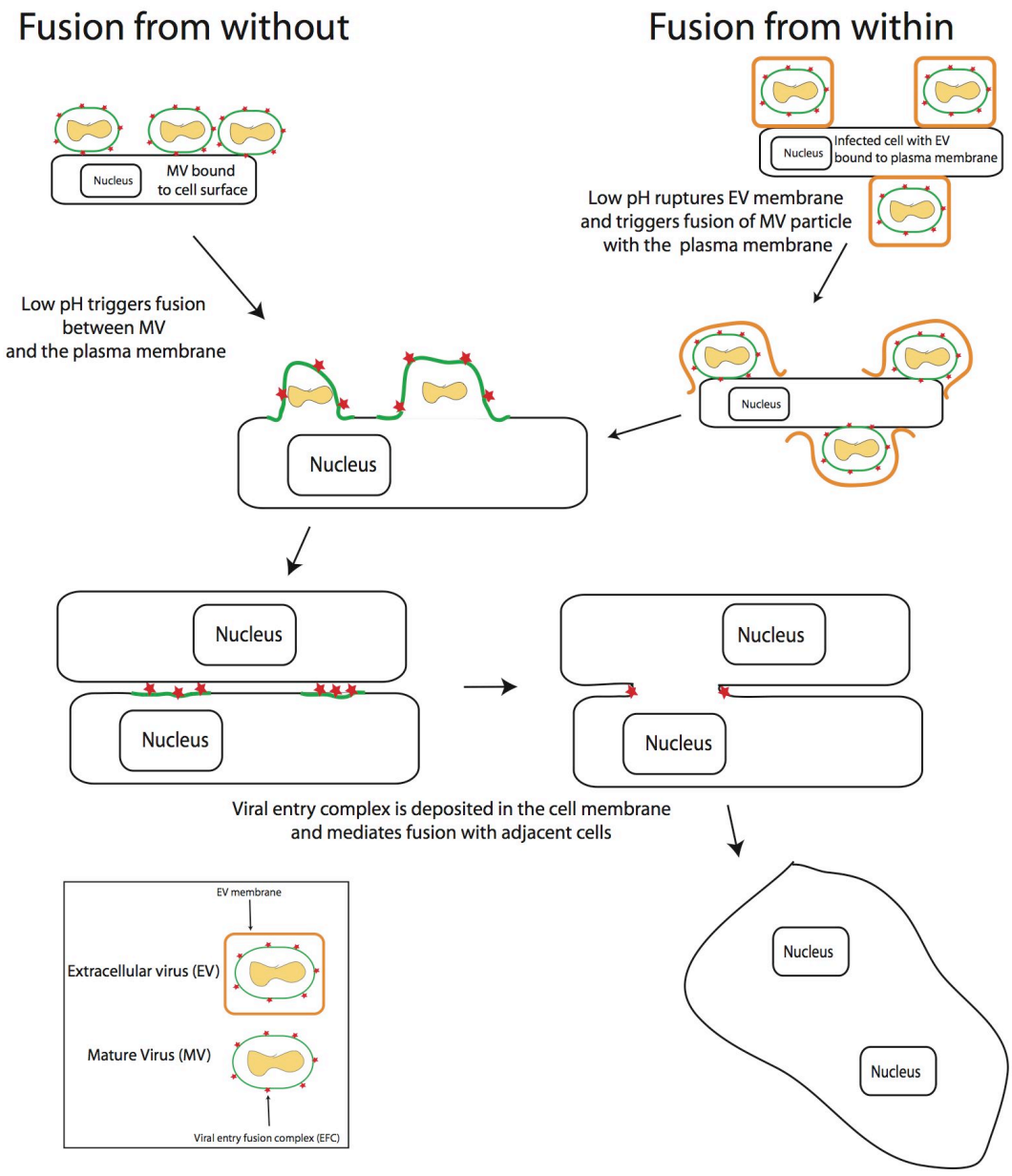

Figure 1-4: Model of VACV induced low pH cell-cell fusion.
(Fusion from without.) A large number of mature virus (MV) particles is bound to the surface of an uninfected cell and then treated briefly with low pH (5.5 or below) medium. Low pH synchronizes MV fusion with the plasma membrane and deposits the viral membrane with the entry fusion complex (EFC) into the cell membrane. The EFC within the cell membrane mediates fusion between adjacent cell leading to formation of multinucleated syncytia. **(Fusion from within.)** Cells infected with VACV possess surface extracellular virus (EV). Following treatment with low pH medium the EV membrane ruptures to expose the MV particle, which fuses with the cell membrane.

whether the EFC directly mediates fusion or instead is important for assembly of the viral fusion apparatus. Never the less, the EFC is required to mediate all forms of low pH cell fusion.

1.3.2 A56R and K2L.

In addition to low pH cell fusion described above, VACV triggers neutral pH cell fusion when either the VACV A56R [167] or K2L [168-170] gene is absent. A56 is known as the viral hemagglutinin for its ability to agglutinate red blood cells of certain species. K2 is one of three viral proteins with homology to serine protease inhibitors (SERPINS) and has been referred to as serine protease inhibitor 3 (SPI-3), [171]. Tissue culture cells infected with closely related cowpox virus with deletions of the cowpoxvirus HA (VACV A56R homologue) or SPI-3 of cow pox (VACV K2L homologue) also develop extensive cell-cell fusion. Neutral pH cell fusion is induced by specific monoclonal antibodies that react with A56 [172] or K2 [173]. The mechanism by which the A56 and K2 antibodies trigger cell fusion is unknown, although the antibodies may perturb protein interactions of A56 or K2 required for inhibition of cell fusion. Neutralizing antibodies (which normally prevent virus infection) added to cultures following infection with VACV lacking A56 or K2 inhibit neutral pH cell fusion [169]. Neutral pH cell fusion depends on EV, which is also required for low pH fusion from within, suggesting the two forms of fusion occur by a similar mechanism [152].

Both A56 and K2 localize to the plasma membrane of infected cells and are incorporated into the membrane of EV [174]. K2 binds to A56 and this interaction is required for proper localization of K2 [173]. K2 lacks a membrane anchor and is

secreted from infected cells in the absence of A56. The K2 protein is glycosylated, however point mutations that abolish the four putative N linked glycosylation sites have no effect on the ability of K2 to inhibit cell-cell fusion [174]. A56 possesses both N and O-linked glycosylation [175], but the role of glycosylation in the anti-fusion activity of A56 has not been studied. Although A56 is an integral membrane protein in the EV particle, the protein has no role in formation of EV and the virus is able to form virus tipped actin tails in the absence of A56 or K2. Immunoelectron microscopy reveals that A56 is inconsistently incorporated into the EV membranes with approximately one third of EV particles lacking A56, although the significance of this is unknown [176]. Deletion of A56 caused a slight attenuation of disease in an intranasal mouse model [177], while no effect on viral virulence was observed when K2 was deleted [168]. Recombinant K2 was shown to function as a SERPIN *in vitro* [178]. The cowpox SPI-3 and myoxoma SERP1 both exhibit similar proteinase inhibition profiles *in vitro*, however the myoxoma SERP1 is unable to complement the anti-fusion activity of SPI-3 [179]. This result may have been predicted as it was shown earlier that point mutations that abolish the SERPIN activity of the cowpox SPI-3 (VACV K2L homologue) *in vitro* have no effect on the anti-fusion activity of the protein [180].

The mechanism by which A56 and K2 regulate cell-cell fusion is poorly understood. Curiously, cell-cell fusion only occurs among infected cells that are deficient for A56. This was established by infecting cells with an A56 deletion virus and a separate cell population with wild-type virus that expressing A56. The infected

cells were mixed and cell fusion was noted to occur only among hemagglutination negative cells [167].

The deletion of A56 and K2 causes neutral pH cell fusion which is thought to develop as a result of reinfection by cell surface EV. There are several stages at which A56 and K2 could prevent EV reinfection. Entry of EV depends on expression of the EFC within the MV membrane and is only exposed upon rupturing of the EV membrane. Since both A56 and K2 are found in the EV membrane the proteins could stabilize the membrane to prevent premature rupturing. However, since the EV membrane appears to be quite fragile even in the presence of A56 and presumably K2 [181] inhibition of fusion is probably due to an alternative mechanism. Both A56 and K2 are abundant in the plasma membrane of infected cells and there is evidence to suggest this localization is important as cell fusion develops with (i) monoclonal antibodies to A56 [172] or SPI-3 [173] or when (ii) the membrane anchor of HA is removed [173] or (iii) the signal sequence of SPI-3 is removed [174]. This thesis will define the protein interactions of VACV A56R and K2L with the aim of elucidating a mechanism by which these proteins regulate infected cell fusion.

Chapter 2: The VACV A56R and K2L associate with proteins of the Multicomponent Entry/Fusion Complex

2.1 Introduction

VACV is the prototypical member of the *Orthopoxvirus* genus. Poxviruses are large DNA viruses with a brick shaped virus particle [3]. VACV replicates in the cytoplasm of the cell and produces several types of infectious particles, the simplest of which is MV. The MV particle is surrounded by a lipid membrane that contains nearly 20 viral membrane proteins [182]. The virus core houses the viral genome along with all of the enzymes and factors necessary for early RNA synthesis. The MV particle remains intracellular until lysis, however a portion of MV acquires a double membrane derived from modified trans-Golgi or endosomal cisternae to form WV [16], which is transported on microtubles to the cell surface and released by exocytosis [183]. The resulting EV particle is essentially an MV with one additional membrane.

Entry of the MV particle has been shown to occur by fusion with the plasma membrane [91], while entry is also enhanced by briefly lowering the pH of the medium below 6, characteristics of entry through a low pH endocytic route [94]. Chemical inhibitors of endosome acidification reduce MV entry by 80%, but can be partially rescued by treatment with low pH [94]. Low pH treatment of infected cells triggers cell-cell fusion [152, 165, 166]. Cell fusion also occurs spontaneously at neutral pH when cells are infected with VACV or the closely related cowpox virus in which the A56R gene encoding the viral hemagglutinin (HA) [167] or K2L encoding a serine protease inhibitor (SPI-3) [168, 169, 170] is mutated or deleted. The A56R

gene of VACV is a type-I membrane protein that localizes to the EV and plasma membrane [172, 184]. The K2L gene (SPI-3) does not contain a membrane anchor and associates with the EV and plasma membrane through an interaction with A56R [173, 174]. The anti-fusion activity requires both proteins to localize to the cell membrane. This is supported by the following evidence as syncytia form when (i) poxvirus-infected cells are incubated with antibodies to HA [172] or SPI-3 [173], (ii) the membrane anchor is removed from HA [173] or (iii) the signal sequence of SPI-3 is removed [174]. A56 possess no putative catalytic motifs and the serine protease inhibitory activity of SPI-3 is not required for fusion inhibition [180].

Virus-cell fusion and low pH-induced cell-cell fusion requires the same EFC found in the MV membrane, which consists of at least the following eight viral proteins: A16, A21, A28, G3, G9, H2, J5 and L5 [84, 94]. The study described in this chapter investigates the role of the EFC in neutral pH cell fusion associated with deletion of A56R or K2L gene. Furthermore, tandem affinity purification is utilized to determine the protein interaction of A56 and K2 which may be important for their anti-fusion activity. This study has been previously described in reference [185]

2.2 Materials and Methods

2.2.1 Cell and virus propagation.

BS-C-1 (ATCC CCL-26) and RK13 (ATCC CCL-37) cells were grown in Minimum Essential Medium with Earle's balanced salt supplement (EMEM; Quality Biologicals, Gaithersburg, MD) containing 2 mM L-glutamine, 10% fetal bovine serum (FBS), penicillin and streptomycin. HeLa S3 (ATCC CCL-2.2) suspension

cells were cultured in Minimum Essential Medium, Spinner modification (Quality Biologicals) with 5% equine serum and L-glutamine. Unless specified, all recombinant viruses were derived from the Western Reserve (WR) strain (ATCC VR-1354; accession number AY243312). Virus stocks were prepared as described [186]. Viral titers were determined by plaque assay using a confluent monolayer of BS-C-1 grown in six-well cluster plate. A 10-fold serial dilution of virus stocks was prepared in EMEM containing 2.5% FBS, glutamine and antibiotics (2.5% EMEM). Medium was removed from the six-well plate and 0.5ml of serially-diluted virus inoculum was incubated 1 h at 37 ^{o}C and 5% CO_2. The cells were then overlaid with 2.5% EMEM containing 0.5% methylcellulose and incubated 48 h. The plaques were visualized by staining with 0.1% crystal violet (w/v) in a solution of 20% ethanol and deionized water.

2.2.2 TAP and mass spectrometry.

HeLa S3 cells (1.5 x10^9) were infected at a multiplicity of 5 plaque-forming units (PFU) and after infection for 24 h the cells were collected, washed once with ice-cold buffer (150 mM NaCl and 50 mM Tris-HCl pH 7.4), and lysed by incubating for 1 h at 4^{o}C in streptavidin binding buffer (SBB) (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl pH 7.4) with complete protease inhibitor (Roche, Indianapolis, IN). The lysate was centrifuged for 15 min at 3000 X g and the clarified supernatant was collected. The latter, except for 0.3 ml reserved for later analysis, was added to 0.5 ml to 1 ml of streptavidin sepharose (GE Healthcare, Piscataway, NJ) that had been washed with SBB and the mixture rotated overnight at 4oC. The beads were washed 3 times with 10 ml of ice cold SBB and the bound proteins eluted by 3 washes with 1

ml of SBB containing 1mg/ml of D-Biotin (USB corporation; Cleveland, OH). The 3 ml of streptavidin eluate was supplemented with Mg acetate, imidazole, and $CaCl_2$ to final concentrations of 1 mM, 1 mM and 2 mM, respectively. Calmodulin Sepharose (0.5 ml to 1 ml of packed resin; GE Healthcare) was washed with calmodulin binding buffer (CBB; which consists of SBB supplemented with Mg acetate, imidazole and CaCl2 at final concentrations of 1 mM, 1 mM and, 2 mM, respectively). Calmodulin Sepharose was added to the supplemented streptavidin eluate along with an additional 2 ml of CBB and the mixture rotated overnight at 4^{o}C. The beads were washed 3 times with 10 ml of CBB and 3 times with 0.75ml of SBB containing 25 mM 2-[2-[2-[2-[bis(carboxymethyl)amino]ethoxy]ethoxy]ethyl-(carboxymethyl)amino]acetic acid (EGTA) to elute proteins. The proteins in the calmodulin eluate were concentrated by trichloroacetic acid precipitation and then resuspended in lithium-dodecyl sulfate (LDS) sample buffer (Invitrogen, Carlsbad, CA) containing NuPage sample reducing agent (Invitrogen) and separated on a 4-12% NuPage gel (Invitrogen) with 2(N-morpholino)ethanesulfonic acid buffer. Gels were stained with Coomassie blue (GelCode blue stain Reagent, Pierce, Rockford, IL) and bands of interest were excised from the polyacrylamide gel and subsequently digested with trypsin. Tandem mass spectrometry and database searching were performed at the National Institute of Allergy and Infectious Diseases core facility.

2.2.3 Recombinant virus construction.

The following recombinant viruses were constructed for this study (Table 2-1): vK2TAP, vA28TAP, and vA56TAP vA28iΔA56 vA28iΔK2 vΔA56 and

vK2TAPΔA56 vΔA56ΔK2, vA56TAPΔK2 and vA28TAPΔK2, vsA56TAPi, vK2iΔA56, vsA56TAPiΔC3, vT7lacOIΔF13

vK2TAP, vA28TAP, and vA56TAP were constructed using DNA encoding (i) K2L, A28L or A56R genes with 300 bps of downstream flanking region, (ii) the TAP tag derived from pCTAP (Stratagene, La Jolla, CA), and (iii) a strong VACV promoter adjacent to the gene for a fluorescent protein namely *Heteractis crispa* red fluorescent protein 1 (HcRed) from Clontech (Mountain View, CA) for K2TAP and enhanced green fluorescent protein (EGFP) from Clontech for A28TAP. The constructs were prepared by overlapping PCR (Accuprime Pfx, Invitrogen, Carlsbad, CA) so that the TAP tag sequence was appended immediately before the stop codon of the modified gene. The gene encoding the fluorescent reporter was inserted between the stop codon and the 300 bps flanking region. The PCR product was cloned into pCR-BluntII-TOPO (Invitrogen) and the TOPO plasmids encoding K2TAP and A28TAP were transfected with Lipofectamine 2000 (Invitrogen) into BS-C-1 cells that had been infected 1 h earlier with 1 PFU per cell of VACV. Parental and recombinant viruses were distinguished by fluorescence microscopy and three rounds of plaque isolation clonally purified the latter. vA56TAP was constructed as above except that vΔA56 was used as the parental virus and the EGFP gene in the A56R locus was replaced with A56TAP; recombinant viruses were distinguished from the parental virus by the absence of green fluorescence.

Deletion of the A56R and K2L genes was achieved by replacing the open reading frames with the DNA encoding EGFP or HcRed, respectively. Briefly, 300 bps of DNA corresponding to the left and right flanks of A56R and K2L were fused

by recombinant PCR to the fluorescent protein gene. To construct vA28iΔA56 and vA28iΔK2, BS-C-1 cells were infected in the presence of 100 μM IPTG with vA28i [85] at 1 PFU per cell and then transfected with the respective A56 or K2 deletion plasmid. Recombinant viruses were distinguished from parental virus by fluorescence microscopy and clonally purified by three rounds of plaque isolation. vΔA56 and vK2TAPΔA56 were constructed by deletion of the A56R gene from VACV strain WR and vK2TAP, respectively, utilizing an approach analogous to that described for vA28iΔA56. The K2L gene was deleted from vΔA56, vA56TAP and vA28TAP as described for vA28iΔK2 and the resulting viruses were designated vΔA56ΔK2, vA56TAPΔK2 and vA28TAPΔK2, respectively

vK2iΔA56 was designed to inducibly over express an influenza HA epitope-tagged inducible K2. The corresponding transfer plasmid was assembled by recombinant PCR from: (i) 200 bps of DNA upstream of the A56R gene, (ii) bacteriophage T7 promoter and encephalomyocarditis virus leader sequence containing an internal ribosome entry site from pVote 1 [187], (iii) K2L with a C terminal influenza HA epitope tag sequence, (iv) HcRed gene regulated by a strong VACV promoter, and (v) 200 bps of DNA downstream of A56R. The final PCR product was cloned into PCR-BluntII-TOPO and sequenced. The K2L expression plasmid was transfected into cells infected with vT7lacOI [66]and the vK2iΔA56 plaques were detected by fluorescence microscopy. vK2iΔA56 was clonally purified by three rounds of plaque isolation.

vsA56TAPi, a virus encoding an inducible A56 that is secreted from cells because of deletion of its transmembrane segment and contains TAP, V5 and 10-

histidine tags, was constructed. The virus vT7lacOIΔF13 was created by first deleting the F13L gene from vT7lacOI in order to provide subsequent plaque selection [188]. A DNA segment was assembled by overlapping PCR using DNA encoding (i) the T7 promoter, encephalomyocarditis leader sequence, and E. coli lac operator from pVote 1 to provide inducible expression and cap-independent translation, (ii) A56R gene with a V5 tag inserted between codons 18 and 19 and replacement of codons 280 to 315 with a TAP tag sequence followed by 10 tandem copies of a histidine codon, and (iii) T7 termination sequences from pVote 1. This DNA was then cloned into pRB21 [188] and the resulting plasmid was used to transfect BS-C-1 cells that had been infected with vT7lacOIΔF13. The new recombinant virus vsA56TAPi formed large plaques and was clonally purified. The C3L gene was deleted from vsA56TAPi and vK2iΔA56 in a similar fashion as described for deletion of A56 to construct vsA56TAPiΔC3 and vK2iΔA56ΔC3, respectively.

2.2.4 Western blotting.

Samples subjected to TAP from 2-3 X 10^8 HeLa S3 cells were separated by loading onto a 10% or 4-12% NuPage Bis-Tris gel (Invitrogen). Following electrophoresis, the proteins were transferred to nitrocellulose membranes and blocked with Tris-buffered saline supplemented with 5% nonfat dried milk and 0.05% Tween-20 (TBST) for 1 h at room temperature. The membranes were then incubated with the appropriate primary antibody, washed, incubated with horseradish peroxidase-conjugated secondary antibodies (GE healthcare, Piscataway, NJ), and analyzed with the SuperSignal West Dura or Femto Maximum Sensitivity Substrate chemiluminescence reagents (Pierce, Rockford, IL).

Table 2-1. Recombinant VACV

Recombinant virus	Parent [Reference]	Description
vΔA56	VACV WR	Deletion of A56R
vΔA56ΔK2	vΔA56	Deletion of A56R and K2L
vA28TAP	VACV WR	A28-TAP[a]
vA28iΔA56	vA28i [80]	Inducible A28-HA[b]; deletion of A56R
vA28iΔK2	vA28i [80]	Inducible A28-HA[b]; deletion of K2L
vA28TAPΔK2	vA28TAP	A28-TAP[a] tag; Deletion of K2L
vA56TAP	vΔA56	A56-TAP[a]
vA56TAPΔK2	vA56TAP	A56-TAP[a]; deletion of K2L
vsA56TAPi	vT7lacOIΔF13	
vsA56TAPiΔC3	vsA56TAPi	Inducible and secreted A56-TAP[c]; deletion of C3L
vK2iΔA56	vT7lacOI [66]	Inducible K2-HA[b]; deletion of A56R
vK2TAP	VACV WR	K2-TAP[a]
vK2TAPΔA56	vK2TAP	K2-TAP[a]; Deletion of A56R
vT7lacOIΔF13	vT7lacOI [66]	Inducible T7 polymerase; constitutive expression of *lac* repression; deletion of F13L

[a] TAP-tag at C-terminus
[b] C-terminal HA tag
[c] Transmembrane and cytoplasmic tail of A56 removed and replaced with TAP-tag

Primary and secondary antibodies were removed from the membrane by incubating with Restore Western Blot Stripping Buffer (Pierce) for 30 min at 55^{o}C.

2.2.5 Antibodies.

Rabbit polyclonal antisera used to detect VACV proteins were: anti-A21 [79], anti-L5 [81], anti-A16 [78], and anti-p4b/4b (R. Doms and B. Moss, unpublished). Antibody to the A28 was prepared by immunizing rabbits with purified recombinant protein provided by Gretchen Nelson, NIAID. K2 and A56 rabbit antisera were raised against a synthetic peptide PFDITKTRNASFTNKYGTKT derived from K2 amino acids 176-195 and SEKPDYIDNSNCSSVF derived from A56 amino acids 151-166 with the addition of a C-terminal cysteine for conjugation to keyhole limpet hemocyanin (Covance Research Products, Denver, PA). A monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Covance.

2.2.6 Synthesis and purification of soluble A56/K2.

Ten roller bottles of RK13 cells were coinfected with 1 PFU per cell each of vsA56TAPiΔC3 and vK2iΔA56ΔC3 for 2 h at 37^{o}C. After virus adsorption the cells were washed twice with Dulbeccos's phosphate buffered saline with calcium and magnesium (Quality Biological Inc.). Then 50 ml of Opti-Mem (Invitrogen) with 2 mM IPTG was added and the infection was allowed to proceed for 48 h. The medium was clarified by centrifugation and any remaining debris removed by filtering through a 0.45 μM membrane. The medium was supplemented with glycerol to 10% final concentration and NaCl to 400 mM. The protein was purified by binding to wheat

germ agglutinin agarose (Vector laboratories, Burlingame, CA) and eluted with 500 mM N-acetyl-α-D-glucosamine (Calbiochem, La Jolla, CA). The eluate was then bound to streptavidin Sepharose, washed with phosphate buffered saline and eluted with 1 mg/ml of biotin.

2.3 Results

2.3.1 Neutral pH Cell-Cell Fusion Requires the Entry/Fusion Complex.

The first goal of this study was to better understand neutral pH cell fusion that occurs following infection with VACV deleted for the A56R or K2L gene. Low pH cell fusion of VACV requires a functional EFC, however this complex has not been demonstrated to be required for neutral pH cell-cell fusion. The function of the proteins of the EFC in virus entry and fusion was initially characterized by constructing conditional lethal viruses in which gene expression was regulated by *E.coli lac* repressor [71, 78, 79, 81-85]. Repressing the synthesis of any of the entry proteins produced viruses with a similar phenotype, mainly an inability of the virus particle to penetrate the cell along with failure to trigger low pH cell fusion. The current strategy was to determine the effect of deleting the A56R or K2L gene from one of the inducible mutants of the entry complex. If spontaneous fusion of infected cells occurred when EFC gene was repressed, it would indicate an alternative pathway of cell-cell fusion.

The vA28i was used as the parental inducible virus because regulation of A28 expression was stringently repressed in the absence of IPTG and assembly of the EFC was prevented [85]. A56R and K2L genes were deleted individually from vA28i by

replacing the viral gene with the coding sequence for EGFP to form vA28iΔA56 and vA28iΔK2, respectively. DNA sequencing confirmed the deletion of the A56R or K2L gene. vA28iΔA56 and vA28iΔK2 stocks were prepared in the presence of IPTG so that virions possessed A28 and were therefore able to infect cells. After infection in the absence of IPTG, however, the progeny virions would lack A28 and be unable to spread to neighboring cells. Both recombinant viruses expressed EGFP regardless of the presence or absence of IPTG but only formed plaques under the former conditions. In the presence of IPTG, HeLa cells infected with vA28iΔA56 or vA28iΔK2 formed large multinucleated syncytia visualized by fluorescence microscopy (Figure 2-1). No cell fusion was observed in the absence of IPTG for either vA28iΔA56 or vA28iΔK2 even though the cells were infected as shown by expression of EGFP (Figure 2-1). The results showed the A28 protein, in addition to its role in virus entry and low pH triggered cell-cell fusion, was required for cell-cell fusion occurring at neutral pH in the absence of the A56R or K2L gene. It seems likely that the other components of the EFC would also be required for neutral pH cell fusion indicating the multicomponent EFC mediates both low pH and neutral pH virus-induced membrane fusion.

2.3.2 The anti-fusion proteins A56 and K2 interact with the viral EFC.

A56 contains no putative enzymatic motifs and the serine protease inhibitor active site of the K2 protein is not required to inhibit cell fusion. This suggested the proteins may regulate cell fusion through protein-protein interactions. To investigate possible protein interaction of the A56 protein, a recombinant VACV called

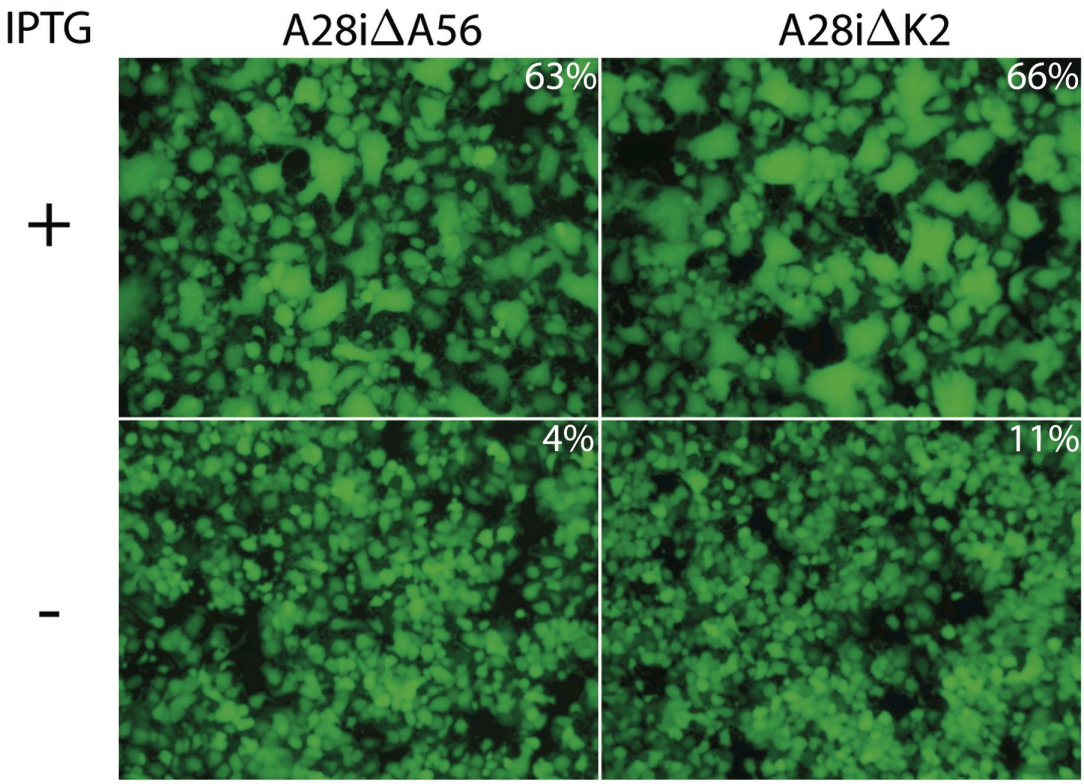

Figure 2-1: The EFC is required for neutral pH cell fusion. HeLa cells were infected with vA28iΔA56 or vA28iΔK2 in the absence (-) or presence (+) of IPTG. After 24 h, the cells were examined under an inverted fluorescence microscope to visualize cells expressing GFP encoded by the recombinant VACVs. The percentage of nuclei in syncytia, as defined by a cell containing 3 or more nuclei, is averaged from two independent experiments.

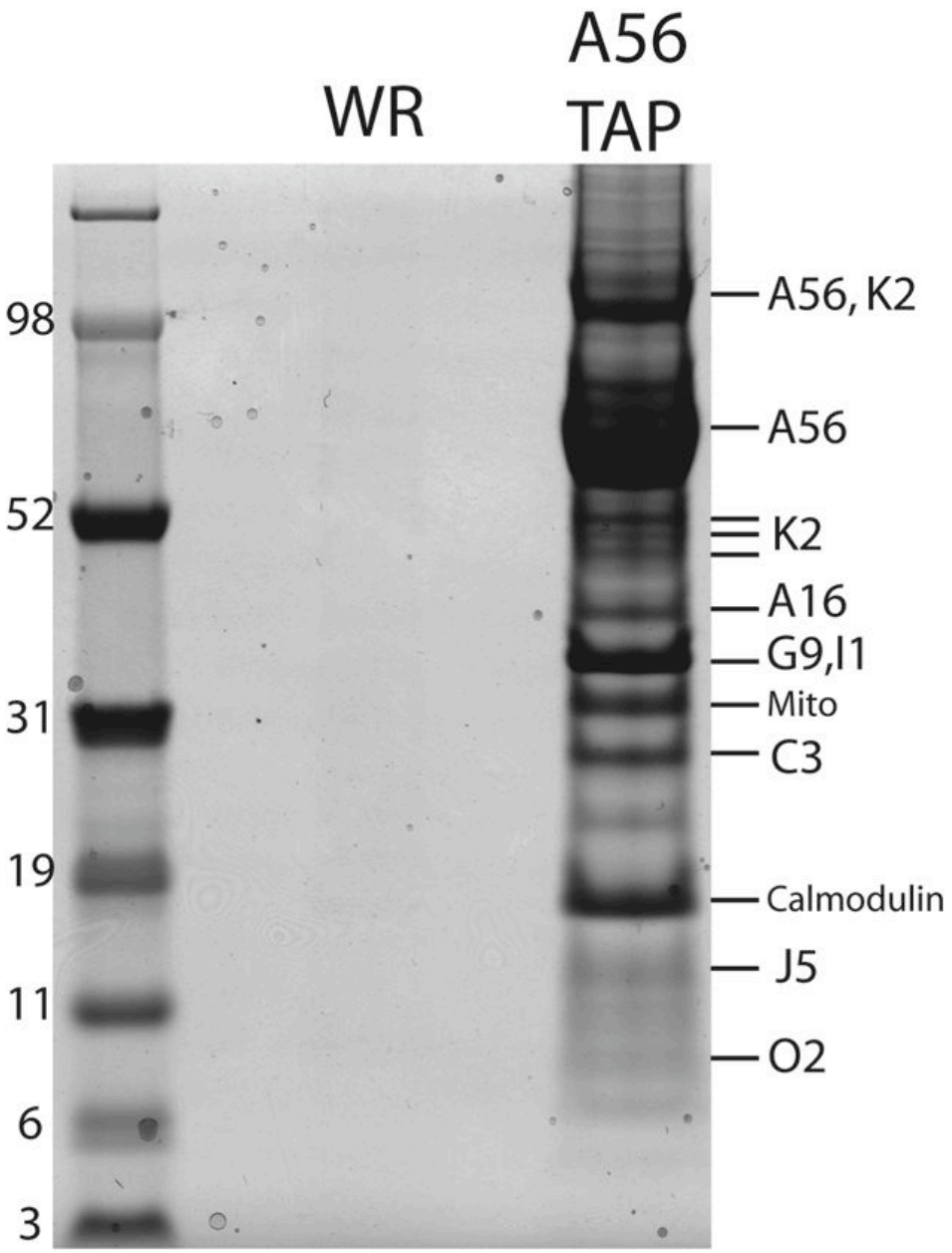

Figure 2-2: Tandem affinity purification of A56.
HeLa cells were infected with VACV WR or vA56TAP and the cells were disrupted with Triton X-100. The post-nuclear supernatants were purified successively on streptavidin and calmodulin affinity columns. The bound proteins were eluted, concentrated, resolved by SDS-PAGE and detected by staining with Coomassie blue. The protein bands were excised from the gel, digested with trypsin and analyzed by mass spectrometry. The identities of the proteins are indicated to the right of the stained bands. In one case, peptides corresponding to two proteins (G9 and I1) were obtained from the same band. Marker proteins with masses in kDa are indicated at the left.

vA56TAP was constructed in which codons for streptavidin and calmodulin binding peptides were fused in frame to the end of the A56R open reading frame. The TAP tag did not compromise the function of the A56 protein, as syncytia did not form when cells were infected with vA56TAP. Infected cells were lysed with Triton X-100 detergent and the post-nuclear supernatant was incubated with Sepharose beads linked to streptavidin. The resin was washed extensively and the bound proteins were eluted from the streptavidin beads by incubation with D-biotin. The eluate was subjected to a second affinity purification by incubating with Sepharose beads linked to calmodulin. The calmodulin Sepharose was washed and the bound proteins were eluted with EGTA. The purified proteins were concentrated, resuspended with a solution of LDS, resolved by polyacrylamide gel electrophoresis, and then visualized by staining with Coomassie blue. Cells infected with VACV lacking a TAP tag were purified in parallel to serve as a negative control. Multiple intensely stained bands were observed in the A56TAP sample, but were absent from the control. The protein bands were excised and then digested with trypsin followed by mass spectrometry to identify the peptides. The proteins corresponding to the observed peptides are indicated next to the bands in figure 2-2. One of the bands corresponded to the K2 protein, confirming an association with A56. The intensity of the K2 band, however, was much less than that of A56, even taking into account the difference in their masses. This suggests either a surplus of A56 relative to K2, not all of the K2 was bound to HA, or the complex partly dissociated during purification. Interestingly members of the EFC A16, G9 and J5 co-purified with A56. Several other proteins, namely calmodulin and the VACV products C3, I1 and O2 also copurified with A56

and were identified by mass spectrometry (Figure 2-2). Calmodulin undoubtedly came from the affinity beads. C3 is a secreted modulator of complement activation [58, 59] I1 is a DNA telomere binding protein [189], [190] and O2 is a non-essential glutaredoxin [191 {Rajagopal, 1995 #554, 192]. Although these additional bands were not detected in the control lane (Figure 2-2), any biological significance of the latter interactions with A56 remains to be determined.

Western blotting with specific antisera to A21, A28 and L5 was used to identify three additional EFC proteins recovered after TAP of A56 (Figure 2-3). It is likely that the other entry proteins G3 and H2 were also present, though antibodies to these proteins were not available to confirm this. Cells infected with wild type VACV lacking a tag on A56 did not associate with A21, A28 and L5 when subjected to TAP, even though these proteins were present in the starting material (Figure 2-3). Furthermore, antisera to K2 and A16 were used as positive controls and antisera to D8, an MV membrane protein not associated with the EFC, and the cellular protein GAPDH as negative controls. In this experiment a small amount of GAPDH was detected; however this was non-specific as it was not dependent on K2 (see next section). Collectively the mass spectrometry and Western blotting data indicated that at least 6 of the 8 EFC proteins were associated with A56 either directly or indirectly.

2.3.3 A56 does not bind the EFC in the absence of K2.

Regulation of infected cell fusion requires K2 as well as A56, yet the role of the individual proteins in the interaction with the EFC was unknown. Therefore the primary binding of the EFC may be mediated by either A56 or K2. To investigate if A56 alone was able to associate with the entry complex, the K2L gene of vA56TAP

was replaced with DNA encoding EGFP. As expected, cells infected with the K2L deleted virus vA56TAPΔK2 fused to neighboring cells. When extracts of cells infected with vA56TAPΔK2 were subjected to TAP in parallel with extracts of cells infected with vA56TAP, EFC proteins were only detected in the latter (Figure 2-3). Cells infected with vA56TAPΔK2 expressed A16, A21, A28 and L5 similar to those infected with either WR or A56TAP (Figure 2-3). The results suggested K2 is important for interaction with the EFC, possibly by direct association.

2.3.4 Both K2 and A56 are needed for association with the EFC.

The inability of A56 to associate with the EFC in the absence of K2 suggested the EFC interacts with A56/K2 through K2 and only indirectly with A56. To test this hypothesis, a recombinant VACV was constructed in which K2 has a TAP tag and A56 was deleted. The virus created in several steps, the first was to make vK2TAP, a recombinant VACV encoding K2 with a TAP tag appended to the C-terminus. A small increase in syncytia formation was noted in cells infected with vK2TAP suggesting the presence of the TAP tag slightly affected the function of K2, though the amount of cell fusion was well below the level of a K2 deletion virus. Western blot analysis of affinity purified K2 showed the protein co-purified with both A56 and components of the EFC and confirmed that the tag did not greatly compromise the function of K2 (shown later). To determine whether K2 was sufficient for interaction with the EFC the A56 gene was removed to construct vK2TAPΔA56. Cells infected with vK2TAPΔA56 formed large multinucleated syncytia consistent with the phenotype observed upon infection with an A56 deletion mutant. Initial experiments suggested the EFC did not associate with affinity purify

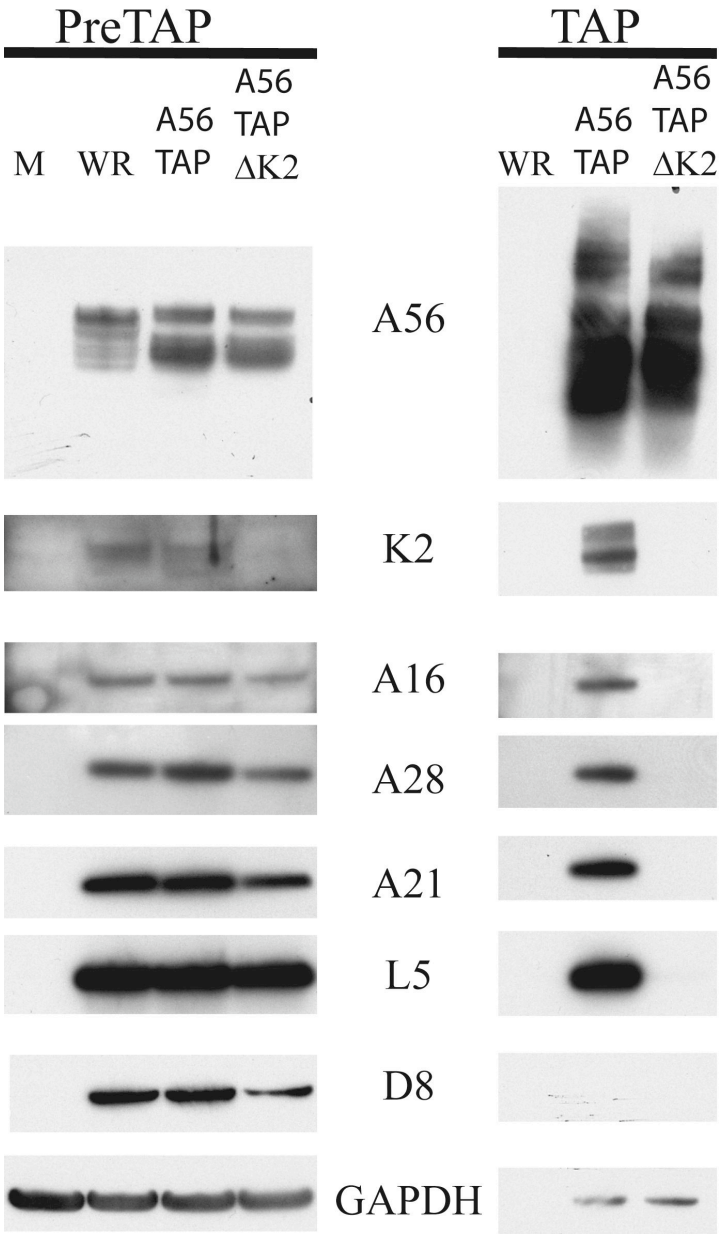

Figure 2-3: Western blot analysis of A56TAP.
Mock infected cells (M) or cells separately infected with VACV WR, vA56TAP or vA56TAPΔK2 were subjected to tandem affinity purification over successive streptavidin and calmodulin affinity resins. The bound proteins were eluted, resolved by SDS-PAGE, and detected by Western blotting with antibodies to the indicated proteins. Components of the EFC are represented by: A16, A28, A21, and L5; D8 is an MV protein and absent from the EFC; GAPDH is a cellular protein used as a control. Starting material is designated PreTAP, samples subjected to dual affinity purification are labeled TAP. Viruses are noted at the top.

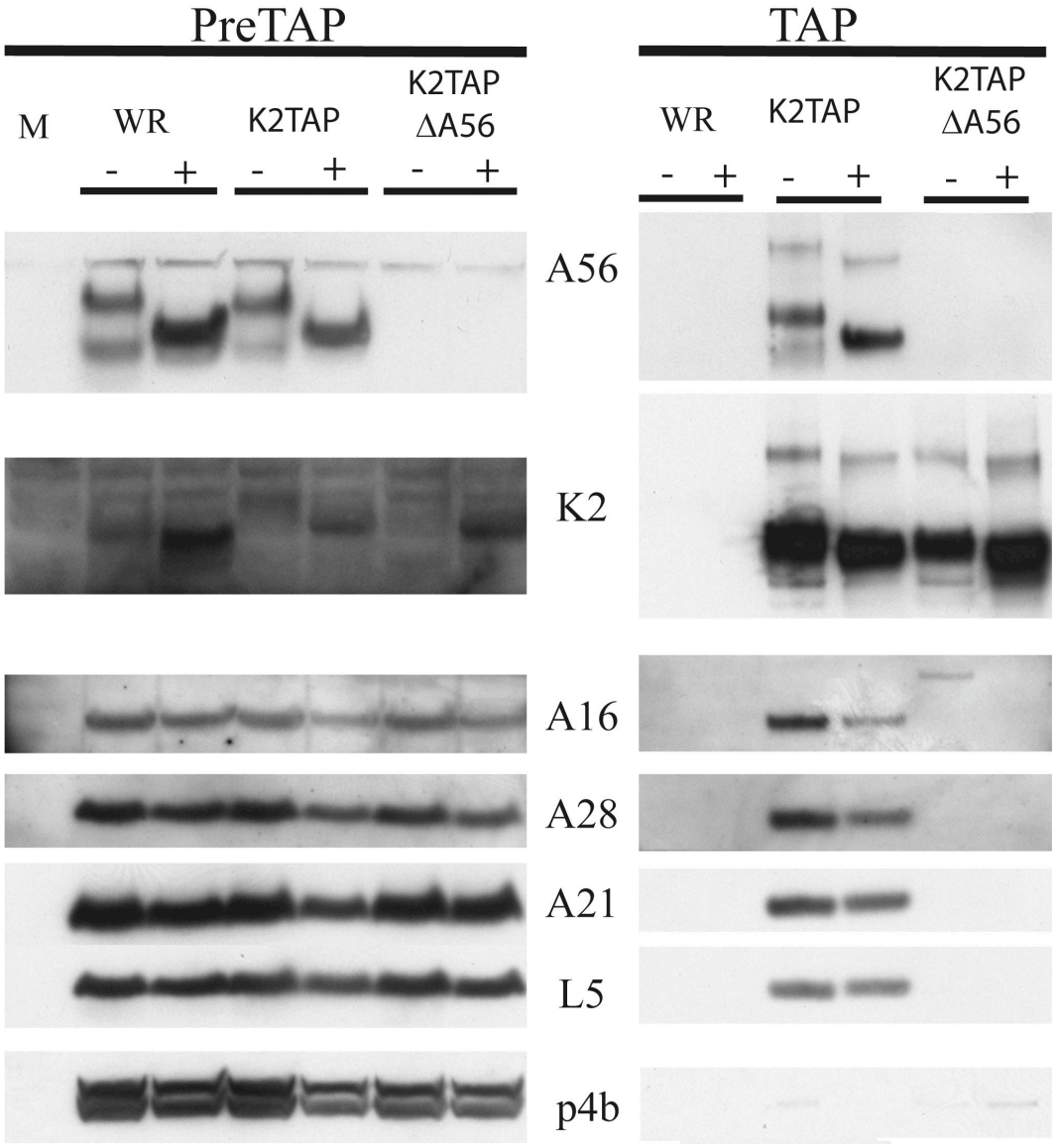

Figure 2-4: Entry proteins co-purify with K2TAP only in the presence of A56. Cells were mock infected or infected separately with VACV WR, vK2TAP or vK2TAPΔA56. The post-nuclear supernatent was affinity purified sequentially over streptavidin and calmodulin Sepharose. The bound material was eluted, separated by SDS-PAGE and analyzed by Western blotting with antibodies to the proteins A56, K2, A16, A21, L5 and p4b as indicated. Starting material is labeled PreTAP. Affinity purified samples referred to as TAP. The - and + signs indicate the absence or presence of brefeldin A, respectively.

K2TAP in the absence of A56. However, in the absence of A56 the K2 protein was partially secreted into the medium, since K2 relies on association with A56 for membrane association. To prevent secretion and potentially allow more opportunity for K2 to interact with the EFC, the antibiotic brefeldin A was used to disrupt the Golgi apparatus and prevent protein trafficking along the secretory pathway [193, 194]. Brefeldin A has been shown to have little effect on the formation of MV, while significantly reducing the amount of EV [195]

To determine whether A56 was required for K2 to bind the EFC, HeLa cells were infected with VACV WR, vK2TAP or vK2TAPΔA56 in the presence or absence of 10 μg/ml of brefeldin A. Cell lysates from the starting material confirmed A56 was not synthesized in cells infected with vK2TAPΔA56 (Figure 2-4). Brefeldin A altered the mobility of glycosylated A56 expressed in cells infected with VACV WR or vK2TAP which was expected due to a failure of the protein to transit through the Golgi apparatus (Figure 2-4). There appeared to be more A56 in the presence of brefeldin A, possibly because shedding of EVs was prevented. Similarly, brefeldin A increased the amount of K2 and changed the mobility of the protein, particularly in the absence of A56. There was no noticeable effect of brefeldin A on the non-glycosylated EFC proteins or on the core protein p4b (Figure 2-4). K2 purified from cells infected with vK2TAP demonstrating an association with A56 and the EFC proteins A16, A21, A28 and L5 in the absence or presence of brefeldin A (Figure 2-4). K2 did not interact with p4b indicating the interaction was specific. In the absence of A56, the K2 protein was unable to associate with A16, A21, A28 and L5

even though brefeldin A increased the amount of K2. These results show both A56 and K2 are needed to association with the EFC.

2.3.5 Association of A56 and K2 with a TAP-tagged EFC.

The EFC has been shown to copurify with both a TAP tagged A56 and a TAP tagged K2 protein. To confirm this interaction, the reciprocal experiment was performed by constructing an additional recombinant VACV with DNA encoding the TAP tag at the 3' terminus of the A28L gene. The A28 protein was chosen as a representative member of the EFC and the protein was previously shown to exhibit normal function with the addition of the influenza HA epitope, suggesting the protein may tolerate the addition of the TAP tag [85]. The recombinant VACV vA28TAP grew to high titers indicating no defect in A28 function. The vA28TAP was used to construct another recombinant VACV in which the K2L gene of vA28TAP was replaced with the gene encoding EGFP. Cells infected with vA28TAPΔK2 formed syncytia at neutral pH. Lysates from HeLa cells infected with VACV WR, vA28TAP or vA28TAPΔK2 were subjected to tandem affinity purification and then analyzed by Western blotting. As expected, the mobility of the A28 protein was reduced in cells infected with A28TAP due to the increase in mass caused by the TAP tag, while K2 was absent in cells infected with vA28TAPΔK2 (Figure 2-5). Both A56 and K2 co-purified with the A28TAP entry protein (Figure 2-5). The interaction between A28 and A56/K2 was specific as neither the core protein p4b nor the cellular protein GAPDH co-purified with A28TAP, in addition no proteins were detected from the affinity-purified lysates of VACV WR (Figure 2-5). The major A56 band did not associate with A28TAP when the protein was purified from the lysate of

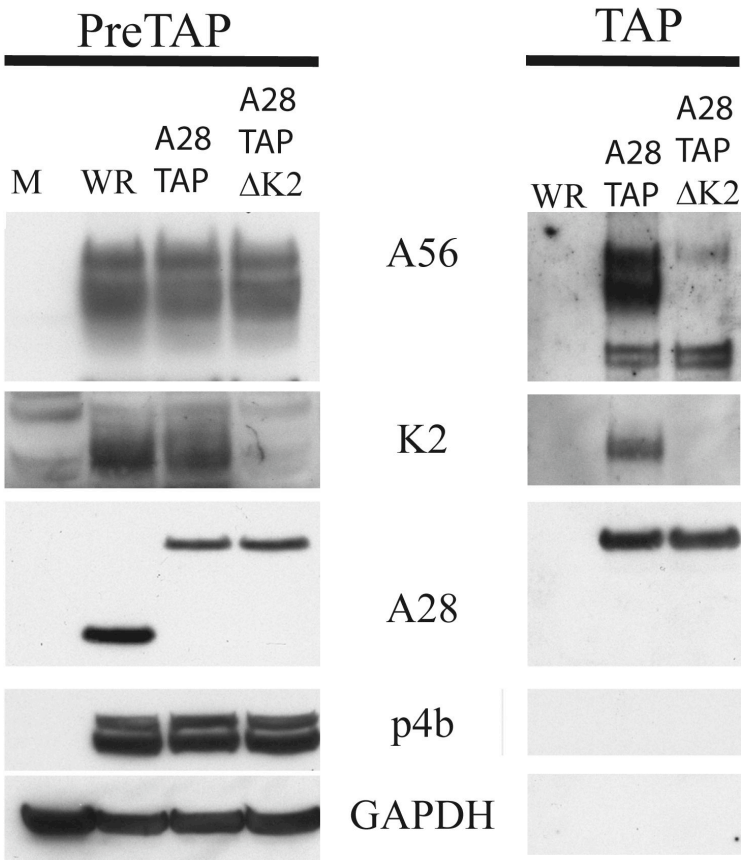

Figure 2-5: A TAP-tagged A28 protein associates with A56 and K2.
Cells were infected individually with VACV WR, vA28TAP or vA28TAPΔK2 as indicated and lysed. The A28 protein was purified sequentially by streptavidin and calmodulin chromatography. The co-purifying proteins were eluted, resolved by SDS-PAGE, and detected by Western blotting with antibodies to the proteins: A56, K2, A28, p4b, GAPDH as indicated. PreTAP, starting material; TAP, affinity purified samples.

vA28TAPΔK2 (Figure 2-5). A much less intense doublet migrating faster than the major A56 bands was observed to copurified with A28TAP in the presence and absence of K2 (Figure 2-5). Initially our thought was that the doublet represented cross-reactivity of the anti-A56 peptide antibody, however the same result was obtained when the Western blot was carried out with a monoclonal antibody against A56. This suggests a minor form of A56 is able to bind A28TAP even in the absence of K2. In the absence of K2, the major bands of A56 fail to associate with A28TAP confirming the importance of K2 for binding the EFC.

2.3.6 Interaction of soluble A56/K2 with the EFC.

To further characterize the association between the fusion regulatory proteins and the EFC, a soluble form of A56/K2 was prepared. The basic idea was to prepare two recombinant VACVs that each inducibly over expressed either K2 or a secreted form of A56 using vT7lacOI [66] as the starting virus. One of the recombinant VACVs had a deleted A56 gene and a K2 gene regulated by the bacteriophage T7 promoter and *E. coli* lac operator. A second recombinant VACV possessed an inducible A56 with the membrane anchor and cytoplasmic tail sequences substituted for codons of the TAP tag. Pilot experiments confirmed secretion of a K2/A56TAP complex from co-infected cells, yet the C3 protein also associated with the complex. (Note that association of C3 with the full-length K2/A56 complex is shown in Figure 2-2). The C3 protein is a non-essential virus-encoded host defense protein and was removed by replacing the gene with one encoding EGFP in both recombinant VACVs to form vK2iΔA56ΔC3 and vsA56TAPiΔC3 which inducibly expressed K2 and a soluble TAP-tagged A56 in the absence of C3.

To isolate a soluble complex of A56 and K2 RK13 cells were coinfected with vK2iΔA56ΔC3 and vsA56TAPiΔC3 in low serum medium supplemented with IPTG. The presence of IPTG was required to induce protein expression, while the low serum reduced the level of contaminating proteins. Both A56 and K2 are glycosylated so an immobilized lectin, wheat germ agglutinin, was used to concentrate the proteins from the medium. Following this step the complex was isolated by an affinity step with streptavidin Sepharose. This two-step purification isolated a soluble complex of A56 and K2 that was free of major contaminating proteins (Figure 2-6A).

To investigate an interaction between purified soluble A56TAP/K2 and the EFC, the infected cell lysate needed to lack endogenous A56 and K2. To facilitate this, a recombinant VACV, vΔA56ΔK2 was constructed by sequential replacement of the A56R and K2L genes with DNA encoding EGFP and HcRED, respectively. Deletion of A56 and K2 was confirmed by Western blot analysis (Figure 2-6). The soluble complex of A56TAP/K2 was incubated with a post-nuclear lysate isolated from HeLa cells infected with vΔA56ΔK2 or mock infected. Affinity purification of the soluble A56TAP/K2 was determined by TAP, utilizing the tag on the recombinant A56TAP protein. Western blot confirmed an association between soluble A56TAP/K2 and A16, A21, A28 and L5, but not with the core protein p4b (Figure 2-6B). The EFC only co-purified with the soluble A56TAP/K2, as none of the entry proteins were detected in its absence. The secreted form of A56TAP appears to be a homogenous, fully glycosylated protein, whereas A56TAP from infected cell lysates (shown on the right of Figure 2-6B) is heterogeneous reflecting varying stages of glycosylation.

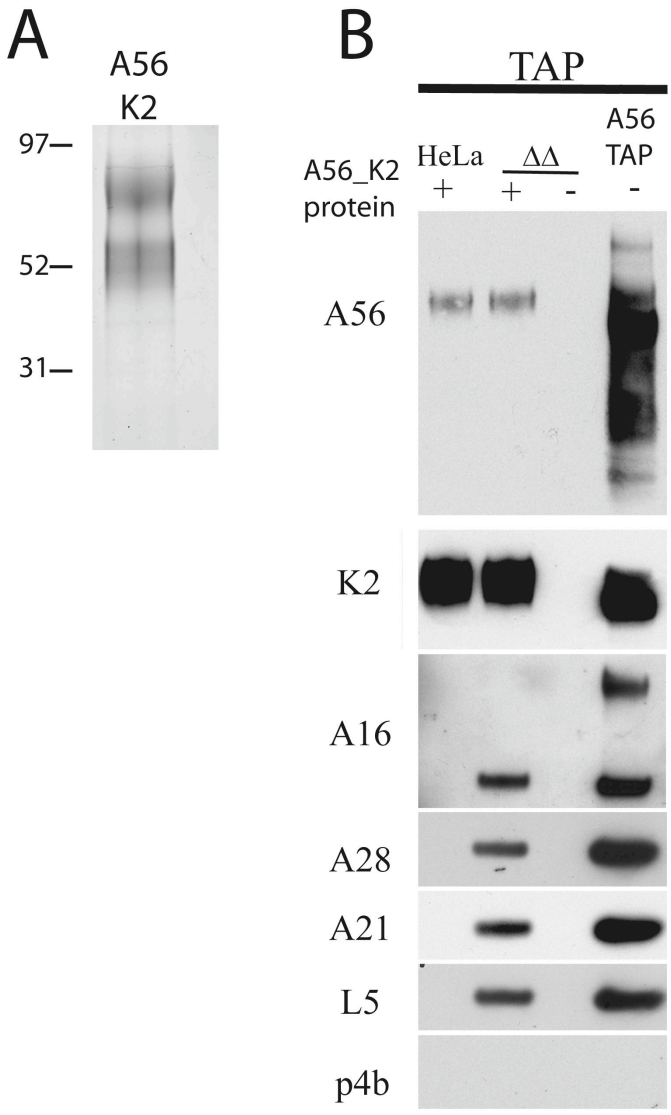

Figure 2-6: Soluble A56/K2 interacts with the EFC.
(A) Soluble A56/K2 was isolated by affinity purification from the medium of cells co-infected with vK2iΔA56ΔC3 and vsA56TAPiΔC3 in the presence of IPTG. A complex of A56/K2 was purified by a 2-step procedure using immobilized wheat germ agglutinin and streptavidin. Following concentration, the purified proteins were analyzed by SDS-PAGE and silver staining. (B) A post nuclear lysate of mock infected HeLa cells or cells infected with vΔA56ΔK2 was incubated with the purified soluble A56/K2. The A56/K2 complex was tandem affinity purified, separated by SDS-PAGE and analyzed by Western blotting by using antibodies to the proteins indicated on the left.

2.4 Discussion

Entry of VACV core is preceded by fusion of the MV membrane with the plasma membrane at neutral pH [91] or with the endosomal membrane at low pH [94]. Fusion of MV at the plasma membrane is greatly enhanced by lowering the pH of the medium below 6, a process that is thought to mimic the decrease in pH within the endosome [94]. At late times during an infection, briefly lowering the pH of the medium triggers cell-cell fusion, a process that depends on cell surface EV (called fusion from within) [152, 165, 166]. Cell fusion also occurs when cells are inoculated with large numbers of purified MV and subsequently treated with low pH (called fusion from without) [166]. Cells infected with VACV normally do not fuse, but deletion of the A56 or K2 protein leads to spontaneous cell fusion of infected cells at neutral pH [167-170].

EV is required for infected cell fusion trigger by low pH as well as cell fusion associated with deletion of A56 (and presumably K2), indicating virus-cell fusion and cell-cell fusion are closely related phenomena. Previous investigations have revealed virus-cell fusion and low pH-induced cell fusion depended on a conserved multiprotein EFC [84, 94]. The study described in this chapter demonstrated neutral pH cell fusion requires the A28 protein. Repressing synthesis of A28 prevents formation of the EFC [84]. It is likely that other proteins of the complex will also be required for neutral pH cell fusion. The first stage in the development of cell fusion is thought to be fusion between MV and cell membrane (Figure 1-4**)**. The absence of a functional EFC would prevent virus-cell fusion and as a result the latter steps would not occur.

The mechanism by which A56 and K2 regulate cell fusion is unknown. The A56 protein has no putative catalytic motifs and mutations in K2 that disrupt the SERPIN activity have no effect on syncytia formation. Given this information it was suspected that A56/K2 might inhibit cell fusion through protein-protein interaction. To investigate this hypothesis a number of recombinant VACV were constructed with the TAP tag attached to A56, K2 or the A28 EFC protein. Both A56 and K2 were observed by a combination of mass spectrometry and Western blotting to interact with proteins of the EFC. A56 was unable to interact with the EFC in the absence of K2, and efficient interaction of K2 with the EFC required A56. A minor form of A56 was observed to copurified with TAP-tagged EFC in the absence of K2, although the significance is not yet understood. The requirement of both A56 and K2 to efficiently bind the EFC correlates with the need of both proteins to efficiently prevent spontaneous fusion of infected cells. The dynamics of the interaction between the EFC and A56 and K2 remain to be studied. It is unknown whether a conformational change occurs within A56 or K2 upon associating with one another. Alternatively, the conformation of the complex of A56 and K2 may be required to bind the EFC.

Both A56 and K2 are present in the EV membrane [184] and are located within the plasma membrane as well [172]. It is suspected that plasma membrane localization of A56/K2 is important for preventing re-infection of cells by progeny extracellular virions. In order for A56/K2 to interact with the EFC within the MV membrane, the outer EV membrane must first be disrupted. It was recently reported that the EV membrane is ruptured by interaction with cell surface GAGs [97].

The interaction of A56/K2 with the EFC was observed to occur post-lysis. This was shown by the ability of soluble A56/K2 to interact with the EFC from an infected cell lysate devoid of endogenous A56/K2. Therefore under the experimental conditions the addition of the detergent lysis the cells and allows A56/K2 to interact with the EFC. Additional investigation will be required to determine the proteins of the EFC that mediate an interaction with A56/K2.

Chapter 3: The VACV fusion regulatory proteins A56 and K2 interact with a subcomplex of A16 and G9

3.1 Introduction

VACV induces cell fusion following low pH treatment of infected cells or spontaneously at neutral pH when either the A56R or K2L gene is absent. VACV entry and fusion requires a conserved multiprotein complex of at least eight proteins that resides within the MV membrane. The EFC is essential for entry of both the MV and EV forms of the virus as is also required for low pH cell-cell fusion, while in the previous chapter the A28 entry protein was shown to be essential for neutral pH cell fusion associated with deletion of A56 or K2. Therefore virus-cell fusion, neutral cell fusion and low pH cell fusion all depends on the viral EFC. Assembly of the EFC does not occur in the absences of a single entry protein, although the remaining entry proteins are stable and localize to the MV membrane.

Little is known about the mechanism by which A56 and K2 regulate cell fusion. Tandem affinity purification of the A56 protein was utilized to identify a novel protein interaction with the EFC, suggesting the anti-fusion activity of A56 and K2 may be mediated through an interaction with the viral entry proteins. Both fusion regulatory proteins were required to bind the EFC; neither A56 nor K2 alone was sufficient. The previous chapter did not determine the protein within the EFC required for an interaction with A56/K2. To determine the minimal components of the EFC required for binding to A56/K2 a series of conditional lethal viruses were constructed in which various entry proteins were repressed and interaction with A56/K2 were assessed. Our analysis revealed both A16 and G9 were needed to bind

A56/K2 as neither A16 nor G9 alone bound efficiently to A56/K2. Furthermore A16 and G9 were shown to interact with one another in transfected cells suggesting the two proteins directly interact within the EFC.

3.2 Material and Methods

3.2.1 Cells and virus.

BS-C-1 (ATCC CCL-26), HeLa S3 (ATCC CCL-2.2) suspension cells were grown as described in chapter 2, section 2.2.1. 293TT cells, stably expressing the large T antigen, were provided by Chris Buck [196], and were grown in Dulbecco's minimum essential medium (Quality Biological) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 400 μg/ml hygromycin (Invitrogen, Carlsbad, CA). The Western Reserve (WR) strain of VACV was used in the construction of all recombinant viruses unless noted otherwise. General procedures for preparing and titrating stocks were done as previously described in chapter 2, section 2.2.1.

3.2.2 Plasmid and recombinant VACV construction.

The recombinant viruses constructed for this study (Table 3-1) were: vA28iA56$_{TAP}$, vA21iA56$_{TAP}$, vA28iA56$_{TAP}$J5$_{Flag}$, vA28iA56$_{TAP}$G9$_{3XFlag}$, vA28iA16$_{3XFlag}$, vA28iG9$_{3XFlag}$, vA16iA56$_{TAP}$G9$_{3XFlag}$, and vG9iA56$_{TAP}$ (where i indicates an inducible gene, TAP refers to a tandem affinity tag, 3XFlag indicates 3 copies of the Flag epitope). Recombinant viruses were screened by PCR to confirm the absence of parental virus and the sequence of the inserted DNA was confirmed. The vA28iA56$_{TAP}$, vA21iA56$_{TAP}$, vA16iA56$_{TAP}$ and vG9i$_{HA}$A56$_{TAP}$ were constructed from vA28i [85], vA21i [79], vA16i [78] and vG9i$_{HA}$ [83], respectively by appending

Table 3-1. Recombinant VACV

Recombinant virus	Parent (Reference)	Description
vA16i	vT7lacOI [66]	Inducible A16
vA16iA56$_{TAP}$	vA16i [78]	Inducible A16; A56-TAP[a]
vA16iA56$_{TAP}$G9$_{3XFlag}$	vA16iA56$_{TAP}$	Inducible A16; A56-TAP[a]; G9-Flag[b]
vA21i	vT7lacOI [66]	Inducible A21
vA21iA56$_{TAP}$	vA21i [79]	Inducible A21; A56-TAP[a]
vA28i	vT7lacOI [66]	Inducible A28-HA[c]
vA28iA16$_{3XFlag}$	vA28i [80]	Inducible A28-HA[c]; A16-Flag[b]
vA28iA56$_{TAP}$	vA28i [80]	Inducible A28-HA[c]; A56-TAP[a]
vA28iA56$_{TAP}$G9$_{3XFlag}$	vA28iA56$_{TAP}$	Inducible A28-HA[c]; A56-TAP[a]; G9-Flag[b]
vA28iA56$_{TAP}$J5$_{Flag}$	vA28iA56$_{TAP}$	Inducible A28-HA[c]; A56-TAP[a]; J5-Flag[d]
vA28iG9$_{3XFlag}$	vA28i [80]	Inducible A28-HA[c]; G9-Flag[b]
vA56$_{TAP}$	vΔA56	A56-TAP[a]
vG9iA56$_{TAP}$	vG9i [83]	Inducible G9-HA[e]; A56-TAP[a]

[a] TAP-tag at C-terminus
[b] 3 copies of Flag tag at C-terminus
[c] 1 copy of C-terminal HA tag
[d] 1 copy of Flag tag at N-terminus
[e] 1 copy of N-terminal HA tag

the codons for a C-terminal TAP tag to the A56 gene. The DNA used to construct the C-terminal TAP tag has been described in chapter 2, section 2.2.3. vA28iA56$_{TAP}$ was the parental virus for the construction of vA28iA56$_{TAP}$J5$_{Flag.}$ Overlapping PCR (Accuprime *Pfx*; Invitrogen) was used to assemble the DNA used for recombination. The layout of the DNA sequence for the J5$_{Flag}$ construct from the 5' to 3' was as follows: (i) 500 bps of DNA sequence upstream of the J5R gene, (ii) EGFP expressed from the I1L promoter, (iii) 70 nucleotides containing the J5R promoter and (iv) the initial methionine of J5R, followed by the DNA sequence for the Flag epitope (DYKDDDK) and then the remaining DNA sequence of the J5R gene. The recombinant PCR product was cloned into pCR-BluntII-TOPO (Invitrogen) and verified by DNA sequencing. The J5$_{Flag}$ plasmid was linearized by cleavage with a unique restriction endonuclease. BS-C-1 cells were infected with vA28iA56$_{TAP}$ for 1 h, then transfected with the linearized plasmid using Lipofectamine 2000 (Invitrogen). IPTG was added to the medium at 100 μM to allow expression of the inducible A28 gene. The parental and recombinant viruses were distinguished by the GFP fluorescence of the latter. Recombinant plaques were clonally isolated through 3 rounds of plaque purification.

vA28iA56$_{TAP}$G9$_{3XFlag}$ and vA28iG9$_{3XFlag}$ were constructed from vA28iA56$_{TAP}$ and vA28i, respectively. vA16iA56$_{TAP}$G9$_{3XFlag}$ was constructed sequentially by first generating vA16iA56$_{TAP}$ from vA16i. The 3XFlag was added to the G9 protein to form vA16iA56$_{TAP}$G9$_{3XFlag}$, vA28iA56$_{TAP}$G9$_{3XFlag}$, vA28iG9$_{3XFlag}$ as follows. The G9R gene was PCR amplified from genomic DNA of the Western Reserve (WR) strain (ATCC VR-1354, accession number AY243312). The 3XFlag epitope was

appended to the C terminus of G9 prior to the stop codon. This was followed by the coding sequence of the *Discosoma sp.* red fluorescent protein (DsRed) expressed from the I1L intermediate promoter for screening of recombinant viruses. The L1R gene was PCR amplified along with 90 bps upstream of the gene to include the L1R promoter. Recombinant PCR was utilized to add L1R with the promoter after the DsRed coding sequence. The promoter for the LIR gene is located within the C-terminal codons of G9. To conserve expression of the L1R gene, the C-terminus of G9 was duplicated causing a direct repeat of DNA sequence before and after the DsRed gene. Direct repeats are unstable in the virus genome [197]. Therefore to prevent the eventual loss of the DsRed gene the codons of the final 33 amino acids from G9 were altered, while conserving the amino acid sequence. The recombinant $G9_{3XFlag}$ PCR was cloned into pCR-BluntII-TOPO and verified by DNA sequencing. Recombinant viruses were generated by infecting BS-C-1 cells with the appropriate parental virus and then transfecting linearized $G9_{3XFlag}$ plasmid. Recombinant viruses were distinguished from parental by DsRed fluorescence and were clonally isolated through 3 rounds of plaque purification.

3.2.3 A16 and G9 Codon Optimization.

The DNA sequence for the VACV WR A16L and G9R genes was optimized (Geneart, Regensburg, Germany) to alter codon usage and G-C content to improve RNA processing and translation. The optimized A16L and G9R genes were PCR amplified with oligonucleotides that contained the sequence of the influenza virus HA or 3XFlag epitope appended to the C-terminus of the respective ORFs. The PCR

products of A16$_{HA}$ and G9$_{3XFlag}$ were cloned into the directional TOPO vector pcDNA3.1 (Invitrogen) and sequenced to confirm proper insertion and sequence.

3.2.3 Affinity purification.

BS-C-1 cells (6 x10^6) were infected at a multiplicity of 3 to 5 plaque forming units per cell in EMEM with 2% FBS. After 24 h the cells were scraped into the medium and subjected to low speed centrifugation. The cell pellet was washed once by resuspending in 150 mM NaCl with 50 mM Tris-HCl (pH 7.5). The cells were then lysed with ice-cold SBB [1% Triton X-100 (Sigma, St. Louis, MO), 150 mM NaCl, 50 mM Tris-HCl (pH 7.5)] supplemented with complete protease inhibitor (Roche, Indianapolis, IN) and rotated at 4 ^oC for 30 min. The lysate was pelleted at 4^oC in a benchtop centrifuge at 20,000 x g for 10 min and the postnuclear supernatent was collected and 50 μl saved for analysis. Tandem affinity purification was carried out as previously described in chapter 2, section 2.2. Single step purifications were as follows. Packed beads (20-30 μl) of either streptavidin Sepharose (Millipore, Billerica, MA) or anti-Flag conjugated agarose (Sigma) were washed once with 1 ml of lysis buffer and the postnuclear supernatant was added to the affinity resin and rotated overnight at 4 ^oC. The affinity resin was washed 5 times with 1 ml of SBB prior to elution. The bound material was eluted from the anti-Flag agarose by added 50 μl of 1X LDS sample buffer (Invitrogen) supplemented with reducing agent (Invitrogen) and incubated at 100 ^oC for 5 min. The beads were pelleted by centrifugation and the supernatent was collected. Bound material was eluted from the streptavidin Sepharose by incubation with 300 μl of lysis buffer supplement with 2 mM d-Biotin (US Biological, Swampscott, MA). The elution was repeated two times

and the eluates were combined prior to concentration by precipitation with trichloroacetic acid. The precipitated material was resuspended in 40 μl of 1x LDS buffer with reducing agent.

3.2.5 Transfection and coimmunoprecipitation.

293TT cells were plated at a density of $2x10^6$ per 9.2 cm^2 the day before transfection in 10% DMEM plus glutamine, but without hygromycin. Cells were transfected with 2 μg of total DNA using Lipofectamine 2000 (Invitrogen). After 24 h fresh 10% DMEM was added and the incubation continued for an additional 24 h at which time cell extracts were prepared and subjected to immunoprecipitation.

3.2.6 Western blotting and antibodies.

Samples were loaded onto a 4-12% Novex NuPAGE acrylamide gel (Invitrogen) and separated by electrophoresis using 2(N-morpholino)ethanesulfonic acid buffer (Invitrogen). The protein samples were transferred to a nitrocellulose membrane and then blocked with 5% nonfat milk in TBST. Primary antibody was incubated a minimum of 1 h prior to extensive washing with TBST. The secondary antibodies (Pierce, Rockford, IL) were diluted in 5% nonfat milk TBST and incubated for at least 1hr. The nitrocellulose membrane was washed and then developed with Dura or Femto Chemilumenscent substrate (Pierce). The antibodies were stripped from the nitrocellulose by incubating 20 min at 55 ^oC with Restore (Pierce). Antibodies to A56 (chapter 2, section 2.5), K2 (chapter 2, section 2.5), A21 [79], L5 [81], A16 [78], and p4a/p4b (R. Doms and B. Moss, unpublished data) were used in Western blot analysis. A monoclonal antibody (conjugated to horseradish peroxidase)

against the influenza hemagglutinin epitope was acquired from Bethyl laboratories (Montgomery, TX). The anti-Flag M2 monoclonal antibody was obtained from Sigma (St. Louis, MO) and a monoclonal antibody to the cellular glyceraldehyde-3-phosphate dehydrogenase was purchased from Covance Research (Princeton, NJ). The rabbit antiserum to H2 and A28 was generated by immunizing rabbits with purified recombinant H2 or A28 proteins, respectively and provided by Gretchen Nelson, NIAID. Peptide antibody was generated to the G3 protein by immunizing rabbits with the synthetic peptide [SLNGKKHTFNLYDDNDIRT] coupled to keyhole limpet hemocyanin through an N terminal cysteine (Covance).

3.3 Results

3.3.1 A56/K2 interacts with a subset of the proteins within the EFC.

The interaction of A56/K2 with the EFC was described in chapter 2. Both A56 and K2 polypeptides were required to interact with the EFC, although the entry proteins required for the interaction were not determined. Our analysis was based on previous observations that (i) the viral membrane is required for assembly of the EFC and (ii) that the EFC fails to form in the absence of A28 or A21 even though the remaining EFC proteins are incorporated into the viral membrane [84]. It may be possible by repressing synthesis of A28 and A21 to identify either individual polypeptides or previously uncharacterized subcomplexes of the EFC capable of interacting with A56/K2. To implement this strategy two recombinant viruses were constructed, vA28iA56$_{TAP}$ and vA21iA56$_{TAP}$, in which A28 and A21 were conditionally expressed, respectively. Conditional expression of A28 and A21 was

regulated by components of the *E. coli lac* operon in combination with the T7 phage DNA-dependant RNA polymerase, such that viral gene expression depended on the addition of IPTG. Both recombinant viruses were constructed with a TAP tag appended to the C-terminus of the A56R gene to allow purification of the A56/K2 heteromultimer along with any associating polypeptides. In addition, A28 had a C-terminal HA epitope tag.

HeLa cells were infected with VACV strain WR, vA56TAP (chapter 2, section 2.3), vA28iA56TAP (+ and – IPTG), vA21iA56TAP (+ and – IPTG), or mock infected. VACV WR, with an untagged A56 was used as a negative control for nonspecific interaction during affinity purification, while vA56TAP with a constitutively expressed A28 was used as a positive control. The stocks of vA28iA56TAP and vA21iA56TAP were prepared in the presence of IPTG so that the virus particles contained A28 and were infectious, but synthesis of A28 or A21 during the next cycle depended on the addition of IPTG. At 24 h post infection, the cells were lysed with Triton X-100 detergent and the post-nuclear supernatant was subjected to TAP on streptavidin and calmodulin Sepharose columns. The proteins in the final eluate were concentrated, separated by SDS-PAGE, and detected by Western blotting with specific antibodies.

Western blotting of the starting material of cells infected with vA28iA56TAP and vA21iA56TAP, confirmed synthesis of A28 and A21, respectively, depended on IPTG (Figure 3-1, PreTAP). The slightly slower electrophoretic migration of A28 in the lysate of vA28iA56TAP was due an HA epitope tag on the C terminus of the

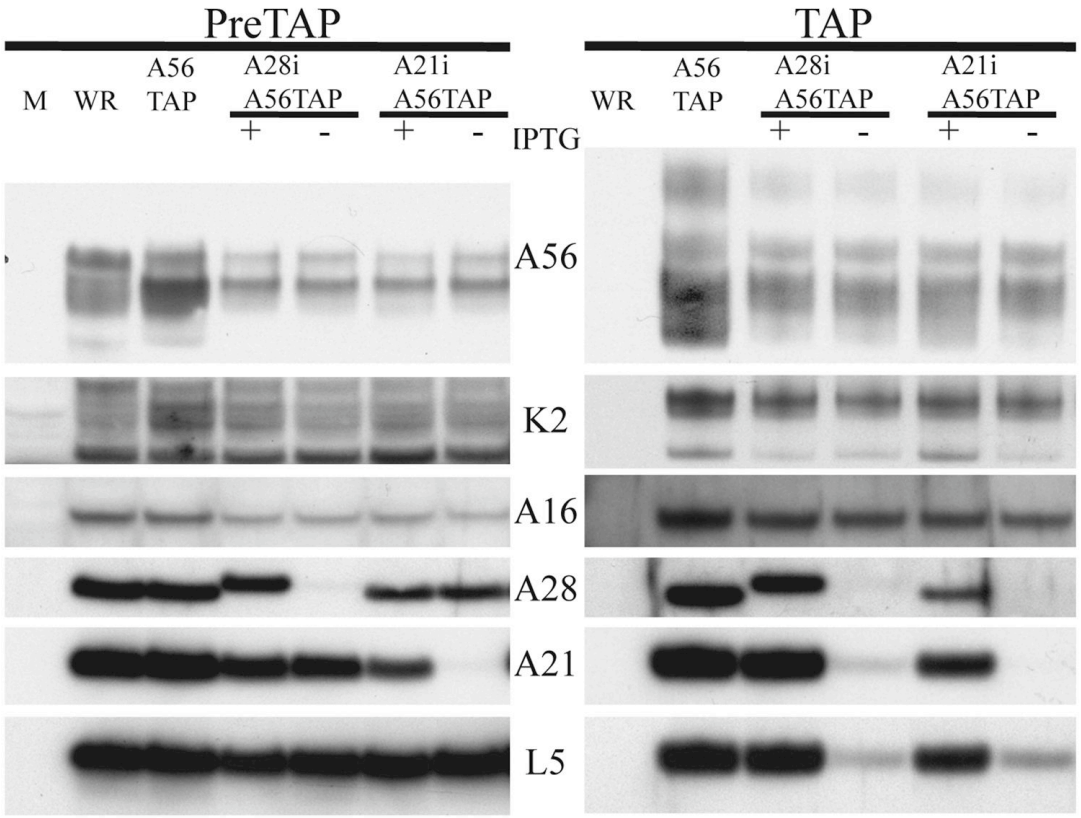

Figure 3-1: A56/K2 physically associate with A16.
HeLa cells were mock infected (M) or infected with VACV WR, vA56TAP, vA28iA56TAP (+ and – IPTG) and vA21iA56TAP (+ and – IPTG). After 24 h the cells were lysed with Triton X-100 and the A56 protein was affinity purified sequentially over streptavidin and calmodulin Sepharose. The starting material (PreTAP) and purified samples (TAP) were separated by SDS-PAGE and analyzed by Western blotting using antibodies to the entry proteins: A16, A28, A21 and L5 as well as antibodies to the A56 and K2 proteins. Secondary antibodies conjugated to horseradish peroxidase were used for detection by chemiluminescence. The recombinant viruses are listed at the top and proteins targeted by the antibodies are listed in the center.

protein. Repression of either A28 or A21 had no effect on the synthesis or stability of the other proteins examined. A56 and K2 were resolved as multiple bands due to alternative initiation codons for A56 as well as glycosylation.

Following affinity purification of A56 from vA56$_{TAP}$, vA28iA56$_{TAP}$ (+IPTG) and vA21iA56$_{TAP}$ (+ IPTG) the A56 protein was observed to interacted with K2 as well as the EFC, as represented by A16, A28, A21 and L5 (Figure 3-1, TAP). However, when A28 was repressed by omitting IPTG, K2 and A16 still co-purified with A56 but only trace amounts of A21 and L5 were detected (Figure 3-1, TAP). Similarly, when A21 was repressed, K2 and A16 were detected after affinity purification of A56 but only trace amounts of A28 and L5. These results likely indicate the fully assembled EFC is not required for interaction with A56/K2 and suggest A16 alone or a subcomplex of polypeptides that includes A16 binds to A56/K2. However, the association of A56/K2 with the four other entry proteins (G3, G9, H2 and J5) was not examined because appropriate antibodies for detection were not available at the time of the experiment.

3.3.2 A16 and G9 selectively copurify with A56/K2.

To determine if G3, G9, H2, and J5 were important for the interaction between the EFC and A56/K2 several additional recombinant viruses were constructed with epitope tags on G9 or J5. In addition, antibodies were acquired to G3 and H2. The recombinant virus vA28iA56$_{TAP}$J5$_{Flag}$ encoded an inducible A28, TAP-tagged A56, and J5 with the Flag epitope fused to the N terminus. Cells were infected separately with VACV WR, vA56$_{TAP}$ or vA28iA56$_{TAP}$J5$_{Flag}$ (+ and – IPTG). After 24 h, the cells were lysed and the starting material was analyzed along with the

affinity-purified samples. Western blots of the starting material confirmed A28 was stringently repressed in the absence of IPTG, furthermore both J5 and G3 were detected using Flag tag and G3 peptide antibody, respectively (Figure 3-2A, PreTAP). The true J5 band could be distinguished from the upper and lower background bands by its absence from cells infected with VACV WR and vA56$_{TAP}$. The core protein A3 (p4b) was used as a negative specificity control for the affinity purification. Bands corresponding to A56, K2, A16, A28, A21, L5 and G3 were detected after affinity purification of A56 from cells infected with vA56TAP (Figure3-2A, TAP). The same proteins, as well as J5 Flag, were detected after affinity purification of cells infected with vA28iA56$_{TAP}$J5$_{Flag}$ in the presence of IPTG (Figure 3-2A, TAP). However, in the absence of IPTG, cells infected with vA28iA56$_{TAP}$J5$_{Flag}$ did not express the A28 protein and only K2 and A16 co-purified with A56. Therefore, neither J5 nor G3 interacted with A56/K2 when the A28 protein is repressed and the EFC does not assemble.

The second virus constructed was vA28iA56$_{TAP}$G9$_{3XFlag}$. As its name implies, expression of A28 was inducible, A56 was TAP-tagged, and G9 had three copies of the Flag tag (at the C-terminus). Cells were infected with VACV WR or vA28iA56$_{TAP}$G9$_{3XFlag}$ (+ and – IPTG). At 24 h after infection, the cells were lysed and analyzed directly or after streptavidin affinity purification, as the calmodulin step was not found to be required. Western blots of the starting material from cells infected with vA28iA56$_{TAP}$G9$_{3XFlag}$ (- IPTG) showed A28 was repressed while both the epitope tagged G9$_{3XFlag}$ and H2 were expressed (Figure 3-2B, PreTAP). The use of 3 copies of the Flag epitope enhanced the detection of G9 over background bands.

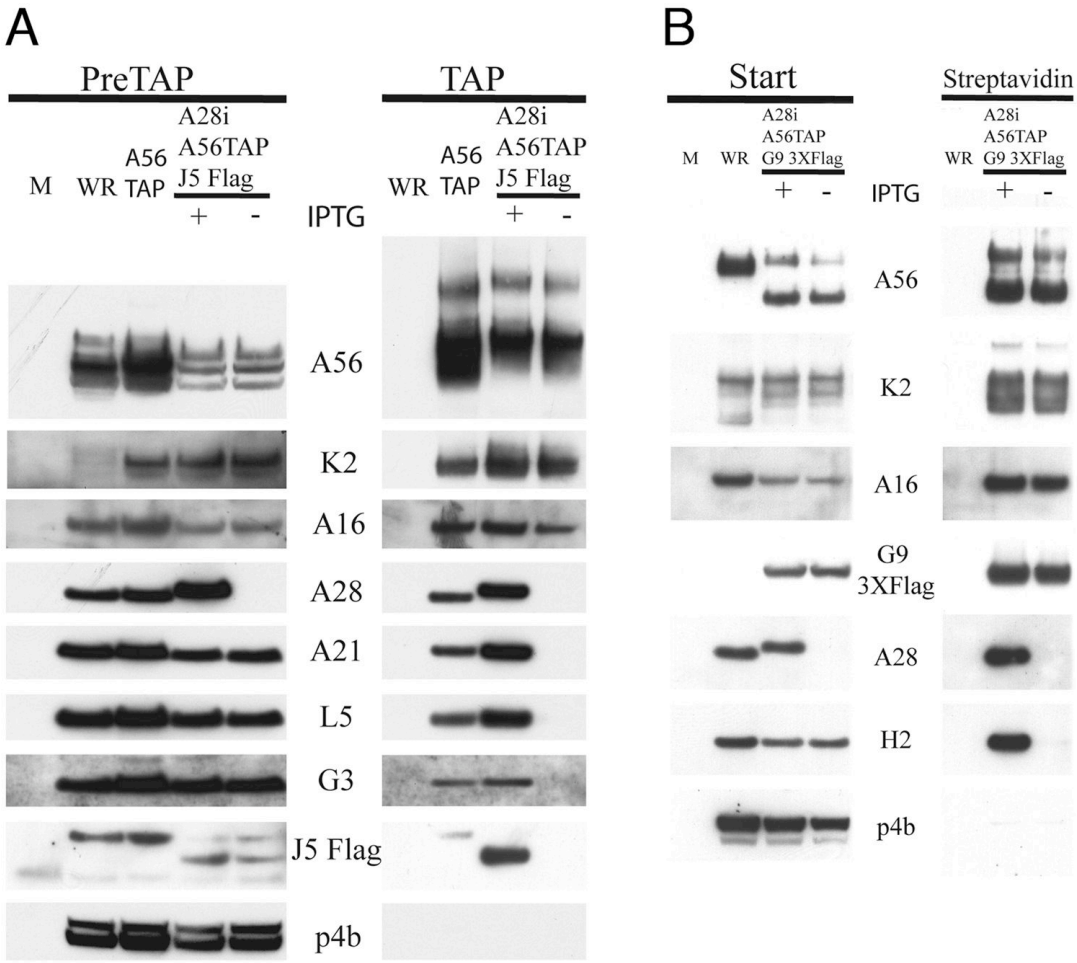

Figure 3-2: A16 and G9 selectively co-purify with A56/K2.
(A) HeLa cells were mock infected (M) or infected individually with VACV WR, vA56TAP, vA28iA56TAPJ5Flag with (+) or without (–) IPTG. After 24 h, the A56 protein was isolated from the infected cell lysates by binding successively to streptavidin and calmodulin beads. Western blotting was performed on the starting material (PreTAP) and affinity purified proteins (TAP). (B) BS-C-1 cells were mock infected (M) or infected with VACV WR or vA28iA56TAPG93XFlag (+ or – IPTG). After 24 h, the A56 protein was isolated by binding to streptavidin Sepharose. Western blotting was performed on the starting material (Start) and affinity purified proteins (Streptavidin) as above.

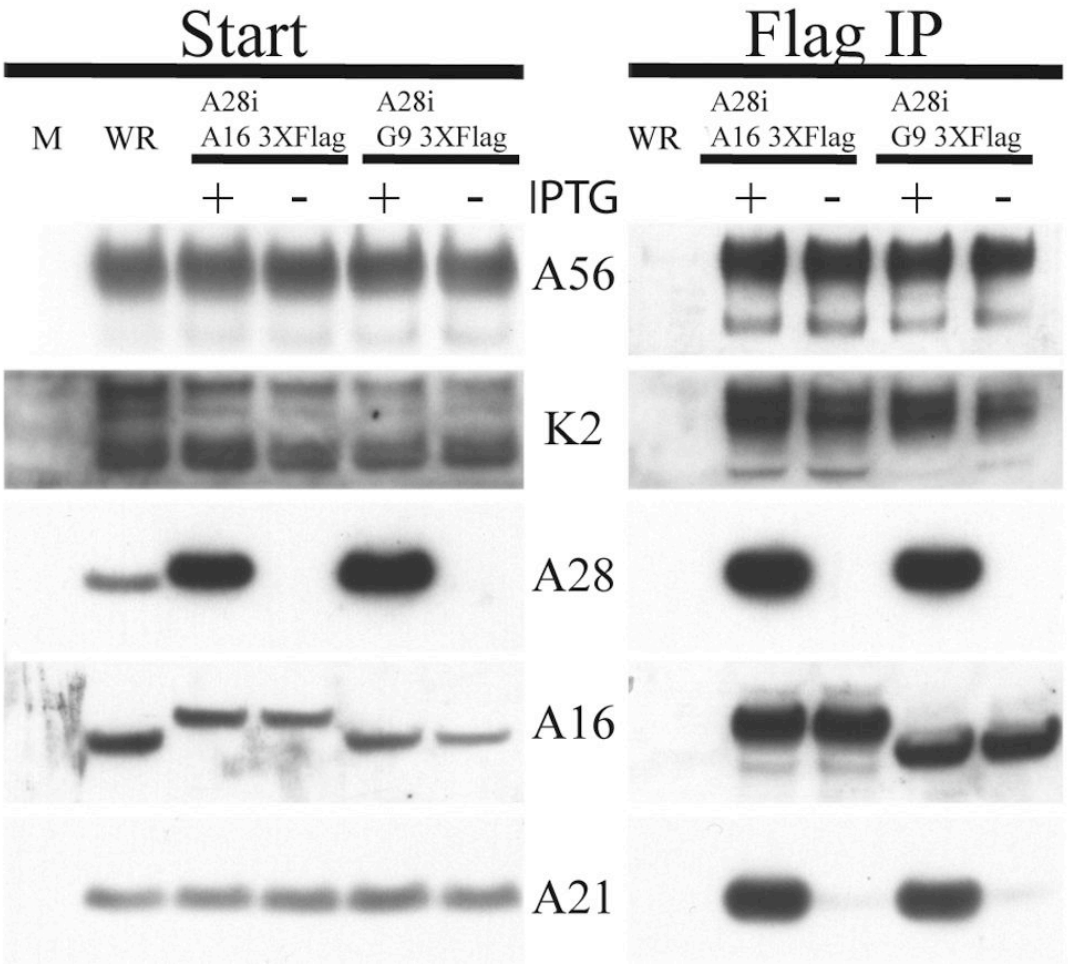

Figure 3-3: A56/K2 binds to affinity purified A16 and G9.
BS-C-1 cells were mock infected (M) or infected with VACV WR, vA28iA16$_{3XFlag}$ (+ or – IPTG), vA28iG9$_{3XFlag}$ (+ or – IPTG). Infected cells were harvested after 24h and lysed with Triton X-100. The lysate was cleared by centrifugation and the A16 and G9 polypeptides were isolated by binding to agarose beads conjugated with Flag antibody. The eluate (Flag IP) and starting material (Start) were separated by SDS-PAGE and analyzed by Western blotting with antibodies to the viral proteins A56, K2, A28, A16 and A21 as indicated in the center. The viruses are indicated at the top of the figure.

A56, K2, A16, G9, A28 and H2 were detected after affinity purification of proteins from cells infected with vA28iA56$_{TAP}$G9$_{3XFlag}$ (+ IPTG), but only K2, A16 and G9 co-purified with A56 in the absence of IPTG (Figure 3-2B, TAP). The A3 core protein (p4b) was not detected in the presence or absence of IPTG. Collectively, the affinity purification experiments indicated that of the eight EFC proteins, A16 and G9 interacted most directly with A56/K2.

3.3.3 A56/K2 copurify with A16/G9.

The reciprocal experiment was carried out to confirm the interaction of A16 and G9 with A56/K2. To implement this, two additional epitope tagged viruses were constructed. Both recombinant VACVs inducibly expressed A28, while G9 and A16 had a 3XFlag epitope attached to the C terminus in vA28iG9$_{3XFlag}$ and vA28iA16$_{3XFlag}$, respectively. Cells were infected with VACV WR, vA28iG9$_{3XFlag}$ (+ and – IPTG) or vA28iA16$_{3XFlag}$ (+ and – IPTG). After 24 h, the cells were collected and the postnuclear supernatant was incubated overnight with anti-Flag antibody covalently linked to agarose beads, which were then washed extensively prior to elution of the bound material. The eluted proteins were resolved by SDS-PAGE and detected by Western blotting. Analysis of the starting material indicated that A28 was stringently regulated and that each of the constructs expressed A56, K2, A16 and A21 (Figure 3-3, Start). The slower migration of A16 from samples infected with vA28iA16$_{3XFlag}$ compared to wild type was due to the 3XFlag epitope. A21 was analyzed as a representative EFC protein to confirm that the complex was not assembled in the absence of A28. As anticipated, A16 or G9 interacted with A21 only when A28 was synthesized (+ IPTG) (Figure 3-3, Flag IP). Nevertheless, A16 and G9

interacted with A56 and K2 even when A28 was repressed (Figure 3-3, Flag IP). Thus, the interaction of A56/K2 with A16 and G9 occurred regardless of the affinity tag and whether it resided on A56, A16 or G9.

3.3.3 Both A16 and G9 are required for their association with A56/K2.

In the above experiments, A16 and G9 always co-purified with A56/K2 and vice versa. Additional recombinant viruses were constructed to determine whether expression of both A16 and G9 were required for a stable interaction with A56/K2. The recombinant vA16iA56$_{TAP}$G9$_{3XFlag}$ expressed an inducible form of A16, TAP-tagged A56 and G9 with a 3X Flag tag. Cells were infected with either VACV WR or vA16iA56$_{TAP}$G9$_{3XFlag}$ (+ and – IPTG) for 24 h and the postnuclear supernatent was analyzed directly or after streptavidin affinity purification. Analysis of the starting material demonstrated the stringent repression of A16 in the absence of IPTG (Figure 3-4A, Start). Importantly, the other EFC proteins examined, namely G9, A21 and L5, were stable even in the absence of A16. Curiously, the faster migrating A56 band predominated in the lysates of cells infected with vA16iA56$_{TAP}$G9$_{3XFlag}$ suggesting initiation predominantly at the second start codon but this was independent of IPTG. In the presence of IPTG, A56 interacted with K2, A16, G9, A21 and L5 as shown by their copurification (Figure 3- 4A, Streptavidin). In contrast, only K2 interacted with A56 when synthesis of A16 was repressed. Therefore, G9 cannot interact independently with A56/K2.

Recombinant vG9iA56$_{TAP}$, which expressed an inducible form of G9 with an HA epitope tag and TAP-tagged A56, was used to test whether A16 alone was able to interact with A56/K2. Western blotting showed that K2, A16, A28, A21, and

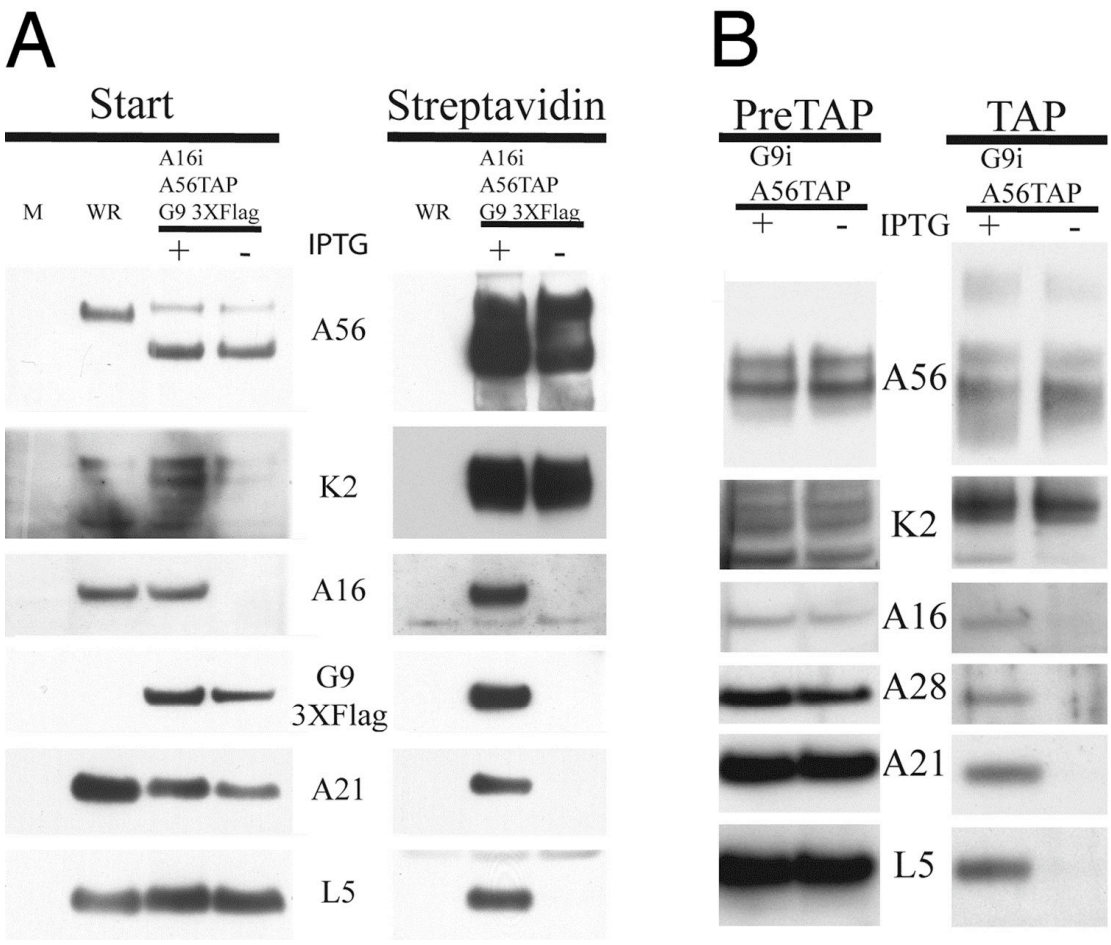

Figure 3-4: Both A16 and G9 are required for binding A56/K2.
(A) BS-C-1 cells were mock infected (M) or infected with VACV WR or vA16iA56TAPG93xFlag (+ or – IPTG). The cells were lysed with Triton X-100 after 24 h and the A56 protein was isolated by binding to streptavidin beads. The starting material (Start) and the affinity purified proteins (Streptavidin) were resolved by SDS-PAGE and analyzed by Western blotting using antibodies to the viral proteins indicated on the side. (B) HeLa cells were infected with vG9iHAA56TAP (+ and – IPTG) and the A56 protein was tandem affinity purified. The starting material (Pre-TAP) and the purified proteins (TAP) were analyzed as in panel A.

L5 co-purified with A56 when cells were infected in the presence of IPTG (Figure 3-4B). However, only K2 co-purified with A56 when cells were infected in the absence of IPTG. Because of the relatively weak signal produced by the antibody to A16, this analysis was repeated in two independent experiments. With a more intense A16 signal in the +IPTG lane, a low amount of the protein was observed to co-purifying with A56 when G9 was repressed. Therefore, both A16 and G9 are needed for efficient interaction of either with A56/K2.

3.3.4 Association of A56/K2 with G9 requires A16.

Next, the reciprocal experiment was carried out to determine if A56/K2 co-purified with Flag-tagged G9 in the absence of A16. Cells were mock infected or infected with VACV WR or vA16iA56$_{TAP}$G9$_{3XFlag}$ (+ and – IPTG). After 24 h, the G9 protein was purified from the postnuclear supernatent by incubating with the Flag antibody conjugated to agarose beads. The beads were washed and the bound proteins were eluted, separated by SDS-PAGE and analyzed by Western blotting. GAPDH served as a control for loading and non-specific binding. Analysis of the lysate prior to immunopurification confirmed the stringent control of A16 expression (Figure 3-5, Start). When A16 was expressed (+ IPTG), the EFC represented by A28 and G9 as well as A56 and K2 co-purified with G9 (Figure 3-5, Flag IP). When A16 synthesis was repressed, however, A28, A56 or K2 failed to co-purified with G9. Therefore A56/K2 did not stably bind to G9 in the absence of A16.

3.3.5 A16 and G9 stably associate with each other in uninfected cells.

The inability of G9 or A16 to independently associate with A56/K2 suggested

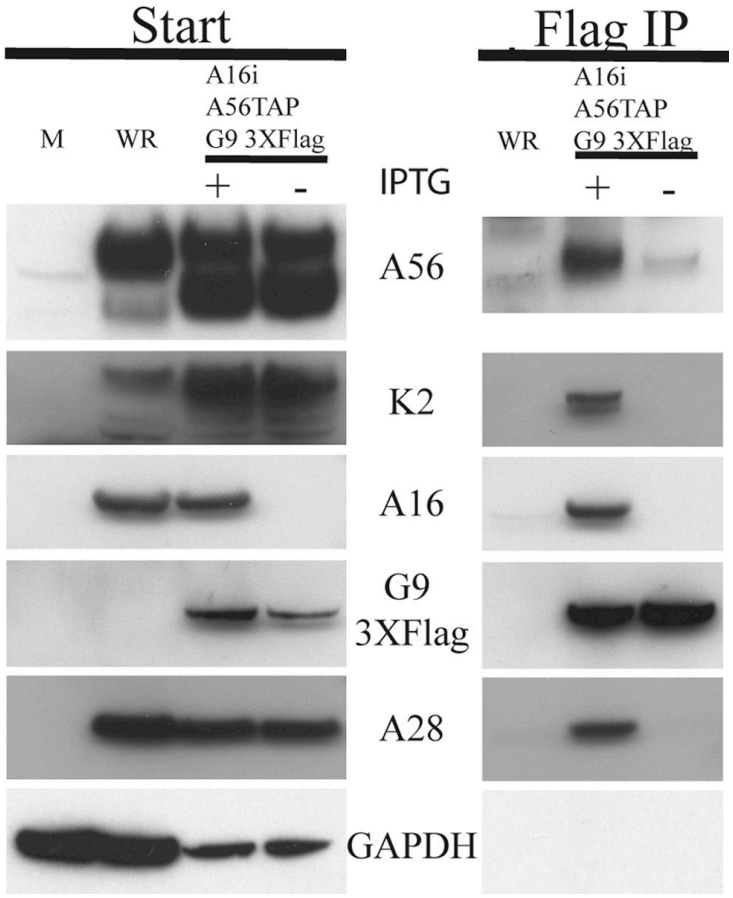

Figure 3-5: A16 is required for G9 to bind A56/K2.
Cells were mock infected (M) or infected with VACV WR or vA16iA56TAPG9$_{3XFlag}$ (+ or – IPTG). Infected BS-C-1 cells were harvested at 24 h and lysed with Triton X-100. Flag antibody conjugated to agarose beads was used to purify the G9 protein. The bound material (Flag IP) was eluted from the agarose beads and separated along with the starting material (Start) by SDS-PAGE and then transferred to nitrocellulose. Western blotting was preformed using antibodies to the proteins A56, K2, A16, A28, the Flag epitope or GAPDH as indicated.

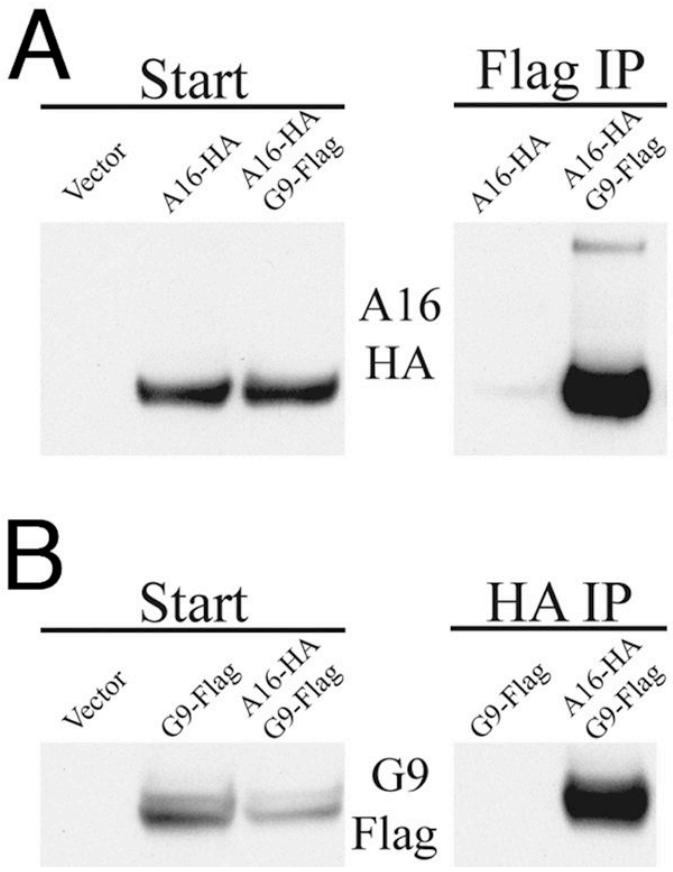

Figure 3-6: A16 and G9 interact in uninfected cells.
(A) 293TT cells were transfected with empty vector or plasmid DNA expressing A16 with a C-terminal influenza HA epitope (A16-HA) or co-transfected with plasmids expressing A16-HA and G9 with a C-terminal 3XFlag tag (G9-Flag). After 48 h the cells were lysed with Triton X-100, the postnuclear supernatant was incubated with the Flag antibody bound to beads, and the captured proteins were analyzed by Western blotting with the anti-HA antibody. (B) Cells were transfected with empty vector, G9-Flag or co-transfected with A16-HA and G9-3XFlag. The lysates were incubated with anti-HA antibody bound to beads and the captured proteins were analyzed by Western blotting with the anti-Flag antibody.

these two EFC polypeptides existed as a heterodimer or higher order multimer. To test this hypothesis, A16 and G9 were codon optimized for expression in human cells and tagged with a C-terminal influenza HA epitope or a 3XFlag epitope, respectively. $A16_{HA}$ and $G9_{3XFlag}$ were cloned separately into pcDNA3.1 under control of the human cytomegalovirus major immediate-early promoter. Human 293TT cells were transfected with the individual plasmids or co-transfected with both. After 48 h, the cells were lysed with Triton X-100 and synthesis of the recombinant proteins was demonstrated by SDS-PAGE and Western blotting of portions of the postnuclear supernatants (Figure 3-6A, Start; Figure 3-6B, Start). Additional portions of the postnuclear supernatants were incubated with agarose conjugated to Flag or HA antibody. The beads were washed extensively and the eluted proteins analyzed by SDS-PAGE and Western blotting. The $A16_{HA}$ protein was detected after the Flag immunoprecipitation only when coexpressed with $G9_{3XFlag}$ (Figure 3-6A, Flag IP). Likewise, the $G9_{3XFlag}$ was detected after the HA immunoprecipitation only when coexpressed with $A16_{HA}$ (Figure 3-6B, HA IP). Thus, A16 and G9 can associate with each other in the absence of other viral proteins.

3.4 Discussion

An unusual feature of VACV reproduction is that the infectious virus particles are assembled in the cytoplasm, rather than at the plasma membrane, and subsequently transported to the periphery and exocytosed. Large numbers of progeny virus particles remain adherent to the cell surface and these are chiefly responsible for virus spread to neighboring cells [198]. It is believed that syncytia form when large numbers of virus particles “fuse-back” i.e. deposit their fusion proteins into the

plasma membrane of the parent cell. The fact that only small numbers of syncytia form normally implies a negative regulation of fuse-back. The EV membrane surrounding the MV may form one barrier to fuse-back, although MVs with broken EV membranes are detected on the surface of cells [181]. The A56 and K2 polypeptides, which form a heteromultimer on the cell surface and EV membrane may provide another barrier since a syncytial phenotype occurs with null mutants of either [167-170, 172, 173]. It is possible A56/K2 could regulate fuse-back by preventing the disruption of the EV membrane and exposure of the MV or the subsequent interaction of the MV and plasma membranes. The ability of A56/K2 to interact with the EFC suggests the latter mechanism is important although the EFC is poorly characterized both structurally and functionally. In fact, it is not known whether the EFC directly mediates fusion or is simply a positive regulator.

There are at least eight proteins within the EFC and numerous protein-protein interactions are needed to form a stable complex [84]. To determine the binding partners of A56/K2 individual proteins of the EFC were repressed to destabilize the entry complex. Both A16 and G9 bound A56/K2, however alone these proteins interacted weakly with A56/K2 suggesting that a complex of A16 and G9 is need for the interaction with A56/K2. The interaction between A16 and G9 was confirmed by coimmunoprecipitation of the two polypeptides following transfection of expression vectors into uninfected cells. A model (Figure 3-7) depicts A56/K2 in the plasma membrane of an infected cell interacting with A16 and G9 of the EFC in the viral membrane.

A16 and G9 appear to have two roles. Each is required for membrane fusion and virus entry as well as for interaction with A56/K2. Since viruses with mutations in A56 or K2 form syncytia, it seemed possible that modifications of A16 or G9 that perturb the interaction with A56/K2 could result in a similar phenotype.

In conclusion, the presence of A56/K2 in the plasma membrane provides a way of differentiating infected from uninfected cells, presumably ensuring that VACVs preferentially fuse with the latter.

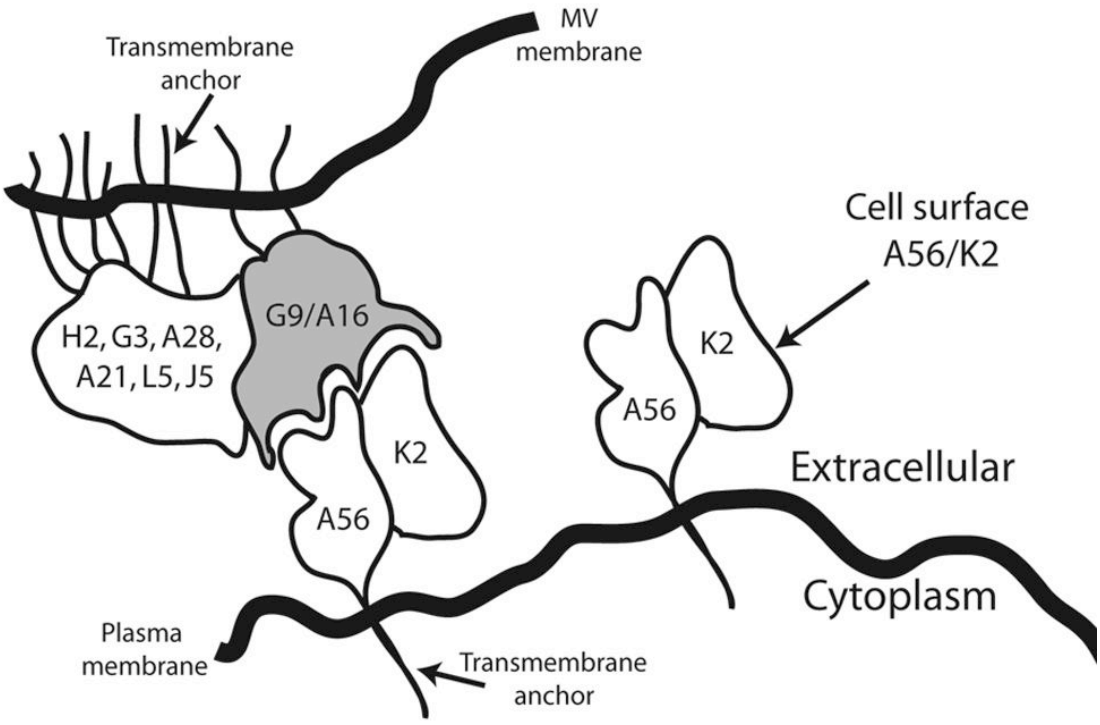

Figure 3-7: Model by which A56/K2 inhibit infected cell fusion.
G9 and A16 are anchored in MV membrane in association with EFC. A56/K2 is anchored in plasma membrane through the transmembrane domain of A56. The interaction of A56/K2 with A16 and G9 is postulated to prevent fusion of the MV particle with the plasma membrane. Fusion may be prevented as a resulting o f the interaction of A56/K2 with the EFC which could prevent activation of the viral fusion proteins.

Chapter 4: Cells expressing A56 and K2 show reduced virus entry and fusion with VACV infected cells

4.1 Introduction

In the absence of either A56 or K2 infected cells fuse spontaneously at neutral pH [167-170, 172, 173]. A56 and K2 are regulators of cell-cell fusion, a process thought to be the consequence of superinfection by cell surface EV [199]. In chapter 2 both A56 and K2 were shown to associate with proteins of the EFC. A56 was unable to bind the EFC in the absence of K2 and vice versa. This suggested a relationship between binding of A56/K2 to the EFC and inhibition of cell fusion. Although the EFC is composed of at least eight proteins; only A16 and G9 were important for interacting with A56/K2 (chapter 3). Both A16 and G9 were needed to interact with A56/K2 as neither protein alone bound efficiently. A16 and G9 interacted in transfected cells independent of any additional viral proteins. These data support a model by which A56/K2 bind to the EFC to prevent infected cell fusion.

When cells are mixed after single infection with IHD-J (A56+) or IHD-W (A56-) cell fusion occurs only between cells infected with IHD-W (A56-) [167]. To provide support for a biological function for the interaction between A56/K2 and the EFC cells expressing A56/K2, or each protein individually were mixed with cells infected with vΔA56ΔK2. Cells expressing both A56 and K2 were resistant to fusion compared to cells expressing A56 or K2 alone. Expression of A56/K2 also correlated with reduced virus entry. These results strongly suggest A56/K2 inhibits cell fusion and virus entry likely by interacting with the EFC.

4.2 Material and Methods

4.2.1 Cells and virus.

BS-C-1 (ATCC CCL-26), HeLa S3 (ATCC CCL-2.2) suspension cells were grown as described in chapter 2, section 2.2.1. Growth of human 293TT cells was described in chapter 3, section 3.2.1. Construction of vΔA56ΔK2 was described in chapter 2, section 2.3. vFire-WR has been described previously in [94]. General procedures for preparing and titrating stocks were done as previously described in chapter 2, section 2.2.1.

4.2.2 Purification of VACV.

HeLa cells were infected at an MOI of 3 vFire-WR and incubated for 2 day at 37 ^{o}C. VACV MV was isolated by mechanical disruption of HeLa S3 cells and subjected to sedimentation twice through a 36% sucrose cushion. Virus was resuspended in 1mM Tris-HCl (pH 9.0) and titered as described in chapter 2, section 2.2.1.

4.2.3 Codon optimization of A56 and K2.

The DNA sequence for the VACV WR A56R and K2L genes was optimized (Geneart, Regensburg, Germany) to improve RNA processing and translation by altering codon usage and G-C content. The codon optimized A56R and K2L genes were PCR amplified. For A56, oligonucleotides were designed to append the DNA sequence for a V5 epitope tag to the C terminus of the ORF. The PCR products of A56$_{V5}$ and K2 were cloned into the directional TOPO vector pcDNA 3.1 (Invitrogen) and sequenced to confirm proper insertion and sequence.

4.2.4 Transfection.

The day prior to transfection $2x10^5$ 293TT cells were seeded per well of a 48 well plate in 300 µL of 10% DMEM lacking hygromycin. A transfection mixture was prepared as follows: 1.5 µL of Lipofectamine 2000 (Invitrogen) was mixed with 50 µL of Opti-Mem (Invitrogen) and incubated 5 min at room temperature. In a separate tube, 50 µL of Opti-Mem was combined with the DNA. After 5 min the two solutions were mixed and incubated a minimum of 25 min at room temperature. The different samples for transfection were configured as follows: 900 ng of total DNA, 100 ng of which is the P11-FFLUC plasmid that encodes the firefly luciferase gene expressed from the late P11 viral promoter. Cells were cotransfected with by adding 400 ng of A56 plasmid and 400 ng for K2. Cells transfected with only a single plasmid consisted of 400 ng of either A56, K2, or VSVG with the remaining 400 ng of DNA supplemented with empty vector. Once transfected the cells were grown for 24 h at 37 ^oC with 5% CO_2 at which point the medium was changed and the cells were incubated for an additional 24h prior to analysis.

4.2.5 Antibody staining and flow cytometry.

The following antibodies were used: anti-A56 monoclonal antibody 1H831 (provided by Alan L Schmaljohn), anti-K2 monoclonal antibody 4A11-4A3 (provided by Richard Moyer), Cy5-conjugated donkey anti-mouse. (Jackson ImmunoResearch). Antibody staining was performed as follows: cells were resuspended and pelleted in bench top centrifuge for 20 sec at 5k x g and supernatent was removed. The primary antibody (anti-A56 or anti-K2), diluted in 10% DMEM, was incubated with cells for 15 mins at which point the cells were washed twice with 500 µL DPBS without Ca

and Mg (Quality Biological). Secondary antibody (diluted 1:300 in 10% DMEM) was incubated 15 min with the cells after which the cell were washed twice with 500 µL DPBS. Samples were acquired on a FACSCalibur flow cytometer (BD Immunocytometry Systems) and analyzed using FlowJo software (TreeStar, San Carlos, CA).

4.2.6 Quantification of cell-cell fusion.

$2x10^5$ BS-C-1 were seeded per well of 24 well plate. The following day, cells were infected at an MOI of 5 in 2.5% EMEM with vΔA56ΔK2. Separately, 293TT cells were cotransfected with A56/K2 or transfected individually with A56, K2, empty vector or the VSVG glycoprotein fused to GFP. All transfections also contained 100 ng of p11-FFluc plasmid. After 48 h the transfected cells were resuspended immediately prior to adding to the infected cell monolayer at a concentration of $5x10^5$/mL in 10%DMEM with 40 µg cytosine arabinose (AraC). The medium was removed from the infected cell monolayers at 18 h postinfection and replaced with 250 µL of medium corresponding to $1.25x10^5$ 293TT cells. The cells were incubated with the infected monolayer for 4 h at 37 ^oC and then lysed by adding 100 µl of 3.5X cell culture lysis buffer (Promega). Cells were incubated with lysis buffer for 10 min at room temperature with constant rotation. A 20 µl portion of the lysate was mixed with 100 µl of luciferase assay substrate (Promega), and activity was quantified on a Berthold Sirius luminometer.

4.2.7 Measuring virus entry

vFire-WR pelleted over 2X sucrose cushions was diluted in 10% DMEM to

$6x10^6$ Pfu/ml. 50 μl of virus inoculum was added per well of a 48 well plate. At 2 h post-infection, medium was removed and cells were lysed by adding 100 μL of cell culture lysis buffer and incubating 10 min at room temperature with constant rotation. A 20 μl portion of lysate was removed and incubated with 100 μl of luciferase assay substrate, Luc activity was quantified on a Berthold Sirius luminometer.

4.3 Results

4.3.1 Expression of A56 and K2 in transfected cells.

The A56 and K2 proteins localize to the plasma membrane of infected cells and are also found in the EV membrane. A56 is required for membrane retention of K2 in both infected as well as transfected cells [173, 174]. A56 and K2 inhibit cell-cell fusion possibly through an interaction with proteins of the EFC. Cell surface localization of A56 and K2 is important for inhibiting cell fusion which is supported by studies which reveal: i) deletion of the membrane anchor of A56 [173], ii) deletion of the signal sequence from K2 [174] or iii) addition of antibodies to A56 [172] and K2 [173] lead to cell-cell fusion.

Cell surface localization of A56 and K2 was analyzed by flow cytometry. Prior to expression in mammalian cells the A56 and K2 genes were codon optimized. VACV genes are normally expressed in the cytoplasm and may contain sequences inhibitory to nuclear expression. The optimized A56 and K2 genes were individually cloned into pcDNA3.1 under control of the human cytomegalovirus major immediate-early promoter. Cells were cotransfected with plasmids expressing A56 and K2, or with a single plasmid encoding A56, K2 or empty vector. At 48 h post-

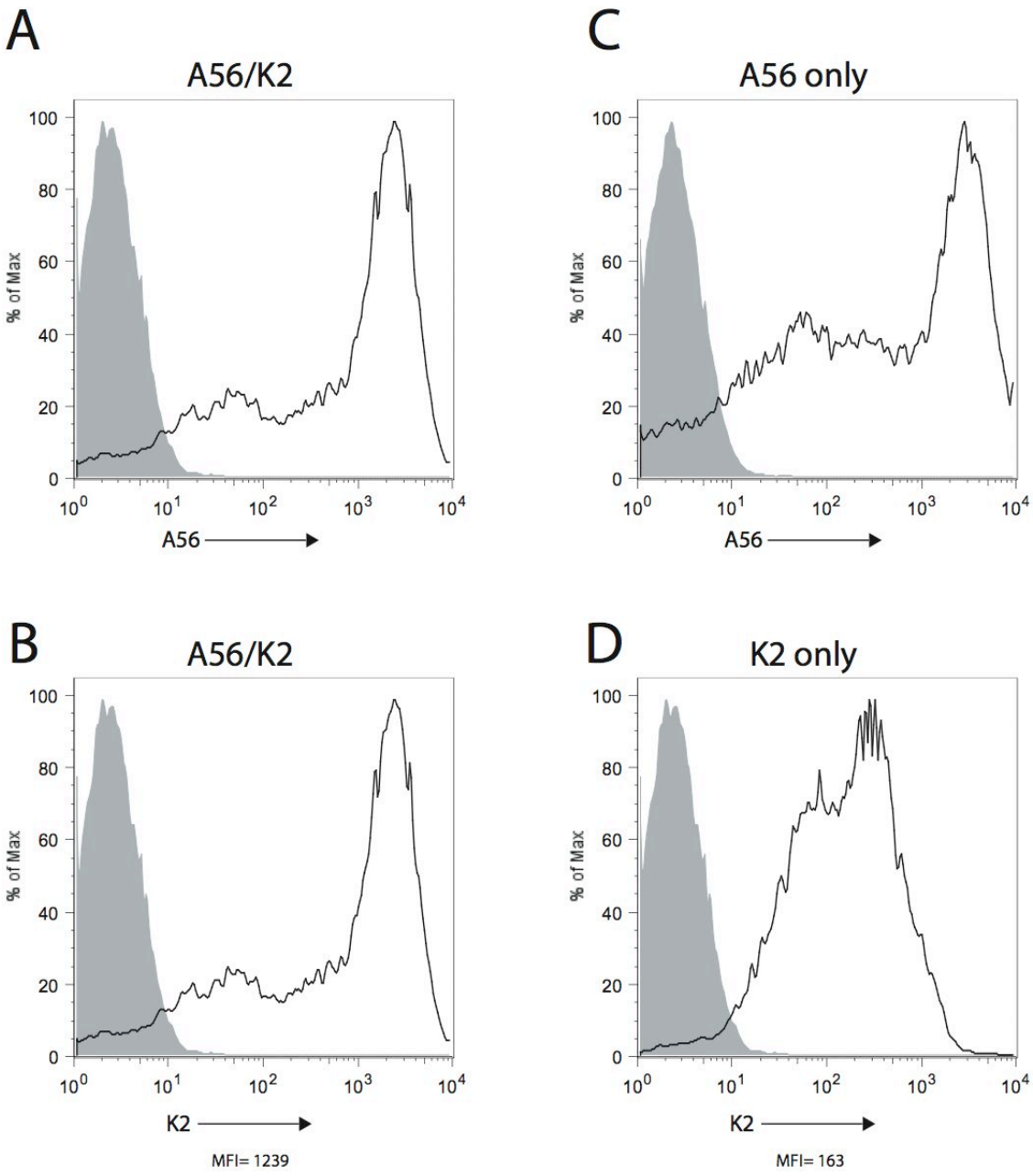

Figure 4-1: Cell surface expression of A56 and K2.
293TT cells were cotransfected with plasmids expressing A56 and K2 (A, B), or transfected with a single plasmid for empty vector, A56 (C), or K2 (D). At 48hr posttransfection cells were stained with A56 antibody (A, C) or K2 antibody (B, D), washed and incubated with secondary antibody conjugated to Cy5 prior to analysis by flow cytometry. Staining of cells transfected with empty vector is represented within the gray shaded area. Mean fluorescent intensity (MFI) is listed below the abscissa.

transfection the cells were stained with antibodies to A56 or K2, washed and then stained with secondary antibodies conjugated with a fluorophore. Cells were not fixed so as to retain the integrity of the plasma membrane and following staining the samples were analyzed by flow cytometry. Both the antibodies used to detect A56 and K2 were of mouse origin preventing simultaneously analysis of A56 and K2. Instead, cells transfected with A56 and K2 were divided, with a portion stained with A56 antibody and a portion with the K2 antibody. Cells cotransfected with both A56 and K2 plasmid displayed abundant surface staining of both proteins (Figure 4-1A+B). Cells transfected with an A56 plasmid also exhibited extensive surface staining for A56. The gray area represents antibody staining of cells transfected with empty vector. The mean fluorescence intensity of cells expressing K2 alone was reduced 7 fold when compared to cells expressing K2 with A56. These results agree well with previous reports that have demonstrated A56 is important for anchoring K2 to the plasma membrane [173].

4.3.2 A56/K2 expression correlates with reduced fusion with virus induced syncytia.

Cell fusion occurs spontaneously at neutral pH among cells infected with viruses that lack either the A56R or K2L gene. If cells are mixed after single infection with IHD-J (A56+) or IHD-W (A56-) cell fusion occurs only between cells infected with IHD-W (A56-) [167]. This indicated acquisition of A56 was associated with inhibiting cell fusion, but did not demonstrate A56 (along with K2) was sufficient to inhibit cell fusion.

To determine if A56 and K2 were sufficient to inhibit fusion, 293TT cells were cotransfected with plasmids expressing A56 and K2, or transfected with a single plasmid encoding A56, K2, VSVG glycoprotein fused to EGFP, or empty vector. A plasmid containing the firefly Luc gene under control of a late viral promoter was included in all transfections to monitor cell fusion. At 48 h post-transfection the cells were resuspended in medium containing the viral DNA replication inhibitor AraC to prevent virus late gene expression which may occur after the cells are mixed with the infected monolayer. The transfected 293TT cells were added to a monolayer of BS-C-1 cells that had been infected 18 h previously with vΔA56ΔK2. Extensive cell fusion had developed in the infected BS-C-1 monolayer by 18 h postinfection. The cells were incubated with the monolayer for 4 h, lysed and Luc activity was measured.

Data is expressed as a ratio of the Luc activity relative to A56/K2. Cells expressing both A56 and K2 displayed the lowest Luc activity, which would be expected if binding to the EFC inhibits fusion. Comparing the Luc value of cells expressing A56/K2 to the values obtained for cells expressing only A56 or K2 indicated a 3.72 and 5.53 fold increase in Luc activity, respectively, indicating cells expressing only A56 or K2 were unable to inhibit fusion (Figure 4-2). These data are consistent A56 or K2 individually being unable to efficiently bind the EFC. The ratio of Luc value for cells expressing just A56 (3.72) was lower than the value obtained from cells expressing K2 alone (5.53) , VSVG (5.61) or empty vector (6.63), however the value was well above what was observed for expression of A56/K2 (1.00). The VSVG glycoprotein and empty vector were used as negative controls

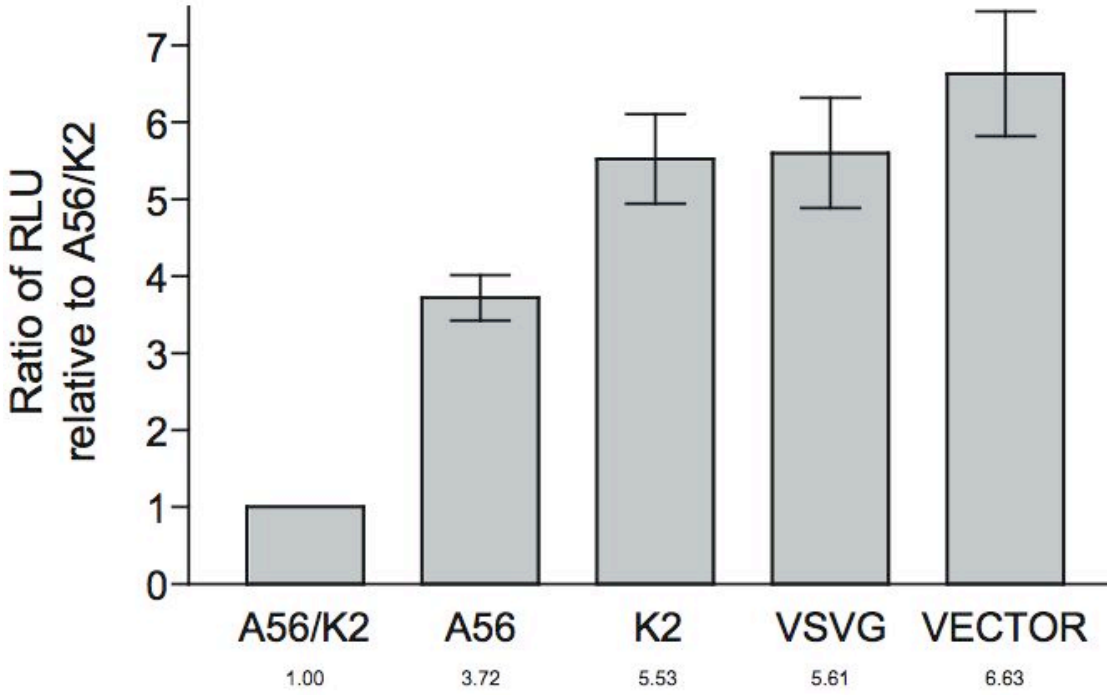

Figure 4-2: Effect of A56 and K2 expression on fusion between transfected cells and VACV induced syncytia.
293TT cells were cotransfected with plasmids for A56 and K2 or transfected with a single plasmid expressing A56, K2, VSVG or empty vector. All transfections included firefly luciferase plasmid as a reporter for cell-cell fusion. At 48 h post-transfection the cells were resuspended and added to a monolayer of BSC1 cells that had been infected for 18 h with vΔA56ΔK2. After 4 hrs the cells were lysed and assayed for firefly luciferase. Data is represented as a ratio of relative light units (RLU) relative to A56/K2 sample. Experiments were preformed in quadruplicate and data points represent the mean (listed below sample names) ± standard errors of the means.

as cells transfected with either plasmid were not expected to inhibit cell fusion. A56 was observed to weakly interact with proteins of the EFC (Figure 2-5), which may account for the slight reduction in Luc activity. Never the less, optimal inhibition of cell fusion required both A56 and K2. These results demonstrate that uninfected cells are able to fuse with infected cells and cells expressing both A56 and K2 reduce the extent of fusion.

4.3.3 A56 and K2 expression reduce virus infection.

The processes of virus-cell fusion and cell-cell fusion require the conserved multiprotein EFC, indicated A56/K2 may be able to regulate virus entry in addition to cell-cell fusion. There currently is no assay available to directly quantify virus fusion, however early gene expression has been used to study entry of VACV. The VACV vFire-WR expresses the firefly Luc gene from an early-late promoter and Luc synthesis begins almost immediate after virus entry. Although this is a post-fusion assay it has been used previously to characterize the effect of low pH on virus entry [94, 95]. 293TT cells were cotransfected with plasmids for A56 and K2 or transfected with a single plasmid for A56, K2, VSVG glycoprotein fused to GFP, or empty vector. At 48 h post-transfection cells were infected at an MOI of 1 with vFire-WR, 2 h later the cells were lysed and the Luc activity was measured.

The data is reported as a ratio of Luc value compared to infection of cells transfected with vector alone. Cells transfected with both A56 and K2 displayed only 27% of the Luc activity of cells transfected with vector alone (Figure 4-3). Cells expressing only A56 or K2 failed to effectively inhibit virus entry to the extent of

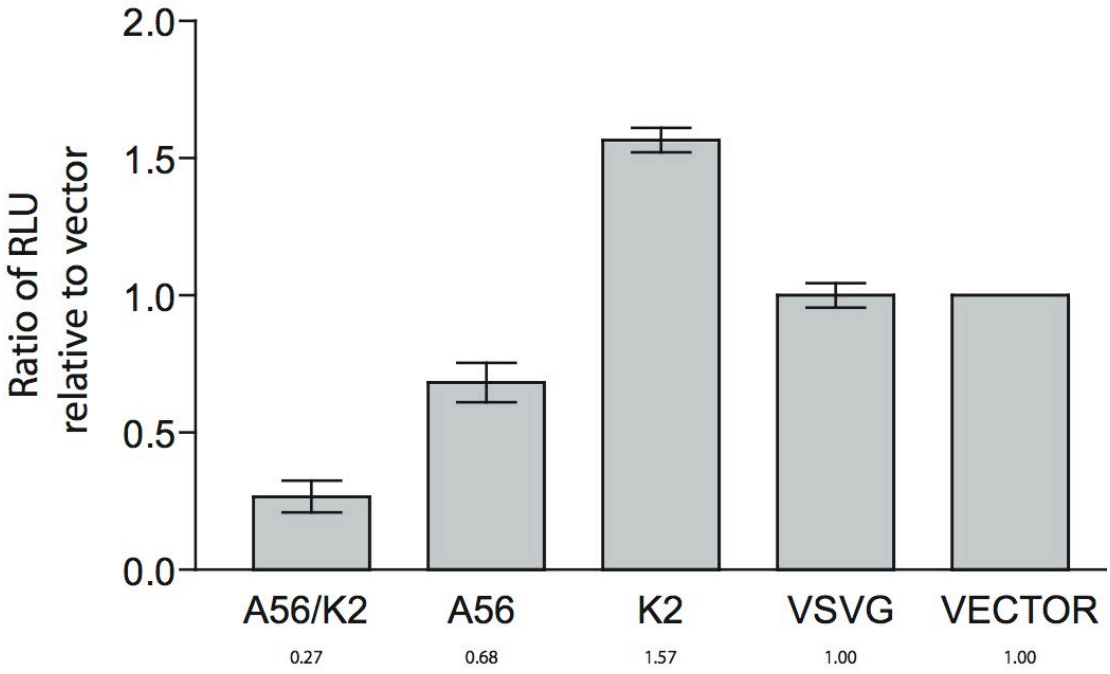

Figure 4-3: Effect of A56 and K2 expression on VACV entry.
293TT cells were cotransfected with plasmids expressing A56 and K2 or transfected with a single plasmid for A56, K2, VSVG or empty vector. At 48hr post-transfection cells were infected at an MOI of 1 with vFire-WR. After 2hrs, luciferase activity was measured and expressed as relative light units (RLU). The data is the ratio of RLU compared to vector. Experiments were done in quadruplicate with the mean (listed below the sample names) ± standard errors of the means.

cells expressing both proteins. In fact, cells expressing K2 showed an increase in Luc activity (1.57 fold), while a slight reduction in Luc expression was noted with cells expressing A56 alone (68%) similar to what was noted for inhibition of cell-cell fusion. Cells expressing the VSVG glycoprotein displayed similar Luc activity (1.00) compared to cells transfected with empty vector. These results indicate that A56/K2 in addition to reducing cell-cell fusion also appear to reduce virus entry as measure by early gene expression.

4.4 Discussion

Cell fusion is triggered by low pH treatment of infected cells [165, 166] or occurs spontaneous at neutral pH with viruses that are deleted for A56R or K2L gene [167-170, 172, 173]. Cell fusion depends on cell surface EV and is hypothesized to involve "fuse back" of EV, in which the viral EFC is depositing into the plasma membrane [199]. Viruses unable to form EV do not trigger cell fusion [152] and fusion fails to occur when components of the viral EFC are repressed [80]. A56 and K2 have been shown to bind the viral EFC. This interaction was shown to depend on expression of both A56 and K2 (chapter 2) and the fusion regulatory proteins (A56/K2) were identified to interact specifically with A16 and G9 entry proteins (chapter 3). Through this interaction a model was developed by which A56/K2 binds the EFC to inhibit cell-cell fusion (Figure 3-7).

Previous experiments involving mixing of cells individually infected with IHD-J (A56+) or IHD-W (A56-) demonstrated cell fusion occurs only between cells infected with IHD-W (A56-) [167]. In these experiments cells expressing A56 (and presumably K2) were resistant to cell fusion. To extend this observation and

demonstrate A56/K2 are sufficient to prevent fusion, cells were transfected and shown to express both A56 and K2 on their cell surface. These cells were added to a monolayer of cells that had formed syncytia and cell fusion was monitored by activation of Luc. Cells expressing both A56 and K2 fused poorly exhibiting only 20% of Luc activity relative to cells transfected with empty vector. In comparison, cells expressing only A56 or K2 were unable to inhibit cell fusion with Luc value 3.7 fold and 5.5 fold higher, respectively, compared to cells expressing A56/K2. These results were consistent with the inability of A56 or K2 alone to bind the EFC and confirmed i) uninfected cells are able to fuse with syncytia form by infected cells and ii) expression of A56 and K2 correlated with reduction in cell fusion with virus triggered syncytia. This provides the first evidence that A56 and K2 are sufficient to inhibit cell fusion. Cell-cell fusion and virus-cell fusion can be view as very similar processes. Entry of VACV was examined in cells expressing A56 and K2 by early gene expression. A56 and K2 reduced early gene expression by 70% compared to cells transfected with empty vector. Expression of A56 showed only a 30% reduction, while K2 actually increased early gene expression. The increase in Luc expression observed for cells expressing K2 was unexpected. Additional experiments are required to explain the increase in Luc activity. Perhaps the increase is related to the SERPIN motif within K2. A minor portion of A56 was observed to bind to the EFC in the absence of K2, which may partially accounted for a slight reducing in virus entry for cells expressing A56 (Figure 2-5). These data offer the first evidence for a biological significance to the interaction between A56/K2 and the EFC.

Chapter 5: Conclusions and Future Direction

5.1 Conclusions

In this dissertation, I have sought to understand the mechanism by which A56 and K2 proteins inhibit infected cell-cell fusion. Deletion of A56 or K2 is associated with spontaneous neutral pH syncytia formation at late times during infection. Spontaneous cell fusion requires EV and is likely mediated by superinfection of cell surface EV. Entry and fusion of VACV requires a conserved multiprotein complex located within the MV membrane.

My dissertation project focused on identification of the protein interactions of A56 and K2 related to the ability of these proteins to regulate infected cell fusion. Tandem affinity purification revealed A56 interacted with proteins of the VACV EFC. A56 associates with K2 so it was conceivable that K2 may also bind the EFC. An interaction between K2 and the EFC was confirmed by co-purification of the EFC with the K2. Further experiments revealed A56 failed to bind the EFC in the absence of K2. Interestingly, K2 displayed a similar dependence on A56 to bind the EFC. To validate an interaction between A56/K2 and the EFC the A28 protein was isolated by tandem affinity purification and Western blot confirmed A56 and K2 associated with the affinity purified EFC. However, in the absence of K2, the A56 protein failed to co-purify with the EFC. The results reported in chapter 2 provided the first evidence for an interaction between A56/K2 and the viral EFC. More importantly, it suggested binding of A56/K2 to the EFC might regulate infected cell fusion. The inability of A56 or K2 to bind the EFC independently agreed well with the requirement for both proteins to inhibit cell fusion suggesting the two processes may be related.

A56 is important for anchoring K2 to the plasma and EV membrane. The two proteins likely interact during transport through the secretory pathway, although the domains involved in their interaction have not been well defined. A region between amino acids 145-373 of K2 is important for binding A56, while mutations that abolish the protease inhibitory activity of K2 have no effect on interactions with A56 [173]. K2 with truncations of the N and C termini failed to inhibit cell fusion, suggesting truncations may prevent the protein from assuming a particular conformation important for regulating cell fusion. In our hands, similar modifications of K2 generally eliminated the anti-fusion activity of the protein.

Neither the stoichiometry of the interaction between A56 and K2 nor whether the two proteins are constantly associated is known. Affinity purification of A56 identified a number of additional proteins that may compete with K2 for binding to A56. In particular the interaction between A56 and C3 could have potential benefit for the virus in avoiding complement-mediated lysis of infected cells or virus particles.

A direct interaction with the EFC could not be attributed to either A56 or K2 individually. Perhaps the conformation of A56 and K2 is altered upon associating with one another. For example, binding of K2 with A56 may alter the conformation of A56 and allow it to associate with the EFC, or the opposite may be true. Alternatively, a conformation formed by the complex of A56/K2 may associate with the EFC. Still another possibility is that A56 and K2 both bind weakly to the EFC so that a complex stable enough for isolation is only detected when the two are associated. A minor form of A56 was observed to associate with the EFC even in the

absence of K2 (Figure 2-5), suggesting A56 may directly bind the EFC. Western blot analysis of A56 indicates the protein migrates as multiple bands, likely related to various states of glycosylation or alterative translation initiation. Whether a particular glycosylated form preferentially associates with K2 and the EFC was not addressed. A soluble complex of A56 and K2 secreted from VACV infected cells was isolated and shown to bind the EFC. The higher molecular weight form of A56 predominated in the soluble complex consistent with fully glycosylated protein being secreted from the cell. This high molecular weight species of A56 was sensitive to digestion with proteinase K, indicating this form is likely present on the cell surface [172]. A56 and K2 may also form higher oligomeric structures on the cell surface that could potentially bind more efficiently to the viral EFC due to increased avidity.

Once it was identified that A56 and K2 interacted with the EFC the next step was to determine which proteins within the EFC mediated the interaction. Two scenarios were considered: i) A56/K2 associates with the entire EFC, perhaps through the interface formed by protein interactions within the complex, or alternatively ii) individual proteins of the EFC interact directly with A56/K2. The viral EFC is composed of at least eight proteins. To determine which of these the protein were important for binding A56/K2, a series of conditional lethal viruses were constructed. The viral EFC has been shown not to form when synthesis of A21 or A28 is repressed [84]. Affinity purification of A56 in the absence of either A21 or A28 revealed interactions with the EFC were limited to A16 and G9. This suggested that only a subset of the EFC was required to interact with A56/K2.

Since both A16 and G9 co-purified with A56/K2 it was unknown which of the two proteins directly mediated the interaction. To assess this, synthesis of A16 or G9 was repressed and affinity purification of A56/K2 was performed. In the absence of A16, the G9 protein did not associate with A56/K2, while in the absence of G9, the A16 protein did not co-purified with A56/K2. The co-purification of G9 and A16 with A56/K2 suggested a direct association between A16 and G9. This was further supported by the inability of either A16, or G9 to bind individually to A56/K2. To investigate the potential interaction between A16 and G9 the two proteins were expressed by transfection in uninfected cells. A16 immunoprecipitated G9, and A16 was shown to co-purified with G9. This result was the first to demonstrate an interaction between two proteins of the EFC. An interaction between H2 and A28 has been inferred, due in part to the ability of the two proteins to associate in the absence of A16, although the proteins had not yet been shown to interact in the absence of a virus infection [84]. A current focus of the lab is to identify additional protein interaction within the viral EFC.

The identification and characterization of an interaction between the EFC and A56/K2 was important for understanding the mechanism for regulation of cell fusion, however biological significance for this interaction still remained to be established. The working hypothesis is cells expressing A56 and K2 may be refractory to cell-cell fusion or infection. A56/K2 are predicted to interact with the EFC at the cell surface. Therefore, cells transfected with A56 and K2 were monitored for surface expression of the two proteins by flow cytometry. Both A56 and K2 localized to the surface of transfected cells. In the absence of A56, a significant decrease was observed in the

amount of cell surface K2, consistent with the requirement of A56 for retention of K2 on the plasma membrane. Therefore the A56 and K2 proteins behave normally in these transfected cells.

Cells infected with vΔA56ΔK2 normally exhibit extensive cell fusion. Our thought was thatcells transfected with A56/K2 would exhibit resistance to fusing with the syncytia in much the same way that cells infected with wild-type virus expressing A56/K2 do not fuse with A56 negative infected cells. Indeed, cells transfected with A56/K2 displayed greatly reduced fusion with the syncytia monolayer (as monitored by Luc activity) compared to cells transfected with an empty vector. In comparison, cells expressing either A56 alone or K2 alone had a 3 to 5 fold increase, respectively, in Luc activity compared to cells expressing both A56/K2. This was consistent with the requirement of A56/K2 to bind the EFC. Expression of A56 and K2 did not eliminate the ability of the cell to fuse with the syncytia, but significantly reduced the amount fusion.

The process of cell-cell fusion and virus-cell fusion both depend on the viral EFC. Since expression of A56/K2 reduced fusion of cells with the syncytia, the infectivity of cells expressing A56/K2 may also be reduced. To test this, cells were infected with a VACV expressing the Luc gene to measure virus entry. Cells expressing both A56/K2 displayed a dramatic reduction in Luc activity compared to cells transfected with vector alone. This indicates cells expressing A56/K2 display a reduction in fusion with syncytia and entry of VACV.

There is no obvious benefit gained by superinfection of cells indicating the ability to regulate infected cell fusion may offer several advantages to the VACV.

Expression of A56/K2 may aid the virus in distinguishing between infected and uninfected cells. Alternatively, the development of large multinucleated syncytia may lead to premature apoptosis. Other viruses, notably HIV and influenza virus, have mechanisms to prevent superinfection by either downregulating or removal of cell proteins important for initiating virus entry and infection. Certain strains of herpesvirus are known to form syncytia in cell culture. Syncytia formation is associated with mutations the viral glycoproteins gK [200] and gB [201] as well as mutations in UL20 [202]. The mechanisms by which the mutations inhibit cell-cell fusion are unknown, perhaps these mutations alter trafficking or enhance surface expression of the viral fusion proteins. It has been proposed that syncytia formation in herpesviruses may be detrimental to the virus *in vivo* [203].

In conclusion, the A56 and K2 proteins are required for inhibition of infected cell fusion. Affinity purification of A56 and K2 identified an interaction with the viral EFC, suggesting this association may be important for inhibition of infected cell fusion. Cells expressing A56 and K2 were resistant to fusion with virus induced syncytia and displayed a similar resistance to virus entry. Additional studies should reveal the mechanism by which binding of A56/K2 to A16/G9 inhibit virus fusion.

5.2 Future Directions

Identification and characterization of the interaction between A56/K2 and the EFC provides the first evidence for a molecular mechanism for the regulation of infected cell fusion. Much remains to be determined with respect to poxvirus entry and fusion, in particular how the interaction of A56/K2 with the EFC regulates cell-cell fusion. Although the EFC is required for virus-cell and cell-cell fusion it is

uncertain whether the EFC directly mediates fusion or has an accessory role, perhaps serving as a scaffold for assembly of the fusion protein(s). Never the less, VACV appears to have evolved a mechanism of regulating cell fusion through the interaction of A56/K2 with EFC.

Antibodies to A56 and K2 trigger cell fusion similar to deletion of the respective protein. The antibodies may trigger cell fusion through several mechanism: i) the antibodies disrupt interactions between A56/K2 and the EFC or ii) the antibodies trigger endocytosis of A56 or K2, down regulating surface expression. The former is the more interesting outcome as the antibody epitopes could be mapped to identify domains within A56 and K2 important for the interaction with the EFC.

A cell line expressing A56 and K2 would be preferred over transfection, which is associated with variation in the number of cells transfected as well as the level of protein expression. A cell line expressing A56 and K2 would be anticipated to display a similar inhibition of virus entry as transfection and could be used to screen for viral mutants. In particular, viral mutants that trigger cell fusion in the presence of A56/K2 or are unable to bind the fusion regulatory proteins may be identified. Alternatively, a soluble complex of A56 and K2, at a high enough concentration, may inhibit virus entry and provide a means of identifying viral mutants resistant to the anti-fusion activity of A56/K2.

VACV has evolved a mechanism for regulation of cell fusion facilitated by interaction with A56/K2. This indicates A16 and G9 may represent a novel target for development of antipoxvirus agents. A better understanding of the interactions of A16/G9 with other proteins within the EFC may shed light on a mechanism by which

A56/K2 inhibit cell-cell fusion leading to development of small molecule inhibitors of virus entry.

Neutral pH cell fusion caused by deletion of A56 and K2 is likely mediated by superinfection of cell surface EV, although direct evidence is lacking. Electron microscopy has been utilized to examine entry of VACV and images have been captured documenting fusion of both MV [91-93] and EV [97] particles with the cell membrane. It may be difficult to capture the presumably rare asynchronous fuse back of EV, however during the course of superinfection the MV membrane merges with the plasma membrane. Therefore, immunoelectron microscopy could be used to examine the plasma membrane for an increase in MV proteins when cells are infected with an A56 or K2 deletion virus.

Many additional questions with regard to A56/K2 regulation remain to be answered, for example. What is the stoichiometry of the interaction between A56/K2? Is glycosylation of A56 important for binding A16/G9? What components of the EFC do A16 and G9 associate with? How does binding of A56/K2 to A16/G9 inhibit fusion (steric hindrance)? How is the EFC involved in virus fusion? Deletion of A56 and K2 is associated with cell-cell fusion in cell culture, but does cell-cell fusion occur *in vivo*? Are there additional mechanisms for inhibiting superinfection? A56 and K2 are primarily expressed during late gene expression, suggesting there may be an additional mechanism for preventing superinfection during early and intermediate gene expression. If this is the case, why are these mechanisms unable to prevent EV superinfection?

Finally, the work that I have presented in this dissertation has provided a foundation to better understand the mechanism by which VACV regulates infected cell fusion. Hopefully, the questions I have addressed over the course of my research have provided the poxvirus field with information needed to further examine the mechanism of virus entry and fusion.

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