ABSTRACT

Title of Document: CHARACTERIZATION OF TWO HIGHLY CONSERVED POXVIRUS TRANSMEMBRANE PROTEINS OF UNKNOWN FUNCTION

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The vaccinia virus I5L open reading frame encodes a 79-amino-acid protein, with two predicted transmembrane domains, conserved among all sequenced members of the chordopoxvirus subfamily. No nonpoxvirus homologs or functional motifs have been recognized, and the role of the I5 protein remains unknown. I5 synthesis was dependent on viral DNA replication and occurred exclusively at late times, consistent with a consensus late promoter motif adjacent to the start of the open reading frame. I5 was present in preparations of purified virions and could be extracted with nonionic detergent, suggesting membrane insertion. Transmission electron microscopy of immunogold-labeled thawed cryosections of infected cells revealed the association of an epitope-tagged I5 with the membranes of immature and mature virions. Viable I5L deletion and frameshift mutants were constructed and found to replicate like wild-type virus in a variety of cell lines, indicating that the

protein was dispensable for in vitro cultivation. However, mouse intranasal challenge experiments indicated that a mutant virus with a frameshift resulting in a stop codon near the N terminus of I5 was attenuated compared to control virus. The attenuation correlated with clearance of mutant viruses from the respiratory tract and with less progression and earlier resolution of pathological changes. We suggest that I5 is involved in an aspect of host defense that is evolutionarily conserved although a role in cell tropism should also be considered.

The vaccinia virus A43R open reading frame encodes a 168-amino acid protein with a predicted N-terminal signal sequence and a C-terminal transmembrane domain. Although A43R is conserved in all sequenced members of the orthopoxvirus genus, no non-orthopoxvirus homolog or functional motif was recognized. Biochemical and confocal microscopic studies indicated that A43 is expressed at late times following viral DNA synthesis and is a type-1 membrane protein with two N-linked oligosaccharide chains. Neither mature nor enveloped virions contained appreciable amounts of A43, which was detected in Golgi and plasma membranes. Loss of A43R expression had no discernible effect on plaque size or virus replication in cell culture and little effect on virulence in a mouse intranasal infection model. Although the A43 mutant produced significantly smaller lesions in the skin of mice than the control, the amounts of virus recovered from the lesions were similar.

CHARACTERIZATION OF TWO HIGHLY CONSERVED POXVIRUS TRANSMEMBRANE PROTEINS OF UNKNOWN FUNCTION

By

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Dedication

I dedicate this work to my nieces and nephews. May they find and pursue their dreams and passions in life.

Acknowledgements

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List of Abbreviations

A	Adenine
Ab	Antibody
bp	Base Pair
BSA	Bovine serum albumin
C	Cytosine
CPXV	Cowpox Virus
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic Acid
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
ECTV	Ectromelia virus
EMEM	Earle's modified Eagle medium
EM	Electron Microscopy
EFC	Entry Fusion Complex
EV	Extracellular Virion
FBS	Fetal Bovine Serum
G	Guanine
GAGs	Glycosaminoglycans
GFP	Green fluorescent protein
HIV	Human immunodeficiency virus
hpi	Hours post-infection

HRP	Horseradish peroxidase
IFN	Interferon
ID	Intradermal
IHD-J	International Health Department strain J of VACV
IN	Intranasal
ITR	Inverted terminal repetition
IV	Immature Virion
kBps	Kilobase pairs
kD	Kilodalton
MAb	Monoclonal antibody
MAC	Membrane attack complex
MEM	Modified Eagle medium
Mg	Milligram
MHC	Major histocompatibility complex
mM	Millimolar
MOCV	Molluscum Contagiosum
MOI	Multiplicity of infection
MPXV	Monkeypox Virus
mRNA	Messenger ribonucleic acid
MT	Mitochondria
MVA	Modified vaccinia Ankara
MV	Mature Virion
NK	Natural Killer

nm	Nanometer
nt	Nucleotide
ORF	Open Reading Frame
PAb	Polyclonal antibody
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate-buffered saline
PFU	Plaque Forming Units
PRR	Pattern Recognition Receptors
RNA	Ribonucleic acid
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
T	Thymidine
TIR	Toll/IL-1 receptor
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TBST	Tris-buffered saline with Tween-20
U	Uracil
µg	Microgram
VACV	Vaccinia Virus
VARV	Variola Virus
WR	Western Reserve
WV	Wrapped Virion
WHO	World Health Organization

Chapter 1: Literature Review

1.1 Poxviridae

1.1.1 Classification

The *Poxviridae* encompass a group of large and complex DNA viruses. They have a single linear double stranded DNA genome with covalently closed hairpin termini, enzymes to synthesize mRNA, and unique to most DNA viruses, they replicate in the cytoplasm of their host cell [1]. There are two subfamilies belonging to the *Poxviridae* family, the Chordopoxviruses which infect vertebrates and the Entomopoxviruses which infect insects. There are eight genera that belong to the Chordopoxvirus subfamily: Avipoxvirus, Capripoxvirus, Leporipoxvirus, Molluscipoxvirus, Orthopoxvirus, Parapoxvirus, and Yatapoxvirus. The Entomopoxviruses have three subfamilies recently renamed as the Alphaentomopoxviruses, Betaentomopoxviruses, and Gammaentomopoxviruses. Members within each genus are genetically and antigenically related, morphologically alike, and have a similar host range [1].

1.2 The Orthopoxviruses

The orthopoxviruses are the most studied of the poxviruses and infect a wide variety of hosts including humans, primates, cows, and rodents. The most well known virus in this subfamily is variola virus (VARV), the etiological agent of smallpox, which was successfully eradicated from nature by vaccination with another orthopoxvirus, vaccinia virus (VACV).

1.2.1 Variola Virus

1.2.1.1 A Historical Perspective

VARV is the causative agent of smallpox, a major scourge in mankind's history. The name of the virus is derived from one of two Latin words, either "varus" meaning pimple or "varius" meaning spotted, for the pustules that form on an infected individual.

Recent findings suggest that VARV likely diverged from an ancestral virus that was endemic in African rodents 16,000-68,000 years ago [2]. It seems reasonable to think that it most likely came into contact with early human hunters. It was probably difficult for the virus to permanently establish itself in those small isolated hunting communities as an infection with the virus would lead to life-long immunity or death. This limited the virus, allowing only for flare ups to occur once enough people in the community were susceptible. The virus had to mutate, slowly, over a very long period of time to successfully integrate itself into the human population. During this long period, while populations were increasing and cities were being established, genetic variants arose and new strains that could spread well predominated and eventually gave rise to the obligate human pathogen known today as VARV.

The earliest known writings to describe smallpox first appeared in the 4th century A.D. in China, the 7th century A.D. in India and the Mediterranean, and the 10th century A.D. in southwestern Asia [3]. In the early 1900s, paleopathologists examined the well kept tissues from mummies dating as far back as the Eighteenth Dynasty (1580 - 1350 B.C.) and proposed that smallpox was the cause of death for a

few of them, including the great pharaoh, Ramses V. They describe the appearance of lesions on the skin that resembled a smallpox rash [3]. This was the first physical evidence of smallpox ever found but written descriptions of the disease from this time, if any, remain to be discovered.

Over the centuries, smallpox spread around the world mostly by the exploring, colonizing, and the conquering of new land and continents. In places like Australia, North, and South America, the disease decimated native populations who had not previously been exposed to smallpox [3]. By the early 1900's two types of VARV could be distinguished based upon one's symptoms. Along with the dreadful disease cause by VARV major, a milder version of the disease appeared. The infecting virus causing the milder symptoms was referred to as variola minor. In the unvaccinated, variola minor had less than a 1% case fatality rate while variola major had roughly 30% case fatality.

1.2.1.2 Variolation to Vaccination

The earliest known practices to actively attempt at defending against smallpox disease was to quarantine individuals with smallpox or to practice a method now known as variolation. Variolation was the technique of deliberate inoculation of dried scab or pustular material from a smallpox lesion to an uninfected individual in the hopes of obtaining a much less severe disease. The two forms of variolation seem to have been developed independent of each other due to the different methods of inoculation. One method of variolation arose in China around 1000 AD and consisted of a nasal route of inoculation by inhalation of the smallpox material. The other method arose in India probably around the same time and utilized a cutaneous route

of inoculation [3]. The cutaneous route of variolation caused an even milder form of smallpox. A primary lesion was formed at the inoculation site with commonly seen satellite pustules and a generalized rash. Variolation by cutaneous route had a .5-2% mortality rate. The inhalation method for variolation was usually more severe and also produced a generalized rash.

In the 18th century, variolation was a well established method for protection against smallpox. However, the material used was not an attenuated version of VARV and a major disadvantage to variolation was that the virus could still cause and spread smallpox [3]. In the late 1700's, Edward Jenner, an English physician, had many patients living in the countryside. He would often perform variolation for his patients and in doing so he observed that dairy maids would not become infected after treatment. He came to find out that these patients had contracted cowpox virus (CPXV) infections in the past. This was interesting to Jenner and he eventually experimented with a young boy by inoculating the boy with CPXV material obtained from a milk-maid. He later challenged the boy with a smallpox inoculation of which had no ill effect on the boy [4]. Jenner performed additional trials and published his work on the successful use of cowpox at preventing smallpox. He also published an account of his experience and the exciting possibility of smallpox eradication in the future [3-5].

Jenner's vaccine provided many advantages and lowered risks over the practice of variolation and his vaccine was widely accepted all over Europe, the United States, and eventually around the world. However, there were many problems associated with the vaccine. Material for vaccination was not always available since

the infection of cows was sporadic. Initial arm-to-arm transfer of pustular material was a way to ensure the availability of virus but it was sometimes contaminated with VARV which could lead to smallpox. This changed once smallpox hospitals were separated from places of vaccine preparation. The arm-to-arm transfer was still a problem, though, as other human diseases were spread upon the vaccination of a new individual. Additionally, it was quite difficult to maintain a series of individuals with pustules in which material could be used for further vaccination and often the virus used in arm-to-arm transfer was found to be less effective. Sometimes the vaccine would be reintroduced into the cow, not as a source for obtaining more vaccine, but to reestablish the virulence of the vaccine. Eventually the virus was produced on the flanks of calves and resuspended as a glycerol stock. This practice helped to maintain the potency and availability of the vaccine as well as rid the possibility of transmitting other human diseases [3].

The more people vaccinated the less the incidence of smallpox and the lower the mortality rate when smallpox did reappear in a population. Epidemics did occur and though less severe, eventually led to government interventions. In some countries variolation was banned, vaccination was made compulsory for infants, and a scheme for revaccination was established. In the late 1800s, the evolving of a scientific community gave rise to better methods for vaccine preparation and distribution. Endemic smallpox was virtually gone in both North America and Europe and long term storage and large-scale vaccine production emerged in the middle 1900s [6].

Unfortunately vaccine preparations of that time often had bacterial contamination. It wasn't until the early 20th century that scientists realized that it seemed more prudent that biological products used in man should be sterile. Vaccine preparation and production continue to become safer and more efficient and the introduction of a liquid vaccine, which contained glycerol and phenol, aided in eliminating endemic smallpox from Europe and North America. However, the liquid vaccine was not suitable for hot and tropical climates. Finally, in the mid 20th century, a freeze dried vaccine that could be mass produced was made by Collier [3, 6] and shown to be efficacious [7].

In the 19th and beginning of the 20th century, before as well as during the time of vaccine development, material for vaccines was obtained as well as maintained in sheep, horses, cows, as well as arm-to-arm transfers. There were plenty of opportunities for the different vaccine stocks used to become mixed or to obtain mutations after many transfers. It appears that it was during this time that VACV also came into use as a vaccinating agent and was referred to as "vaccine virus." Early literature describes vaccination with CPXV and it seemed that the virus being used for some time had not been cowpox at all, but most likely some other orthopoxvirus [3]. The original source of VACV is still not known and, of course, there is much speculation as to its origin. Two things are certain; vaccinia is a distinct orthopoxvirus and, like CPXV, provides a high degree of protection against smallpox.

1.2.1.3 Smallpox Eradication

In 1958, at the Eleventh World Health Organization (WHO) assembly, Viktor M. Zhdanov submitted a proposal detailing a program to carry out the global

eradication of smallpox. All of the delegates agreed and a global eradication campaign began in 1959. Over a period of seven years the eradication campaign had many problems. It took a back seat to the malaria eradication campaign, it lacked funding and execution, and there was little to no administrative oversight provided by the WHO. It wasn't until 1967 when the WHO started an intensified program with increased funds as well as implementation of the important concept of surveillance [8]. The involvement of and commitment from the Communicable Disease Center (CDC) in the United States also influenced the outcome of the program [3].

At the conception of the intensified program, smallpox was endemic in Africa, Asia, Indonesia, and South America. The global eradication initiative campaign strategy consisted of (i) mass vaccination and (ii) surveillance & containment. Both of these strategies were necessary and within a 5 year time frame over 100 million vaccines were given. The benefit of mass vaccination is that it provided herd immunity. Eventually surveillance and containment became crucial for identifying and controlling outbreaks. It consisted of searching for smallpox cases and vaccinating any and all contacts close to the infected individual [9]. The last naturally occurring case of smallpox was in Somalia, in 1977. Finally, in 1980, Edward Jenner's prediction came true, smallpox was declared eradicated by the WHO.

Many factors added to the success of ridding the world of such a devastating disease. First, VARV is an obligate human pathogen with no known non-human reservoir. If transmission from human-to-human was interrupted the disease could be eliminated. Technical advances, like the bifurcated needle and freeze dried vaccine, also aided in the eradication of smallpox. The ability to diagnose and confirm

smallpox cases and interrupt transmission was crucial to the eradication. Importantly, an effective vaccine that led to years of protection in an individual also aided in the interruption of transmission.

1.2.1.4 The Threat of Smallpox as a Biological Weapon

The eradication of smallpox was a truly amazing feat and although it is almost 30 years later, its reintroduction by way of a biological weapon is considered a possibility. Following the smallpox eradication campaign there was a continuing debate over the deliberate destruction of all VARV stocks. It was suggested by a WHO committee that all stocks of VARV either be transferred to 1 of 2 WHO appointed laboratories, either the Center for Disease Control in Atlanta, Georgia, or the Institute of Virus Preparations in Moscow, Russia [10]. All countries complied. There is, however, a first-hand account from a Russian defector, Ken Alibek, a former deputy director of the Soviet Union's bio-weapons program, regarding a program that started in the USSR in 1980 to make and use smallpox as a biological weapon [11]. It is postulated that smallpox and the technology to grow it could have been acquired from the Soviet Union, during a time of financial need, by groups with ill will. Of course, there also remains the possibility that some countries did not comply with the WHO and did not hand over all of their VARV stocks.

There are a number of reasons to believe and anticipate the use of smallpox as a bioterrorist agent. Its 30% fatality rate among unvaccinated individuals is one of the primary reasons it is considered a serious threat [10]. It cannot be denied that the human population would be highly susceptible to the release of such an agent since vaccination against VARV stopped about 30 years ago and herd immunity would

most likely not help the non-vaccinated individuals. Historical accounts of the introduction of smallpox to naïve populations are proof that it has been used in the past to successfully decimate large populations [3]. In addition to the fact that the virus particle is stable and easily transmitted via aerosol from person-to-person, most health care workers are not experienced in the diagnosis of human smallpox which could cause delay in determining the cause of illness. There would also be confusion and eventually wide spread panic.

With this and other threats in mind, the US signed into law Project Bioshield. Project Bioshield was enacted in 2004 in an effort to increase emergency preparedness in the United States against any major threat to the public. Project Bioshield was designed to hasten research and development, secure funding to facilitate appropriate purchases, and aid in the development of convenient and useful approaches to protect against harmful agents and attacks [12].

1.2.2 Monkeypox Virus and Cowpox Virus

Monkeypox virus (MPXV) is a member of the orthopoxviruses and though initially discovered in 1958, it was not believed to be an important human pathogen until the early 1970's [13]. Monkeypox is endemic in central and western Africa and mainly infects rodents and sometimes non-human primates [13-14]. Zoonotic human infections typically come about upon the intrusion of humans into tropical rainforests and possibly from children playing with contaminated carcasses. The United States has also witnessed zoonotic episodes with PXV in recent years due to the importation of exotic animals from Africa [15-17]. Most zoonotic infections are acquired through cuts, abrasions, or bites from an infected animal but transmission may also occur by

inhalation [18]. Typically transmitted from animal to human, however, human-to-human transmission has also been observed [19]. Zoonotic monkeypox infections are clinically indistinguishable from smallpox and though less fatal than a smallpox infection, there is no proven treatment for human monkeypox [15]. There is concern that monkeypox could become a more efficient human pathogen under favorable circumstances. There are several reason to support this hypothesis: the genetic makeup of the virus, the ecological changes, changes in host behavior, and routine smallpox vaccinations are no longer administered [14].

CPXV virus is another member of the orthopoxviruses best known for its use by Edward Jenner as the first vaccinating agent to smallpox. CPXV was named for its isolation from lesions on infected cattle but the likely reservoir for this virus is wood mice or voles [20]. CPXV is distributed in Europe, Russia, the western states of the former USSR, and adjacent areas of northern and central Asia [21]. CPXV can cause a localized zoonotic infection; however, the occurrence for this is rare. The virus is not often found in cattle and many of the zoonotic cases came about by contact with infected cats [22] and most recently in France from pet rats [21].

1.2.3 Other Poxviruses that Cause Human Infection

There are four poxviruses genera that can infect humans, exclusively or zoonotically: *Orthopoxvirus, Parapoxvirus, Molluscipoxvirus,* and *Yatapoxvirus* [18]. Five members of the orthopoxviruses have been known to cause human infections. VARV, as previously discussed is strictly a human virus and causes the disease smallpox. VARV has been eradicated from nature. Also mentioned above, CPXV and MPXV infect humans, as do buffalopox virus, and VACV [23].

Buffalopox virus, another orthopoxvirus similar to VACV, causes a mild illness with few lesions on the hands and arms. Its disease symptoms are similar to that of CPXV and though less severe it can leave minor pox-like scars. The reservoir host is the water buffalo residing in India. Human-to-human transmission to family members from those zoonotically infected has been reported [24].

The genus *Parapox* has four members that are known to cause infection in humans, orf virus, pseudocowpox virus, bovine popular stomatitis, and seal parapoxvirus. Human infection with a parapoxvirus is typically due to occupational hazard and seldom from fomites. Orf virus is transmitted from sheep and goat, pseudocowpox virus is transmitted from dairy cattle, bovine popular stomatitis virus is transmitted from beef cattle and seal parapoxvirus is transmitted from seal and sea lion. A human infection is acquired through a cut or scrape in the skin. The virus then replicates locally in the regenerating keratinocytes and lesions come about by hypertrophy and proliferation of epidermal cells. Most human infections with parapoxviruses produce one lesion, which can resolve over a period of a few weeks [23].

Like VARV, molluscum contagiosum virus (MOCV) infections are restricted to humans and there is no evidence of transmission from humans to animals and no known animal reservoir. Infection with MOCV is thought to be transmitted directly through a break in the skin though there are some cases where the virus has been transmitted by fomites. Once the virus infects and replicates it initially forms a small papule which matures into a 2-to-5-mm smooth dome shaped nodule that appears tumor-like. The number of lesions that appear can vary from 1-20 and tend to be

located in the trunk and proximal extremities. While lesions may only last for two months the infection can last for up to nine months [23].

Two viruses belong to the *Yatapoxvirus* genus and both are able to cause infection in humans: tanapox virus and yabapoxvirus. The first recognized human infection with tanapox virus was first discovered in Kenya near the Tana River and is restricted to Africa. Tanapox virus is not known to be transmitted from human-to-human but there is evidence it may be transmitted by an insect vector. Though rare, animal handlers have also contracted the virus directly from primates. Once infected with tanapox, a systemic infection ensues with fever, headache, and backache. These symptoms diminish as the formation of the one typical lesion transpires. Resolution takes approximately six weeks. Yabapoxvirus is not a naturally occurring infection in humans but causes infection if injected intradermally or subcutaneously [23].

1.3 Vaccinia Virus

The majority of research on orthopoxviruses has been carried out on VACV. It is a member of the orthopoxviruses and shares genetic and antigenic similarities with other orthopoxvirus members which is the reason VACV was effectively used as the vaccine to help rid the world of smallpox [1]. It continues to be used in the laboratory as a tool to understand the basic virological properties of the orthopoxviruses. VACV is a convenient and safe alternative to the more pathogenic viruses, including VARV and MPXV, and therefore is the most commonly studied orthopoxvirus.

1.3.1 Structure: Morphology and Composition

There are two morphologically distinct infectious particles: the mature virion (MV) and the extracellular virion (EV) (Fig. 1). Unlike most other viruses, the MVs are large and asymmetrical, and seemingly brick shaped with rounded edges. The MV has a single lipid bi-layer containing irregular protrusions that encases a complex inner structure. The core of the virion is electron dense, which is presumed to be DNA-protein complexes, dumbbell shaped and flanked by two lateral bodies of heterogeneous material. It is approximately 360x270x250 nm in size and has an approximate mass of 9.5fg. The virion is primarily protein, lipid, and DNA. Recently, a combination of cryo-microscopy and electron tomographic reconstruction was utilized to aid in determining the inner and outer structure of the MV, which is the most abundant particle form [25]. The EV is essentially an MV with an additional membrane derived from trans-Golgi or endosomal membrane [1].

1.3.2 Genome Organization and Nomenclature

Poxvirus genomes are linear, double stranded DNA with covalently closed hairpin termini. Poxvirus genomes range in size from 134 kBps (parapoxviruses) to 300 kBps (avipoxviruses). All poxviruses have inverted terminal repetitions (ITRs) at each end of their genome which are identical in sequence but opposite in orientation [26]. ITRs are not completely based paired as they contain the hairpin loops that join the two DNA strands [27]. Also within the ITRs are several open reading frames (ORFs), a series of variable length tandem repeats, and a highly conserved sequence of approximately 100 base pairs which is required for resolution of concatemeric genomic DNA (Fig. 2A) [28-31] .

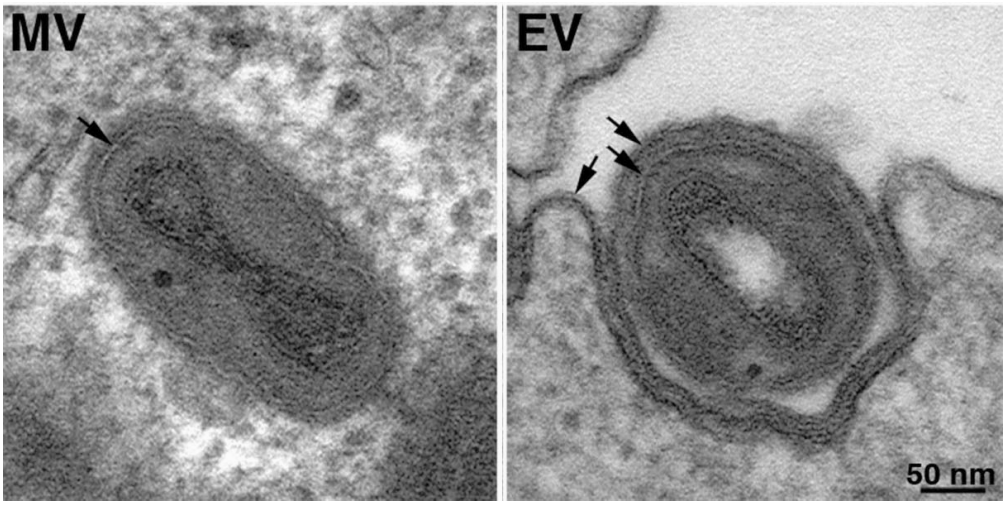

Figure 1-1. Morphological forms of infectious vaccinia virus.

VACV infected cells were ultra-cryosectioned and viewed by transmission electron microscopy. The arrows point to the single membrane in the image of the MV and the two membranes as well as the cell membrane in the image of the EV. Image provided by Andrea Weisburg.

Poxvirus open reading frames (ORFs) are non-overlapping and tend to occur in blocks pointing toward the closer end of the genome. Either an early, intermediate or late promoter sequence is present for each ORF. The more highly conserved genes are located in the central region of the genome and tend to be involved in essential replication functions. The more variable genes are found at the ends of the genome and tend to be involved in host-interactions. The method of naming genes was adopted from the VACV strain Copenhagen before the full sequence of the virus was known and is based on the HindIII restriction digestion of the viral DNA. Open reading frames are designated based on the size of the restriction fragment in which they are found, the largest fragment was designated A, and the smallest fragment was designated P. The letter of the fragment is then followed by the ORF number indicating the position in the fragment and an L (left) or R (right) to show the direction of the ORF (Fig. 2B). The gene product is named similarly omitting the directional indicator. Though other poxvirus genomes have been completely sequenced and genes numbered in successive order, this initial naming method was preserved to maintain consistency in the literature. There are approximately 90 genes conserved in all chordopoxviruses with roughly half of those conserved in all *Poxviridae* [32].

1.3.3 Viral Replication Cycle

1.3.3.1 Attachment

Poxviruses have an elaborate life cycle (Fig. 3) complicated by the existence of more than one type of infectious particle, the MV and the EV. Another snag in

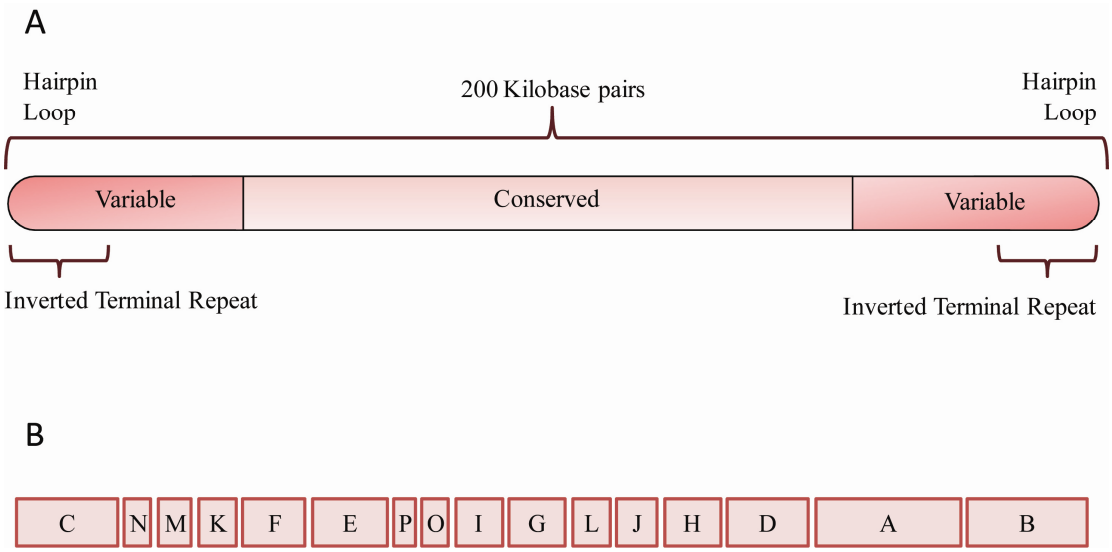

Figure 1-2. Vaccinia virus genome organization. A. Depiction of the VACV 200 kb double stranded DNA with inverted terminal repeats, hairpin loops, variable, and conserved regions. B. Depiction of VACV DNA fragments following digestion of the VACV genome with HindIII restriction enzyme. Naming started with A through P beginning with the largest to smallest fragment.

the issue of attachment and entry is that VACV has a broad tropism making it quite difficult to identify a specific cell surface receptor. The EV particle is involved in cell to cell spread and is essentially an MV particle with an additional outer membrane acquired during the wrapping process. Most studies are done with the MV since it is stable, the more abundant form, and is easily purified. Purification of the EV is also possible but the outer most membrane is quite fragile and can easily be disrupted. The protein composition of the EV and MV membranes are different and this is the most likely reason for the difference in cell interactions.

Another point is that MVs remain in the cell until lysis occurs, but EV is released from the cell and therefore the spread of VACV relies on EV particle. Given that the proteins involved in VACV entry and fusion are found on the MV, the EV membrane must be removed or disrupted for entry of the MV to occur. This disruption of the EV envelope involves two of the EV proteins, A34 and B5, and their interactions with cell surface polyanions [33]. Once the EV membrane is disrupted the MV particle is exposed to the cell surface allowing for MV-cell surface interactions to occur. Three MV proteins A27, D8, and H3 [34-36] have been implicated in viral adsorption to the cell by binding cell surface glycosaminoglycans (GAGS). A27 and H3 bind heparin sulfate while D8 binds chondroitin sulfate. It is possible that these interactions may be a precursor to fusion, however, individually the proteins were not found to be essential for viral replication [37]. Another fascinating point is that MV can bind and enter Sog9 cells, which are deficient for GAGS. Laminin may be important for binding since soluble laminin competitively inhibits MV binding to both Sog9 and BSC40 cells [38]. Interestingly, VACV is

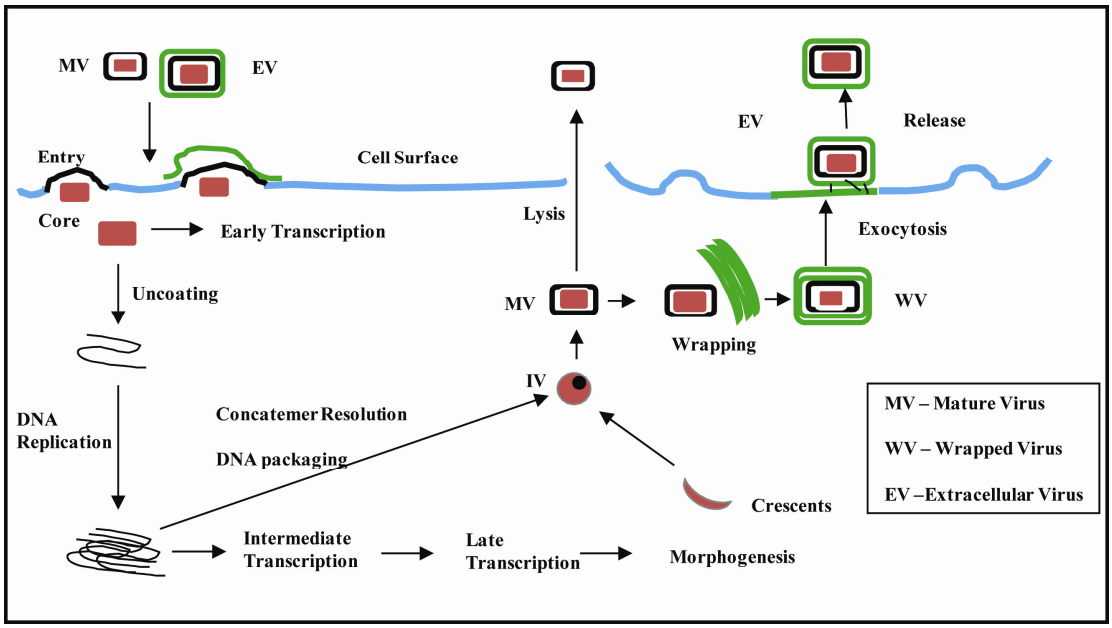

Figure 1-3. Vaccinia virus life cycle. Attachment occurs and MV particles fuse with the plasma or endosomal membrane. Viral cores are released into the cytoplasm and early transcription begins. Next the core is uncoated allowing for viral DNA replication and concatemer formation. Intermediate genes are then transcribed and translated allowing for late gene transcription and translation. Morphogenesis begins with the formation of membrane structures called crescents. Concatemeric DNA is resolved and packaged into the immature virions (IVs), which condense to form the intracellular MV. Most MV stay in the cell until lysis occurs. Some MV are wrapped by trans-Golgi network or early endosomal cisternae forming a wrapped virion (WV). The WV is transported to the periphery of the cell and WV fuses with the plasma membrane exposing an EV on the cell surface.

unable to infect resting T-cells but can infect activated T cells. This could mean resting T cells are deficient in cell specific factors that allow for infection of activated T-cells [39].

1.3.3.2 Entry

After attachment, most enveloped viruses either fuse with the plasma membrane or the membrane of an endocytic vesicle [40-41]. VACV, the prototype poxvirus, has been shown to utilize a few different strategies to enter cells. VACV can fuse at the plasma membrane (Fig. 4), independent of pH [42], and some VACV strains can also enter certain cells lines via the endocytic pathway (Fig. 4) utilizing a low pH induced entry mechanism [43-44]. Additionally a recent report suggests VACV uses apoptotic mimicry and induces cell signaling so that entry of the virus occurs by macropinocytosis [45]. Lipid rafts have also been implicated in MV entry [46]. The MV particles fuse with the plasma membrane or the membrane of endocytic vesicles via a multi-protein fusion complex referred to as the entry-fusion complex (EFC) [47]. The EFC comprise at least 11 proteins: A16, A21, A28, G3, G9, H5, J5, L5, I2, and associated proteins F9 and L1 [48-56]. The EFC is not required for attachment which was demonstrated by the ability of EFC mutants to bind, but not enter cells. The genes encoding the EFC proteins are conserved within all poxviruses suggesting the individual EFC proteins have non-redundant functions. It also suggests that the mechanism of entry had to have evolved before a division between vertebrate and invertebrate poxvirus species.

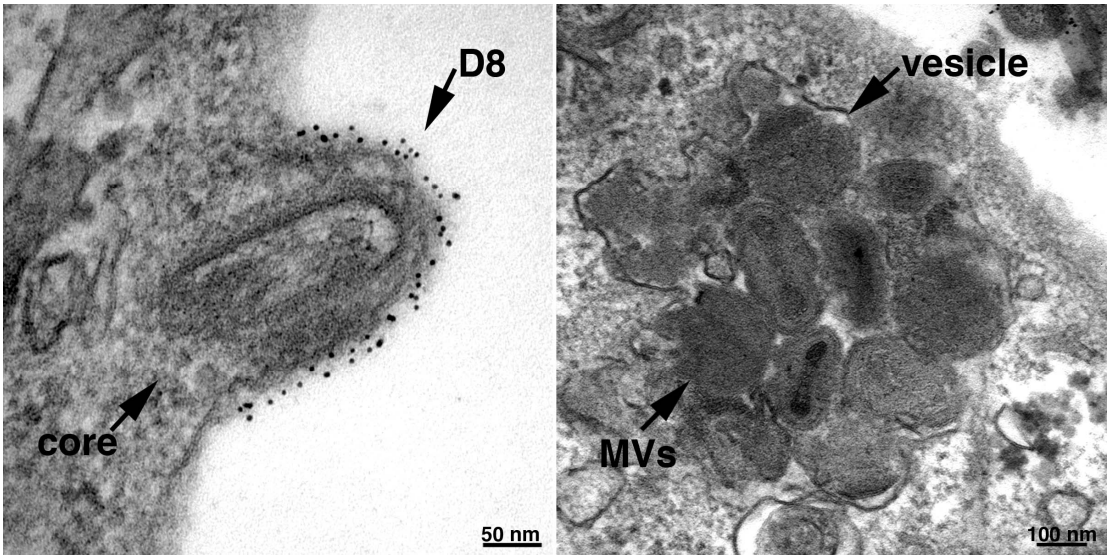

Figure 1-4. Vaccinia virus entry mechanisms. Immunoelectron microscopy was used to visualize MV fusing at the plasma membrane at neutral pH (left) and within endosomal vesicles where fusion occurs at a low pH (right). Image provided by Andrea Weisburg.

1.3.3.3 Gene Expression

After fusion of the MV membrane with the plasma membrane, viral cores are found within the cytoplasm of host cells where they are transported on microtubules to the site of transcription [57]. There are three distinct temporal stages in VACV transcription, early, intermediate, and late, and each stage has a sequence specific promoter found immediately upstream of each gene transcribed in that stage [58]. The VACV genome is transcribed by a virus-encoded multi-subunit DNA-dependent RNA polymerase in union with early, intermediate, and late stage specific factors. The transcription factors for early gene expression are synthesized at late times in an infection and packaged in the virion. The transcription factors needed for intermediate gene expression are synthesized at early times and the transcription factors needed for late gene expression are synthesized at intermediate times. This mechanism, utilizing protein products from one stage of transcription to regulate the next stage of transcription, is referred to as a cascade mechanism of transcription. The cascade mechanism is used by many viruses to allow for the coordination of individual genes with specific processes, like DNA replication and virion assembly.

VACV particles are packaged with a single copy of the genome and necessary transcription factors and enzymes for early stage of transcription which can synthesize mRNA that is capped, methylated, and polyadenylated [58]. The enzymes found within the particle that carry out early transcription include: a multi-subunit DNA-dependent RNA polymerase, RNA polymerase associated polypeptide of 94kDa (RAP94), VACV early transcription factor (VETF), capping and methylating

enzymes, poly A polymerase, nucleotide phosphohydrolase (NPH I), and topoisomerase I encoded by the gene D6L [1]. The viral RNA polymerase is a complex of 8 subunits: RPO147encoded by the J6R gene, RPO132 encoded by the A24R gene, RPO35 encoded by the A29L gene, RPO30 encoded by the E4L gene, RPO22 encoded by the J4R gene, RPO19 encoded by the A5R gene, RPO18 encoded by the D7R gene, and RPO7 encoded by the G5.5R gene. About 20-30% homology exists between the large viral subunits, RPO147 and RPO 132, and cellular RNA polymerases. There is about 23% homology between RPO30 subunit and the eukaryotic transcription elongation factor SII. RPO7 also shares about 23% amino acid homology with the smallest eukaryotic RNA polymerase subunit. Unlike the core RNA polymerase subunits which are synthesized throughout the entire infectious cycle, RAP 94 (encoded by the gene H4L) is only synthesized at late times in infection and is exclusively involved in early mRNA synthesis [59]. VETF is a heterodimer encoded by the late transcribed D6R and A7L genes [60-61]. VETF directly interacts with the viral early promoter at the core region upstream and an additional, non-sequence specific, region downstream of the RNA start site [62]. The capping enzyme is a multifunctional heterodimer encoded by the VACV genes D1R and D12L. The cap is added to the nascent RNA approximately 30 nucleotides into transcription [63-66]. The cap structure is needed by the viral message for transport, stabilization, and recognition of the transcript by cellular ribosomes. The capping enzyme is also required for transcriptional termination [67-68]. The poly (A) polymerase is encoded by the E1L gene (large subunit) and the J3R gene (small subunit). The large subunit catalyzes the addition of 30-35 adenylate residues while

the small subunit acts as a processivity factor for the poly (A) polymerase [69]. Intriguingly, the J3 protein methylates the capped end of the mRNA and is also involved in transcription elongation [70-73].

The early stage of transcription begins within the core soon after they gain access to the cytoplasm of the host cell. Early promoters can be recognized by a single essential element consisting of a 16 base-pair core consensus sequence, AAAAgTaGAAAataTA, located -13 to -27 nucleotides upstream of the transcriptional start site flanked by a variable but highly A-T rich region. A purine found 12 to 17 nucleotides downstream of the core sequence is where transcription initiation occurs [74]. VETF interacts with the early promoters and recruits the RNA polymerase to the initiation site to begin transcription. VACV transcribes approximately half of the genes during the early stage of transcription [75-76]. Early mRNAs can be detected as early as 20 minutes post infection [58]. Early transcripts terminate approximately 20-50 base pairs downstream of the sequence TTTTTNT which is actually mediated by the RNA transcript sequence UUUUUNU [77-78]. The early transcription termination signal is found at the end of most VACV early genes. Two factors are required to induce termination and release of the transcript: NPH I and the capping enzyme.

Since early mRNAs are synthesized within the core they have to be extruded out of the core and into the cytoplasm for translation [79-80]. Early mRNAs have been found in unique structures in the cytoplasm associated with microtubules and EM was able to depict these structures surrounded by ribosomes [81].

Along with the transcription factors and enzymes required for the intermediate stage of transcription, early stage gene products are also involved in DNA replication, such as the DNA polymerase, nucleoside triphosphatase, uracil DNA glycosylase, and the DNA polymerase processivity factor. Many proteins involved in host interactions, such as immune modulation proteins, are also transcribed during the early stages of transcription.

Intermediate genes are expressed after both uncoating of the core and viral DNA replication. Inhibitors of DNA replication have been shown to block intermediate gene expression [82]. This suggests that a DNA template for intermediate transcription is unavailable to the newly synthesized enzymes and intermediate transcription factors. The most obvious reason is that the infecting particle's genome is sequestered within the core. This idea is also supported by the study that when purified VACV DNA is transfected into infected cells it can serve as a template for intermediate and late transcription without the replication of the infecting viral DNA [82].

Intermediate stage promoters include two important regions, a 14-base paired core element and a 4 base paired initiator element. The core and initiator element are separated by a 10-11 base pair region. The necessary enzymes involved in intermediate stage transcription include: *de novo* synthesized viral RNA polymerase [83], viral capping enzyme (used for a mechanism other than capping) [63], VITF-1 encoded by the E4L gene [84], VITF-3, a heterodimer of A8 and A23 [85], and host cell encoded VITF-2 [86]. VITF-2 is a heterodimer of the cellular protein Ras-GTPase activating protein SH3 domain-binding protein (G3BP) and p137 [87].

Interestingly, a recent report shows intermediate transcription factors along with viral mRNA, cellular translational factors, and ribosomal proteins localizing to the viral factories suggesting viral transcription and translation is coordinated within the viral factories [88].

There are five known intermediately expressed genes, however, it is possible there may be others [89]. Three of the five intermediate expressed proteins are late stage transcription factors: A1, A2, and G8 [82]. The other two intermediate genes, I8L and I3L, encode for the RNA helicase NPH II and the ssDNA binding protein, respectively.

Late genes are expressed after intermediate genes and continue to be expressed until the end of the viral life cycle [90]. Late stage promoters include a 20-base paired core sequence with some consecutive T or A residues, a 6 bp separator, and highly conserved initiator element with sequence TAAAT [91]. Often the initiator element TAAAT is followed by a G to form TAAATG where the ATG, recognized in mRNA as AUG, serves as the site for translation initiation. Enzymes involved in late stage transcription include: the viral RNA polymerase, vaccinia late transcription factor (VLTF)-1 encoded by the G8R gene, VLTF-2 encoded by the A1L gene, VLTF-3 encoded by the A2L gene, and a host protein VLTF-X that consist of the heterogeneous nuclear ribonucleoproteins A2/B1 and RBM3 [92-93]. The early expressed H5 protein, also known as VLTF-4, has been implicated in stimulating late transcription [94] and is also involved in elongation of late transcription [95-96].

All of the VACV virus mRNAs are capped but in contrast to early mRNAs, intermediate and late mRNAs have a heterogeneous poly (A) leader sequence at the 5' end and both lack a defined 3' end. The 5' heterogeneous leader is due to the slippage of the RNA polymerase while trying to initiate transcription [97-98]. There doesn't appear to be specific intermediate and late transcriptional termination signals but a mechanism is likely and a few VACV proteins are seemingly involved: A18, G2, and J3.

Late expressed proteins have quite an array of functions. The structural proteins for both the EV and the MV are expressed late. Many late proteins are required for virion morphogenesis and transport, and the early transcription machinery is expressed late and packaged in the virion.

1.3.3.4 Uncoating

Core uncoating and release of the genome into the cytoplasm is dependent upon transcription and translation of VACV early genes [99]. Disruption of the core coincides with the end of the early transcription stage and it is the early gene products that aid in core disassembly. This has been shown by the use of protein synthesis inhibitors during the early stages which results in increased and prolonged synthesis of early mRNA [100]. This proposes that when the core falls apart disruption of the early transcription machinery transpires [1].

1.3.3.5 DNA Replication

VACV DNA replication occurs in the cytoplasm of their host cells. These cytoplasmic foci of replication, coined "viral factories," are found in the peri-nuclear region of the cell [101]. Each factory is the product of one infectious particle [102].

Early mRNA transcription and translation is needed before DNA replication can occur since many factors involved in DNA replication are synthesized at early times in infection. This accounts for the reason the purified viral DNA alone is non-infectious. There is still much to learn regarding VACV viral DNA replication as there are many gaps in the current model.

DNA replication begins within one to two hours after cells are synchronously infected with VACV, though, the timing may vary with different poxviruses [1]. Although attempts have been made to find a specific origin of replication, none have been successful. It is thought that specific origin sequences reside at the ends of the genome, however, plasmid DNA lacking VACV sequence was able to replicate in cytoplasmic viral factories [103].

It is thought that the VAVC DNA replication utilizes a rolling hairpin strand displacement mechanism similar to that used by parvovirus [104]. Replication perhaps begins with a nick adjacent to one or both of the hairpin termini. The nick presents the 3' end for priming the replication complex which then synthesizes new DNA. The newly synthesized DNA folds back on itself and the replication complex proceeds to synthesize the rest of the viral genome. Large head-to-head and tail-to-tail concatemeric structures arise during DNA replication and remain as so until after the onset of late transcription when unit length genomes are resolved by the viral Holliday junction resolvase encoded by the A22R gene [105]. This strand displacement mechanism is supported by the presence of ssDNA; however there are reports of small DNA fragments covalently linked to RNA suggesting a lagging strand synthesis mechanism [106].

Replication of the VACV genome is dependent upon five virally encoded proteins: DNA polymerase encoded by the E9L gene [107], a protein kinase encoded by the B1R gene, nucleic acid-independent nucleoside triphosphate encoded by the D5R gene [108], uracil DNA glycosylase encoded by the D4R gene [109], and a DNA processivity factor encoded by the A20R gene [110]. Attaining optimal levels of precursors for DNA metabolism is sometimes necessary and a variety of additional proteins are encoded by the orthopoxviruses for such activity. The J2R gene encodes a thymidine kinase [111], the A48R gene encodes a thymidylate kinase [112], the I4L and F4L genes encode for a ribonucleotide reductase [113-114], and the F2L encodes for a dUTPase [115].

1.3.3.6 Virion Assembly and Maturation

Assembly of the virus particle occurs after late stage transcription and many of the late synthesized proteins partake in morphogenesis. Many late stage proteins are also involved in early transcription and must be packaged in the particle during virion assembly. In addition, the concatemeric DNA must also be resolved into unit length genomes for packaging, which requires the late expressed protein A22. Assembly of the virion is halted if unit length genomes are not processed [105].

Electron microscopy (EM) was instrumental in providing the earliest evidence of viral assembly [116]. Assembly begins in the viral factory with the formation of membrane structures called crescents. Studies with conditional lethal mutants revealed that late proteins F10 [117-118], H5 [119], G5 [120], and A11 [121] are all implicated in initial steps of crescent formation. Interestingly, none of these proteins are found in the immature virion membrane. Two proteins that are found in the

membrane and are also involved in membrane biogenesis are A14 and A17 [122-123].

In EM images, crescent structures appear to be two layers with a single lipid bi-layer coated by regular spaced projections referred to as spicules. It is the spicules that seem to provide the rigid arched shape to the crescents. These spicules, which are actually trimers of the D13 protein[124], appear to form a honeycomb lattice as shown by deep etch EM [124-125]. It also appears that these single open lipid bilayers formed within the factories are without any connection to other cellular membranes and are presumably formed *de novo* [126]. Later studies suggest that the crescent membranes are formed from the flattened membrane cisternae of the secretory pathway and therefore must be double membrane structures [127]. Freeze fracture studies are suggestive of a one lipid membrane model [125] and more recently that the membrane of crescents is derived from the endoplasmic reticulum and is not formed *de novo* [128].

Crescents form around a dense area of the factory referred to as the viroplasm. The viroplasm is uniform, appears denser than the factories, and can be found as large subdomains within the factories, called virosomes. The virosomes are often surrounded by many crescents that seem to take up portions of the virosome [129]. Several core proteins have been shown to be involved in the interaction of the crescents with the viroplasm: A15, A30, D2, D3, F10, G7, and J1 [130-134]. The crescents appear to grow in length and at the same time maintain the curvature provided by the spicules. Just before the crescent closes to form the IV a

nucleoprotein is inserted [135]. The core proteins, I6 and A32, and the membrane protein, A13, appear to be necessary for proper packaging of the DNA [136-138].

After insertion of the nucleoprotein, crescents eventually evolve into the spherical IV. This process can be blocked by the drug rifampicin [139] and mutants resistant to rifampicin were mapped to the D13L gene, whose gene product is responsible for the honeycomb lattice scaffold needed for assembly [140]. The appearance of the mature form of the virus (MV) follows after the formation of the IV. The important steps required for MV formation are not totally understood but involve assembly of the transcription apparatus, proteolytic processing of both core and membrane proteins [141-142], addition of several surface proteins, reorganizing of the particle to form a defined core and lateral bodies, and loss of the D13 scaffold. Assembly of the transcription apparatus is necessary for virion maturation as shown by the formation of noninfectious virus particles when RAP94 is repressed [143], as well as by accumulation of IV when VETF is repressed [144-145].

Vaccinia encodes two proteins that have homology to known proteases, I7 and G1. The proteolytic processing of both core (A17) and membrane proteins (A4, L10 and L4) occurs most likely by the virally encoded I7 proteases. I7 has been show to be important in formation of the MV since morphogenesis is blocked with a temperature sensitive mutant of I7 [146]. Though G1 is not required for processing of the membrane or core proteins, conditional lethal mutants of G1 are also blocked in morphogenesis [147].

VACV encodes a complete pathway for protein disulfide bond formation system which is comprised of the proteins E10, A2.5 and G4 [148]. Disruption of the

system abrogates viral morphogenesis. Several transmembrane proteins in the MV contain disulfide bonds in their cytoplasmic domain some of which are mediated by the VACV encoded disulfide bond pathway.

The loss of the D13 scaffold is mediated by the proteolytic processed form of A17, a protein that has been shown to interact with D13. A17 is processed by the viral protease, I7. When I7 is repressed, disassembly of the D13 scaffold does not occur and aberrant virions are formed [149]. The addition of several surface proteins is seen after formation of MV from IV occurs and they include: A27, H3 [150], A26 [151], and L1[152]. Four core proteins seem to be involved in the transition from IV to MV: I1 [153], F17 [154], A4 [155], and A3 [156]. And three membrane proteins are known to be dispensable for IV formation but are required for MV formation: A9 [157], L1 [152], and H3 [150].

1.3.3.7 WV Formation, Transport, EV Release, and Dissemination

Most MVs stay in the cell until lysis occurs and typically localize in clusters within the cytoplasm of the infected cell. Some MVs are transported on microtubules to be wrapped by virally modified trans-Golgi membrane or early endosomal membrane. The acquisition of the two extra membranes around the MV is known as wrapping and results in the wrapped virion (WV) [158-159]. The WV has nine additional VACV expressed proteins: A33, A34, A36, A56, B5, E2, F12, F13 and K2 [160-168]. Wrapping of the MV requires several proteins and is inefficient in mutant viruses lacking A27 [169], B5[170], or F13 [171]. The WV is transported on microtubules to the periphery of the cell and the EV is exposed on the cell surface after fusion of the outer most membrane with the plasma membrane. This step results

in the loss of the A36 and F12 proteins which are exclusively associated with the WV. The EV displayed on the cell surface can induce the formation of actin tails. This mechanism is presumably used to push the particles away from the infected cell and toward the adjacent uninfected cell and mediate efficient cell-to-cell spread.

1.3.4 Vaccinia Virus as a Tool

One poxvirus phenomenon is the ability of viral genome to recombine. Homologous recombination can occur in cells infected with more than one virus or with a virus and other sub-genomic DNA [172]. Exploiting this mechanism to insert foreign genes makes VACV very attractive as an efficient expression system for many types of studies [173]. VACV has many advantages over other expression systems such as the ease of isolating recombinant viruses, the broad host range, the ability to insert large amounts of DNA, a high rate of protein expression, and its relative safety.

Some examples include immunologic studies with immunogens such as that of herpes virus [174], hepatitis C [175], human immunodeficiency virus (HIV) [176-177], and influenza virus [178-179]. VACV is also quite useful in the large scale production of recombinant proteins [180]. VACV may also be used in gene therapy [181], in the treatment of some cancers [182], as a vector for rabies wild life vaccines [183].

1.4 Orthopoxvirus Pathogenesis

Pathogenesis is the mechanism involved in the production of disease, spread, and the physiological response of the host to the infecting agent, to include the host

immune response (discussed in the following section). Different species within the orthopoxviruses as well as the different routes of entry can yield either a systemic or localized infection. Details regarding VARV pathogenesis are inferred from descriptions previous to the eradication of VARV as well as from animal models infected with other orthopoxviruses that produce a systemic infection similar to that produced by VARV. Mousepox, the disease in mice caused by ectromelia virus (ECTV), is quite similar to the smallpox disease in humans. The study of ECTV is a very good means towards understanding the pathogenesis of orthopoxviruses. VACV is the prototype poxvirus and has been an excellent and safe tool for studying many aspects of orthopoxvirus pathogenesis.

1.4.1 Variola Virus Pathogenesis

It is thought that a naturally acquired VARV infection occurs after transmission of droplets from an infected individual reach the respiratory mucosa of an uninfected individual [23]. The dose required to establish a primary infection is believed to be just a few virions [10]. The virus migrates to the regional lymph nodes and is amplified. Three to four days post infection an asymptomatic viremia ensues followed by a latent period of 7-17 days in which the virus continues to replicate. Another phase of viremia occurs and is followed by the prodromal phase. The prodromal phase is the first phase of symptoms and lasts approximately 2-4 days. Prodromal symptoms include malaise, headache, backache, fever, vomiting, and muscle pain. Mucous membrane lesions, caused by the localization of virus within the small blood vessels of the dermis and oral/pharyngeal mucosa, appear before the skin rash appears and 2-4 days after onset of fever. An individual is not infectious

until the appearance of lesions within the mouth. Lesions evolve into macules, first on the face followed by all parts of the body within 24 hours. Macules turn into raised papules by the 2nd or 3rd day. Papules turn into vesicles which form pustules. Formation of pustules coincides with another phase of fever. Eventually crusts form over all of the lesions. Crusts eventually fall off and the skin is left with depigmented pitted scar. If the infection is fatal, death typically occurs between the 10th and 16th day. Both, variola major and variola minor, cause smallpox. Variola major exhibits a more severe form of disease with a case fatality rate of about 30%. Variola minor tends exhibit a less severe disease with a mortality rate of ~1% [3, 10, 184].

1.4.3 Ectromelia Virus Pathogenesis

ECTV was identified in an infected laboratory-mouse colony in 1930. Additional strains have been isolated since then with different disease severities [185]. Epidemiologic studies suggest that ECTV is transmitted by abrasions in the skin from infected animals or fomites [186]. This mode of transmission can be easily imitated by infecting via the footpad. The virus replicates at the site of entry in the epidermis. Virus then migrates and replicates in the local lymph nodes and primary viremia is established. Viremia allows the virus to reach and replicate in the spleen, liver, and other organs. A second viremia develops when replicated virus is release into the blood stream from the infected organs. Secondary viremia leads to the infection of the skin, distal to the primary infection which typically becomes inflamed due to the immune response. Infection with certain strains of ECTV can also lead to a rash as seen with smallpox [185].

1.4.2 Vaccinia Virus Pathogenesis

The VACV vaccine is given with a bifurcated needle and currently only given to those working with orthopoxviruses in research labs, public health care workers, or to U. S. military personnel [187]. In August 2007, ACAM 2000, the current smallpox vaccine, was licensed for use in the United States by the Food and Drug Administration. The vaccine is live VACV and replicates immediately at the primary site of infection. A red papule appears at the vaccination site 3-5 days after vaccination. The papule turns into a pustule and typically reaches it maximum size by day 10. The pustule eventually dries and crusts over. Crusts eventually separate 14-21 days after vaccination. The infection remains localized and often regional lymphadenopathy and fever will occur [188].

There are complications that sometimes arise after a vaccination. These include: progressive vaccinia, generalized vaccinia, post vaccination encephalomyelitis, eczema vaccinatum, and accidental infection [23]. Progressive vaccinia is rare and occurs in persons with severe cell immunity deficiencies. Progressive vaccinia is the growth and spread of the virus beyond the site of inoculation and is usually fatal. Generalized vaccinia is the appearance of a pustular rash and there is no known predisposition to attain this rare reaction. Post vaccination encephalomyelitis seems to occur by direct viral invasion of the nervous system or it is possibly post-immunization demyelinating encephalomyelitis. It is a rare complication and again there are no predisposing factors known. Eczema vaccinatum occurs in those with a history of eczema, also known as atopic dermatitis. After vaccination the virus disseminates from the primary site of infection and a local or

general rash appears. Accidental infection happens when a vaccinated individual transmits the virus to an additional site on themselves or to another individual.

1.5 The Innate Immune Response to a Viral Infection

The innate immune response plays a critical role in the host response to a viral infection. It is the initial, fast acting, non-specific response that detects and keeps an infection in check until the adaptive (specific) response can kick in and help clear the infection from the host. The major players of the innate immune response to a viral infection include: apoptosis, pattern recognition receptors (PRR), natural killer (NK) cells, plasmatoid dendritic cells, macrophages, neutrophils, complement, RNA interference (RNAi) and various cytokines released by cells including the pro-inflammatory cytokines, chemokines, interferon (IFN), and tumor necrosis factor-α (TNF-α).

1.5.1 Apoptosis

Apoptosis is the process of programmed cell death and is a tightly regulated intrinsic cellular mechanism involved in maintaining homeostasis, cell development and differentiation, and detection and protection from invading pathogens. Its description is included in this section since virally infected cells can trigger a variety of signals leading to apoptosis. Apoptosis can be triggered by both external and internal stimuli such as TNF or DNA damage, respectively (Fig. 1-5). Cellular recognition of these stimuli results in a signal transduction which activates a set of cellular cysteine proteases known as the caspases. Once activated, caspases cleave a

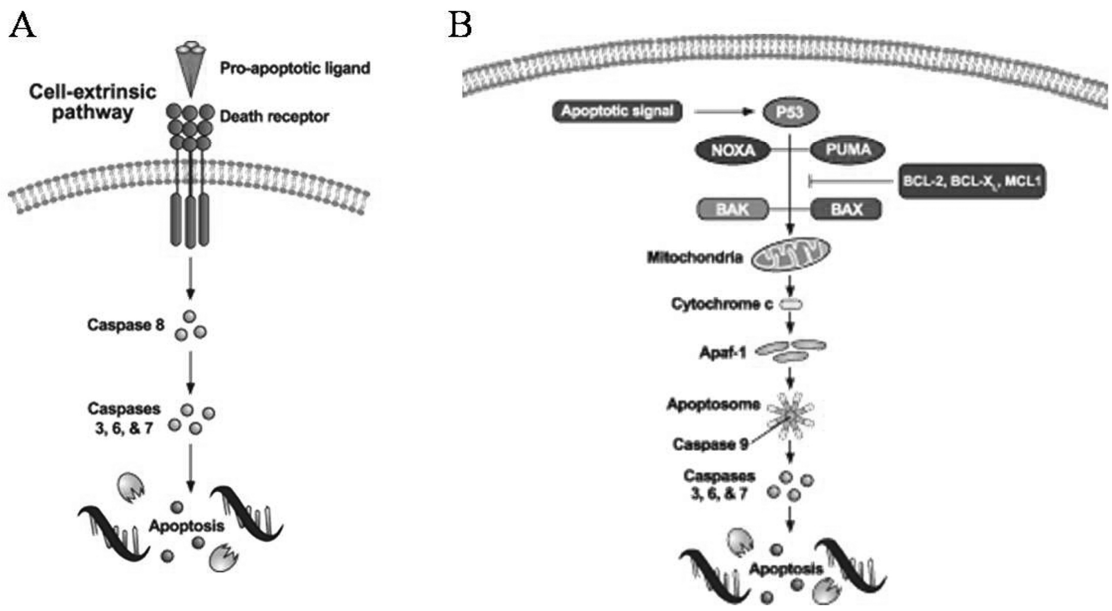

Figure 1-5. Apoptotic pathways. (A) The extrinsic pathway is induced by death receptor signaling when cytokines (e.g., TNF or Fas ligand) bind to death receptors. This leads to the activation of caspase-8 which leads to the sequential activation of effector caspases, 3, 6 & 7, and ultimate cell death. (B) The intrinsic pathway is controlled by B-cell lymphoma protein-2 (Bcl-2) family members. The intrinsic pathway is stimulated by cell stress and results in loss of mitochondrial membrane integrity, release of cytochrome, formation of the apoptosome, activation of caspase 9, activation of effector caspases 3, 6, &7, and ultimate cell death. Figures adapted from Ashkenazi, *Nature Review Cancer*, 2002.

select set of cellular target proteins; this proteolytic cleavage can either inactivate or activate a target protein by cleaving off regulatory domains [189]. Two well studied extrinsic pathways are Fas- and TNF-receptor induced apoptosis. These plasma membrane receptors have death effector domains (DEDs) found in their cytoplasmic region. Binding of their appropriate ligands initiates a signal transduction beginning with the recruitment of cytoplasmic adapter proteins that also contain DEDs which in turn leads to the recruitment and activation of pro-caspase-8. Caspase-8 cleaves and activates pro-caspase-3, -6, and -7. These effector caspases proteolytically cleave target proteins responsible for the execution of the cell [189]. The extrinsic pathway can also indirectly involve the mitochondria (MT) and its permeabilization, a step involved in cell death. The intrinsic pathway directly involves the MT and its permeabilization and ultimate release of pro-apoptotic factors that normally localize to the MT. The Bcl-2 proteins regulate mitochondrial membrane permeabilization (MMP) either by inhibition or promotion; Bcl-2 proteins can be pro- or anti-apoptotic [190]. Another pro-apoptotic protein released from the MT due to the change in MMP is cytochrome c (cyt c). When released from the MT, cyt c interacts with Apaf-1 and pro-caspase 9 which in turn triggers the activation of caspase-9. Caspase-9 can then initiate apoptosis by cleavage of the effector caspases -3, -6, and -7 [189].

1.5.2 Pattern Recognition Receptors

PRRs are a group of receptors found on the cell surface, in cellular compartments, as well as within the cytoplasm of the cell. They are involved in the early detection of a viral infection by recognizing certain pathogen associated molecular patterns (PAMPs) unique to an invading virus. Toll-like receptors (TLRs)

are evolutionarily conserved, membrane bound PRRs that recognize many different PAMPs such as dsRNA, CpG DNA, and other pathogen associated motifs found on or as a product of viruses [191]. Many viruses enter cells via the endocytic pathway therefore it is not surprising that many TLRs involved in viral recognition are found in the endosome such as TLR3, TLR7, TLF8, and TLR9 [192]. TLRs have an intracellular TIR (Toll/IL-1 receptor) domain which is a motif present in the TLR superfamily of receptors. These receptors are key to innate immunity and inflammation. Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are cytoplasmic PRRs that include RIG-I and melanoma differentiation-associated gene 5 (MDA5) [193] and recognize viral nucleic acids [194-195]. Upon binding of their PAMP, PRRs initiate intracellular signaling through the recruitment of one or many adapter molecules which then culminates in the activation of transcription factors NF-κB, IRF3, and IRF7 (Fig. 1-6). These transcription factors induce both Type 1 IFN and pro-inflammatory cytokine production.

Recently cytosolic DNA-sensing systems have been identified and the proteins directly involved in sensing DNA are known as DAI (DNA activator of IRFs) and AIM2 (absent in melanoma 2). DAI upregulates Type-1 IFN and activates NF-κB while AIM2 activates caspase-1 and NF-κB [196-199].

1.5.3 Cells of the Innate Immune Response

NK cells are large granular lymphocytes that patrol blood and lymphoid tissue and provide potent anti-viral activity. They comprise about 20% of all circulating lymphocytes and that number increases upon detection of a viral infection until the adaptive immune response is launched. NK cells utilize two types of receptors to

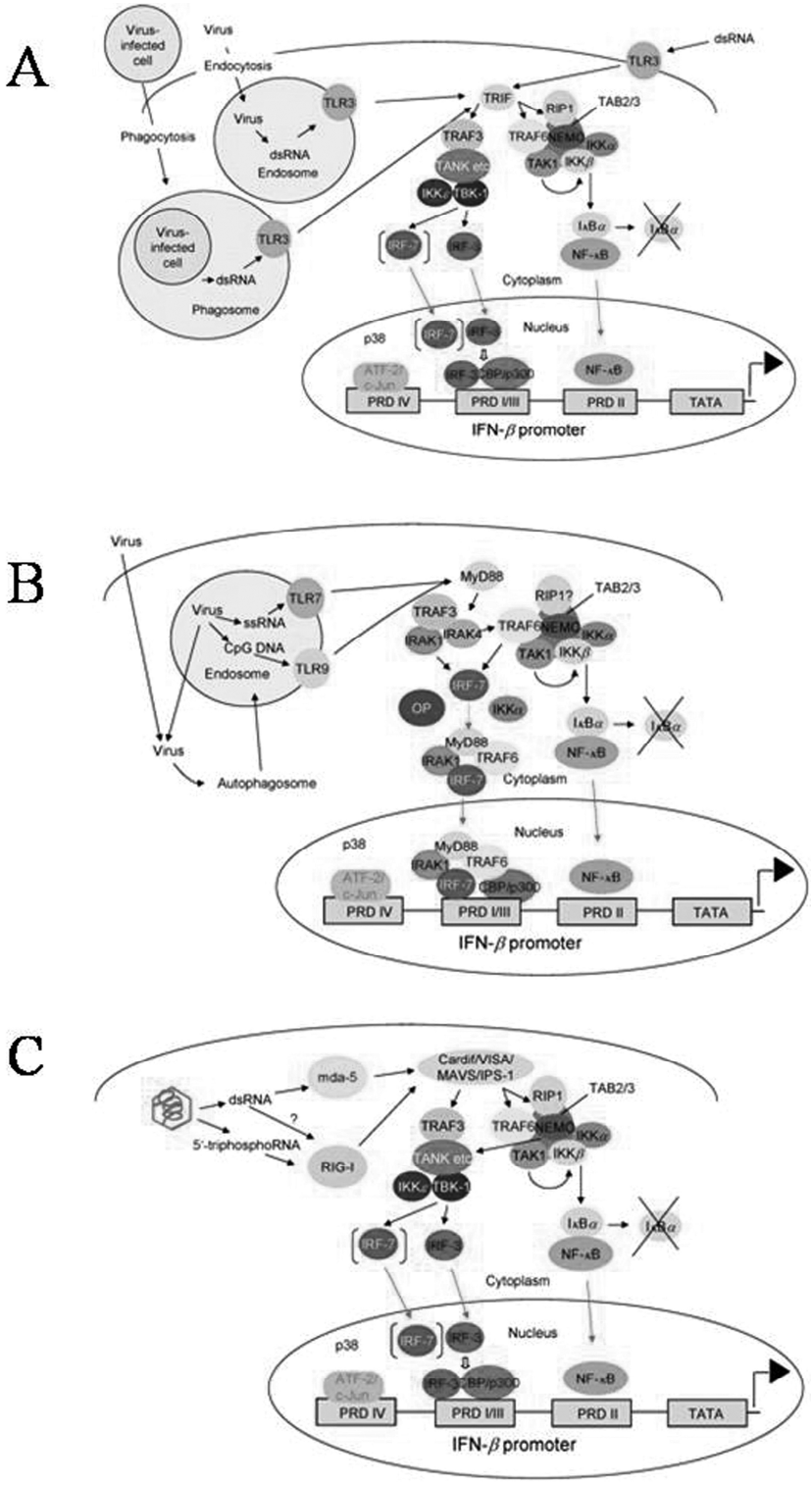

Figure 1-6. Examples of pattern recognition signaling. Signaling pathways via (A) TLR-3, (B) TLR-7 or -9, and (C) RLRs, MDA5 and RIG-1. Figure adapted from Randall, *Journal of General Virology*, 2008.

distinguish whether they should destroy their target cells or not: an activation receptor and an inhibition receptor [200]. Though the mechanism is not completely understood, virus infected cells are recognized by the activation receptor. Once the receptor binds to an infected cell the NK cell kills the infected target cell. It does so by using granzymes and perforins, which perforate the membrane of the infected cell inducing cell death. This outcome is only true if the inhibitory receptor is not co-stimulated when the activation receptor is stimulated. The inhibitory receptor is provided as MHC class I and its presence or absence dominates the fate of the cell. If MHC class I is downregulated on the target cell then the NK cell will kill its target. However, the NK cell will still only be stimulated if the activation receptor binds to its ligand since inhibition dominates over activation. NK cells are also known to release cytokines to add to the inflammatory response (Fig. 1-7)

Dendritic cells are sentinel cells that reside in the skin and mucosal membranes. They have PRRs as well as receptors that bind the cytokines released from infected cells. They in turn release large amounts of cytokines (primarily Type-1 IFN) to help combat a viral infection. They are also involved in antigen presentation which activates the adaptive immune response. There are two subsets of dendritic cells, plasmacytoid dendritic cells and myeloid dendritic cells, each recognizing different pathogens via their distinct set of receptors and each inducing different types of immune responses depending on the existing extracellular environment [201]. Plasmacytoid dendritic cells (pDC) are immature dendritic cells that house TLR7 and TLR9 in their endosomes. These cells are known for releasing

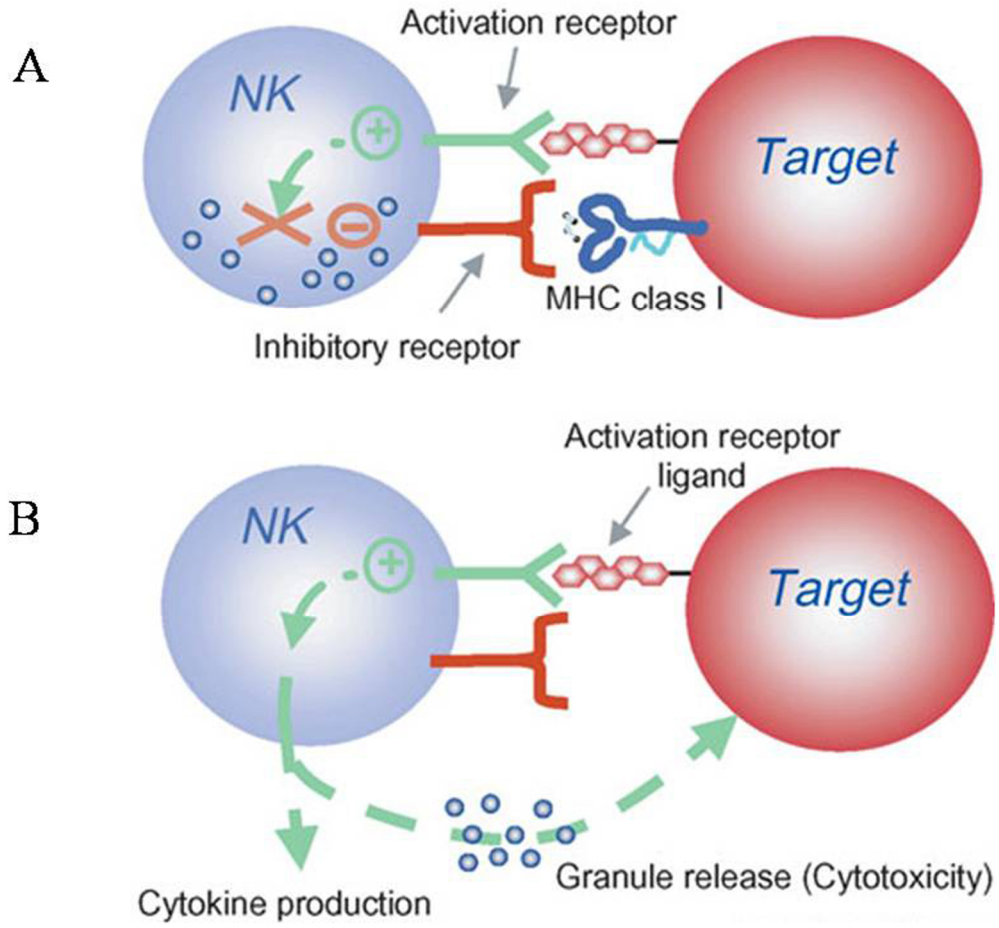

Figure 1-7. Mechanism of NK cell activation or inhibition. (A) Inhibition of NK cell kill signal due to recognition of self MHC class I. (B) Activation of NK cell signal since the inhibitory receptor is not stimulated but the activation receptor is stimulated by binding to its ligand. NK cell responds by releasing granules responsible for cytotoxicity of target cell. Figure from French and Yokoyama, *Arthritis Res. Ther*. 2004.

large amounts of Type I IFN as well as pro-inflammatory cytokines, TNF-α and IL-6, in response to a viral infection. pDC have a role in both the innate and adaptive immune response since they mature into potent antigen presenting cells.

Monocytes and macrophages are a significant source of a variety of soluble immune mediators, including cytokines and chemokines. Along with the possibility of differentiating into dendritic cells, monocytes can also differentiate into macrophages. Macrophages and neutrophils respond to chemotactic signals and are involved in the inflammatory response. Both macrophages and neutrophils are the main phagocytic cells during an immune response to a viral infection and remove dead, damaged, and infected cells [202].

1.5.4 The Complement System

The complement system is a significant mediator of the innate as well as the adaptive immune response to a viral infection. Complement consists of a family of approximately 30 serum and membrane proteins. These proteins work in a sequential fashion to target and destroy bacteria, viruses, and infected cells. There are three complement pathways: the classical, the alternative, and the lectin-binding pathway (Fig. 1-8). The classical pathway is activated by the initial binding of C1q (either to antibody-antigen complexes or to pathogen surfaces) and the subsequent formation of the C1 protein complex (C1qr2s2). The C1 protein complex cleaves complement components C4 and C2 into C4a & C4b and C2a & C2b. C4b and C2a bind to each other to form the classical pathway C3-convertase (C4b2a complex). C3-convertase cleaves C3 into C3a and C3b. The C3 cleavage product, C3a, is a powerful mediator

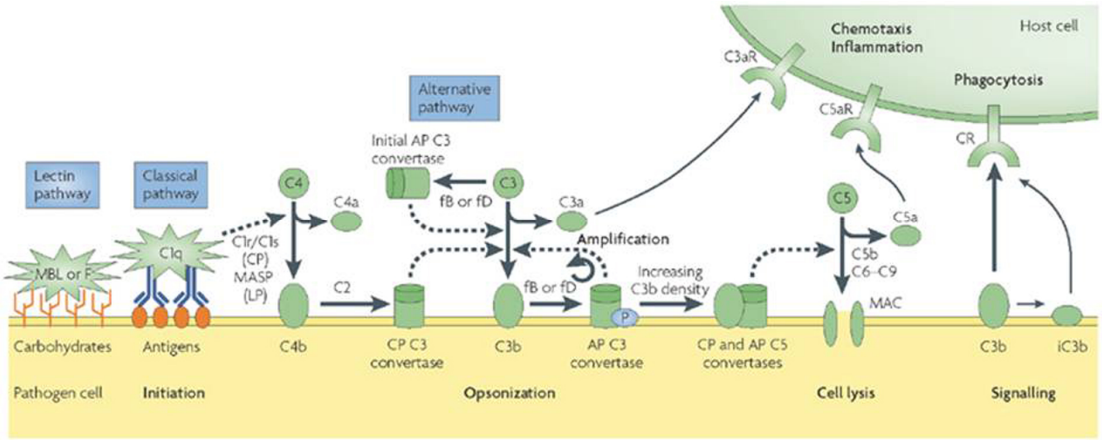

Figure 1-8. Complement pathways. Activation of the complement system via the classical pathway (CP), the lectin pathway (LP), or the alternative pathway (AP). C3 convertases cleave C3 to C3a and C3b. Accumulation of C3b leads to the assembly of C5 convertases which cleave C5 to C5a and C5b. C5b initiates the formation of the lytic membrane-attack complex (MAC). The MAC is comprised of the complement components: C5b, C6, C7, C8 and C9. C3a and C5a induce pro-inflammatory and chemotactic responses by binding to their receptors. Figure from Lambris et all, *Nature Reviews Microbiology*, 2008.

of inflammation while C3b is the primary effector protein for all three complement pathways. C3b remains attached to an infected cell or invading pathogen where it recruits additional components of complement that eventually form a channel in the membrane known as the membrane attack complex (MAC). The MAC is comprised of the complement components: C5b, C6, C7, C8, and C9. C5 is first recruited to the surface and cleaved into C5a and C5b by the complex C4b2a3b (C5 convertase). C6 and C7 bind to C5b and with recruitment of C8 they form a small hydrophobic complex which inserts into the membrane as a small pore. C9 interacts with the C5b678 complex and expands the pore. This perturbation of the membrane typically results in death of the microbe or infected cell due to osmotic lysis.

In the alternative complement pathway C3 is spontaneously cleaved to C3a and C3b. C3b can then bind to the membrane of an infected cell or pathogen. Bound C3b interacts with complement component factor B to form C3bB. In the presence of complement factor D, C3bB will be cleaved into Ba and Bb. C3b binds to Bb to form C3bBb, the alternative pathway C3-convertase. In the lectin-binding pathway, a protein similar to C1q starts the cascade when bound to mannose residues. This mannose binding lectin is a PRR and belongs to the family of collectin proteins. Collectins bind polysaccharides found on many different microbes including many enveloped viruses.

1.5.5 RNA Interference

RNAi is a significant contributor to the innate defense against virus infection. The RNAi pathway can act via endogenous micro RNAs (miRNA) or via small interfering RNA (siRNA) (Fig. 1-9). The siRNA pathway involves cleavage of viral

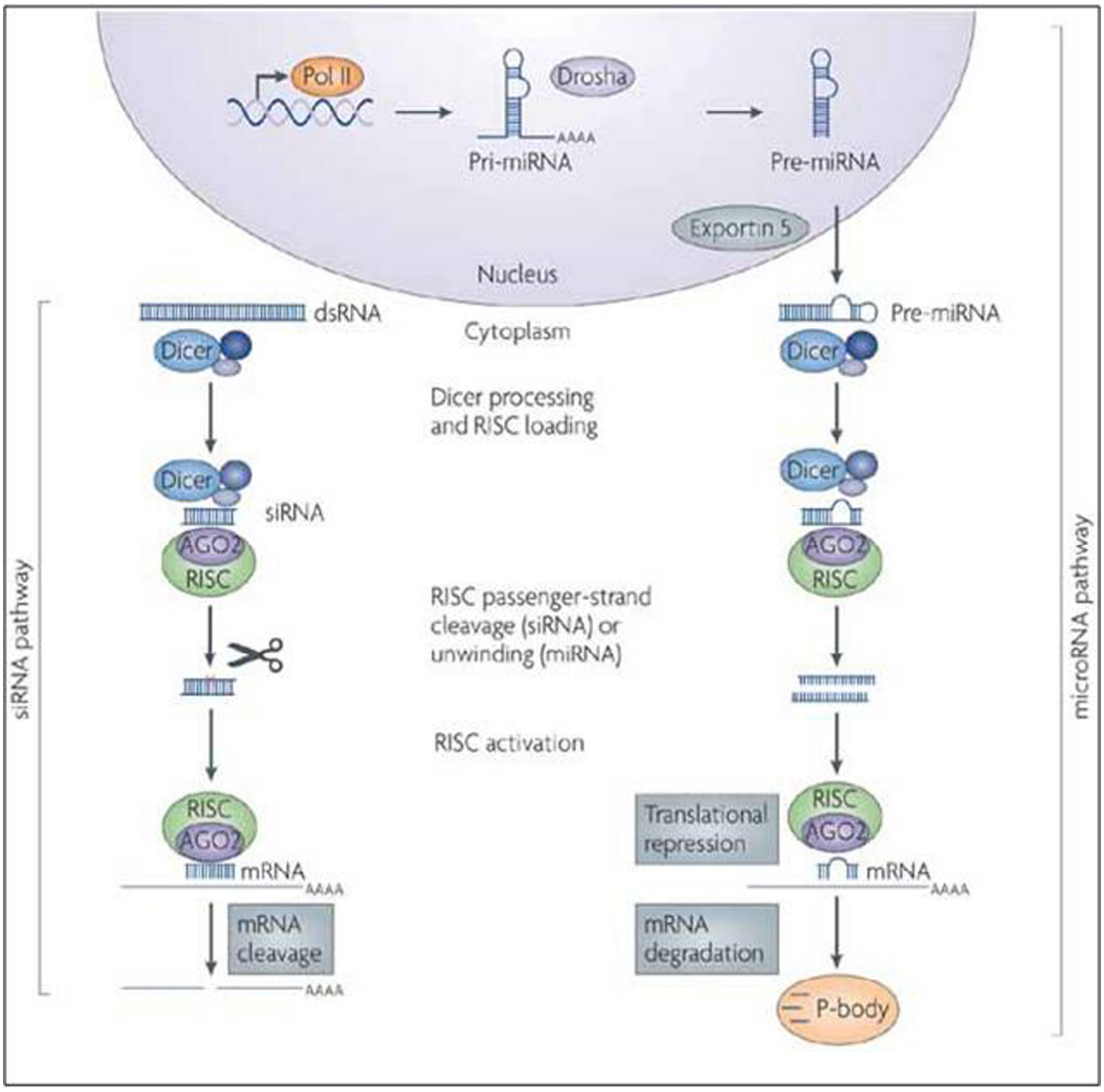

Figure 1-9. RNAi pathways. The siRNA pathway utilizes the enzyme Dicer to cleave long double stranded RNA (dsRNA) into siRNA. The siRNAs generated after cleavage are incorporated into AGO2 and RISC. AGO2 cleaves the sense strand leaving RISC with the antisense strand which recognizes target sites to direct mRNA cleavage. In the microRNA pathway, primary microRNA (pri-miRNAs) are transcribed by RNA Pol II and processed by the enzyme Drosha to yield pre-miRNAs which are exported to the cytoplasm by exportin 5. Dicer binds and processes pre-miRNA which is then loaded onto the AGO2–RISC complex as a mature miRNA. The miRNA recognizes target sites in mRNA which leads to translational inhibition or mRNA degradation. Figure from de Fougerolles et all, *Nature Reviews Drug Discovery*, 2007.

double-stranded RNA (dsRNA) into short 21-24 nucleotide fragments referred to as siRNA (or viRNA when derived from a virus) by the enzyme Dicer. The siRNAs are loaded into an Argonaute 2 (AGO2) containing RNAi-induced silencing complex (RISC) and one strand of the RNA is cleaved and degraded. The remaining siRNA strand recognizes and binds target sites on mRNA to direct cleavage by AGO2 thus inhibiting translation [203-204]. miRNAs are encoded by the host cells RNA polymerase as primary miRNA transcripts (pri-miRNAs). Pri-miRNAs are processed by the enzyme Drosha to yield short stem loop precursor miRNAs (pre-miRNAs). pre-miRNAs are exported to the cytoplasm and processed by Dicer to form miRNA. Similar to siRNA, miRNA is loaded onto the AGO2–RISC complex where the passenger (sense) strand is lost. The mature miRNA, bound to active RISC, can recognize target sites in mRNA and direct translational inhibition by binding of miRNA to target mRNA, which leads to translation repression and possibly to mRNA degradation [203-204]. The RNAi pathway acts directly on viral RNA to inhibit translation of viral proteins and since host miRNAs do not have to rely on complete base pairing they also act indirectly to target viral RNA and inhibit translation.

1.5.6 Cytokines, Chemokines, and Interferon

Cytokines are soluble products secreted from many different cells and are mediators of cell communication. They are absolutely critical in the host immune response to a viral infection since they are immediate players upon a viral infection and can control inflammation, induce an antiviral state, and regulate the action of the adaptive immune response. An infected cell will produce and release cytokines that in turn bind the receptors on neighboring uninfected cells as well as on dendritic cells

and macrophages. The binding of these cytokines results in the synthesis and release of more cytokines from the uninfected cells and a continuous amplification of the initial response to infection transpires. Many cytokines, such as IFN, are pleiotropic and are influenced by the concentration as well as the other cytokines present in the surrounding milieu. Tumor necrosis factor-α (TNF-α) is a cytokine released from activated monocytes and macrophages. Along with its involvement in apoptosis, TNF is also involved in inflammation. Other important pro-inflammatory cytokines include IL-1 and IL-6.

Chemokines are chemotactic cytokines which are important for guiding leukocytes to the sites of infection [205]. Chemokines bind to glycosaminoglycans (gags) on the surface of cells and establish a concentration gradient which aids in the activation and chemotaxis of leukocytes and macrophages. The attracted cells move through the gradient to the higher chemokine concentrations.

IFNs are critical cytokines in the initial response to a viral infection. There are three classes of IFN: Type I (e.g. IFN-α, IFN-β), Type II (e.g. IFN-γ) and Type III (IFN-λ1, - λ2, - λ3) [206]. Type I IFN is the first cytokine to emerge in high concentrations upon a viral infection. It can be made by all nucleated cells and can be induced as easily as a virion binding to a cell [207]. Type II IFN is exclusively secreted by T-cells and NK cells. When secreted, IFN binds to an appropriate receptor on nearby cells and induces a signal transduction through the JAK/STAT pathway (Fig. 1-10) which culminates in the upregulation of hundreds of genes referred to as IFN- stimulated genes (ISGs). ISGs are functionally diverse and many

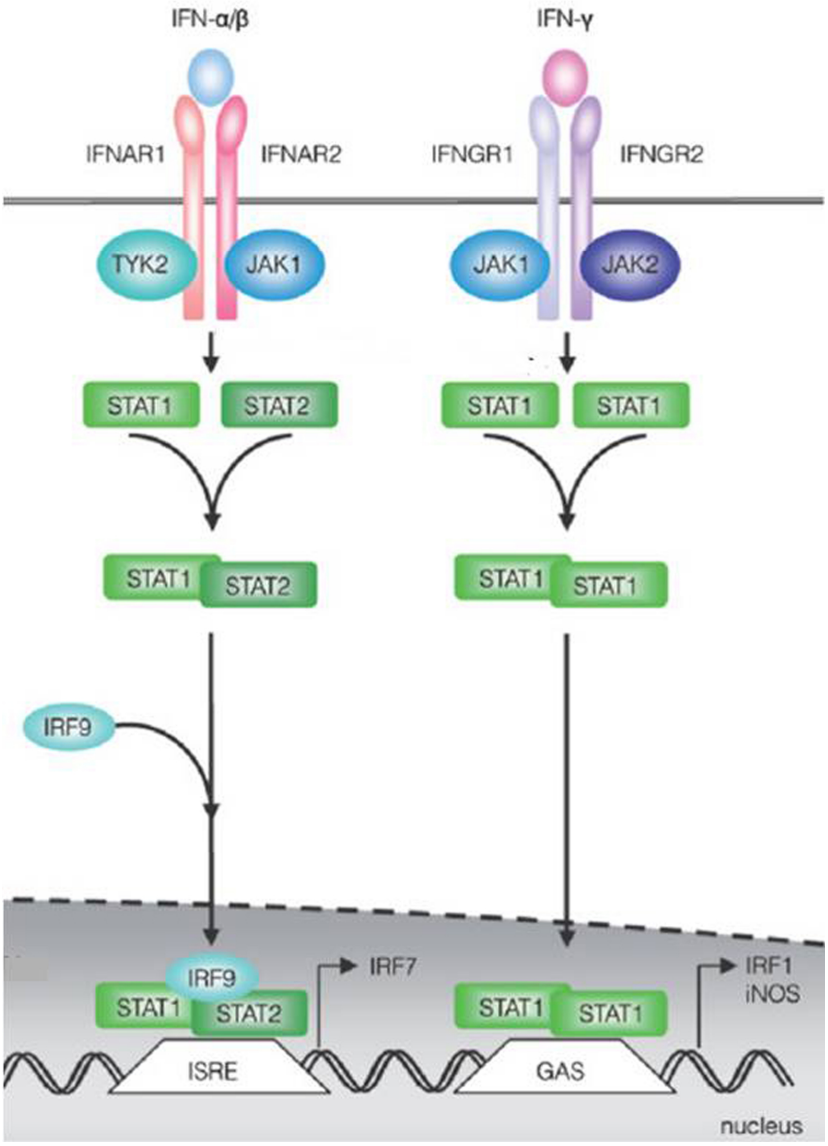

Figure 1-10. IFN signaling pathways. IFN-α/β bind the Type-1 IFN receptor which is composed of two subunits. The subunits, IFNAR1 and IFNAR2, associate with TYK2 (tyrosine kinase 2) and JAK1 (Janus activated kinase 1), respectively. IFN-γ binds a separate receptor, also composed of two subunits, IFNGR1 and IFNGR2, which associate with JAK1 and JAK2, respectively. Binding of IFN to appropriate receptor leads to TYK and JAK phosphorylation which in turn leads to recruitment and phosphorylation of STAT proteins. Once phosphorylated, STATs dimerize, enter the nucleus, and bind to transcriptional control sequences such as ISRE (IFN stimulated response elements) and GAS (gamma-activated site). Figure adapted from Stein et all, *Genome Biology*, 2007.

of these ISGs act directly on the viral life cycle or add to the host's ability to detect virus. The upregulation of ISGs can lead to an antiviral state, in which cells are much more resistant to a viral infection. IFN can also induce apoptosis as well as activate both NK and dendritic cells [208-209].

Often cells halt protein synthesis of both viral and cellular mRNA upon viral infection. Frequently, this defense mechanism is mediated by the upregulation of the ISG, double stranded RNA-activated protein kinase (PKR). Inactive PKR is a serine/threonine kinase that is normally present in the cell in low concentrations. Higher concentrations of inactive PKR appear after IFN binds its receptor on an uninfected cell. PKR will subsequently become active upon binding dsRNA in an infected cell. While bound to dsRNA, activated PKR phosphorylates eIF-2α. The phosphorylated form of eIF-2α binds eIF2B, a protein involved in the catalytic recycling of eIF2-GDP to eIF2-GTP. eIF2-GTP is necessary for translation initiation. Phosphorylated eIF-2α has a high affinity for eIF2B and once bound, eIF-2α sequesters eIF2B from recycling eIF2-GTP ultimately inhibiting translation.

Another upregulated ISG defense mechanism used to prevent synthesis of both viral and cellular proteins by degradation of cellular and viral RNA is the RNase L and 2'-5' oligo (A) synthetase. Some examples of other important ISGs include the Mx family of genes, promyelocytic leukemia (PML) nuclear bodies, APOBECs and Trims, adenosine deaminases, viperin, miRNAs, IFN regulatory proteins (IRFs), and Nitric oxide synthase. Although many ISGs have been well studied there are many more whose functions are not completely understood [210].

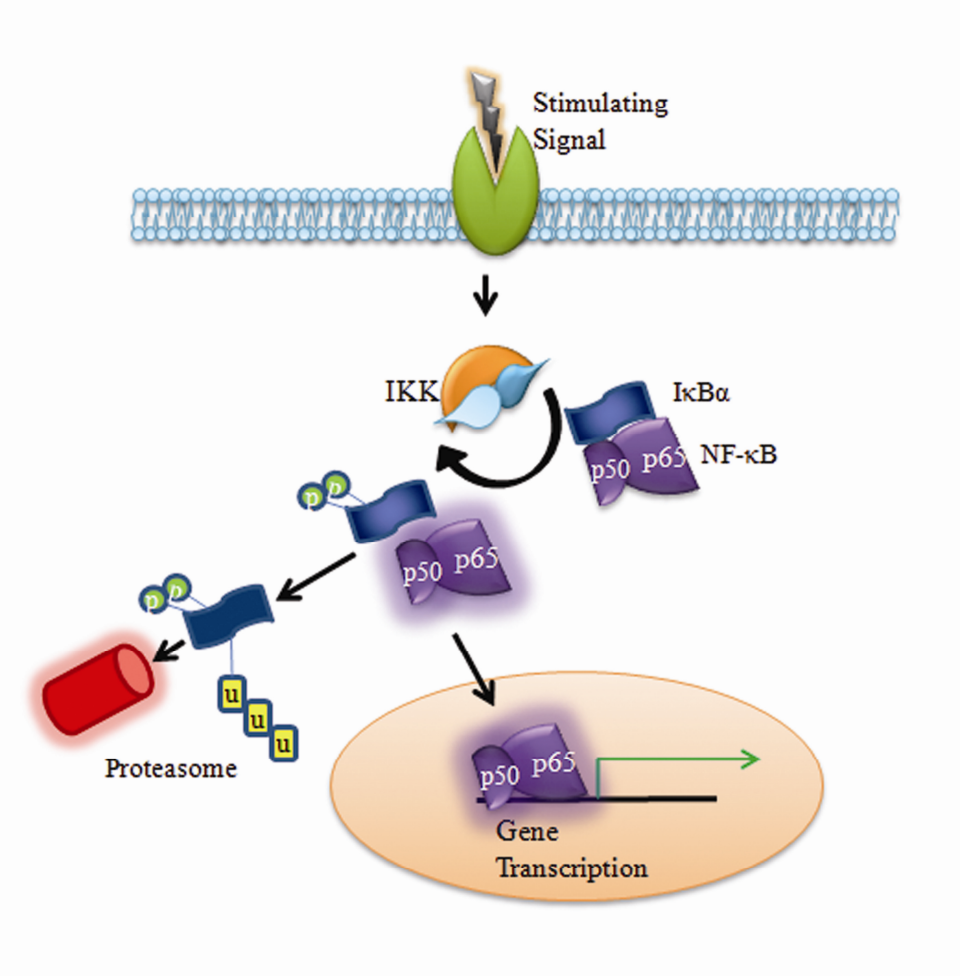

Figure 1-11. Activation of NF-κB. NF-κB is found in the cytoplasm of cells bound to IκBα which renders the transcription factor inactive. A variety of extracellular signals activate IKK, which, in turn, phosphorylates the IκBα protein. Once phosphorylated, IκBα is ubiquitinated and degraded in the proteasome. Activated NF-κB is then available for translocation into the nucleus.

NF-κB is a key, rapid-acting, transcription factor involved in many pathways induced upon a viral infection. It contributes to inflammation and is involved in the induction of IFN. NF-κB resides in the cytoplasm of cells bound to IκBα which renders it inactive. Activation of NF-κB occurs once IκBα becomes phosphorylated and subsequently ubiquitinated and degraded by the proteasome. Activated NF-κB translocates into the nucleus of cells and the induction of many antiviral genes ensues. IκBα phosphorylation is mediated by the activated IKappaB kinase protein (IKK). Viruses stimulate cells to induce many signal transduction pathways that culminate in the activation of IKK, ultimate degradation of IκBα, and translocation of NF-κB into the nucleus of the cell (Fig 1-11).

My discussion has primarily focused on the innate response to a viral infection since most VACV immune modulators target those initial fast acting host reactions. It is important to mention that both arms of the immune response, innate and acquired, are required for full viral clearance. VACV genes directly modulating the adaptive immune response have yet to be discovered.

1.6 Viral Virulence

The virulence of a virus is measured by the ability of that virus to cause disease. The objective to researching viral virulence is to find and understand mechanistically how viral and cellular genes contribute to virulence. Findings can possibly lead to attenuated strains for vaccine use or targets for drug therapies. Many factors can affect the virulence of a virus including inoculation dose, route of infection, as well as additional host factors.

Viral virulence can involve one or a combination of many different gene products. There are gene products that are toxic to the host, aid in viral spread in and among hosts, increase the replication of the virus within the host, or modulate the immune response of the host [207]. Modification of intrinsically toxic gene products typically results in diminished viral virulence in both cell culture and animal hosts. Genes needed for the virus to spread within the host may not always be apparent. For example, a virus carrying a mutant gene involved in spread within the host may appear normal in cell culture but the same virus may not be able to disseminate to its target organ(s) within the host. There are genes that can also affect both viral replication and viral virulence. There are two categories for these types of genes. First, there are the viral mutants that exhibit very low to no replication in vivo or cell culture. These mutants typically are unable to produce enough virus to cause disease which means virulence is reduced. Second, there are viral mutants whose replication is not hindered in cell culture, however, the virus is unable to replicate and cause disease in an animal host. The latter is an example of genes that are of great interest as they represent the genes that specifically cause disease.

In order for a virus to survive and replicate in a host it must be able to counter the vigorous host immune response. Many viruses have evolved anti-immune mechanisms that enable them to be successful pathogens and survive in the hostile environment of their host. These anti-immune mechanisms utilize viral gene products that are expressed as receptors either on cell surfaces or virion surfaces (viroceptors), expressed and secreted from infected cells (virokines), or are expressed intracellularly. VACV virus employs many different strategies to evade the innate

Gene	Time of Expression	Localization	Function
B13R	Early	Intracellular	Inhibits apoptosis by preventing cleavage of Caspase-1 (ICE)
F1L	Early	Mitochondrial	Inhibits apoptosis by interfering with release of cytochrome C
N1L	Early/Late	Intracellular	Inhibits apoptosis and NFκB activation
A46R	Early	Intracellular	Inhibits TLR signaling by a range of TIR domain containing receptors
A52R	Predicted Early/Late	Intracellular	Inhibits TLR signaling by interrupting interaction between IRAK and TRAF6
C21L	Predicted late	Secreted	Complement control protein; inhibits classical and alternative pathways
C12L	Early	Secreted	IL-18 binding protein decoy
B15R	Predicted Late	Secreted	Soluble IL-1β receptor decoy
A41L	Early	Secreted	Binds and inhibits CC chemokines
B8R	Early	Secreted	IFN-γ receptor decoy
B18R	Early	Soluble/Cell Surface	IFN-α/β receptor decoy
E3L	Early	Intracellular	dsRNA bp - prevents activation of IFN induced PKR
K3L	Early	Intracellular	PKR bp - prevents PKR auto-phosphorylation
K1L	Early	Intracellular	Prevents IκBα degradation thus inhibiting NFκB activation
B14R	Early	Intracellular	Inhibits NFκB activation by preventing IKK activity
M2L	Early	Intracellular	Inhibits NFκB activation via ERK2 phosphorylation
H1L	Late	Intracellular	Inhibits STAT1 and STAT2 phosphorylation

Table 1-1. VACV virulence genes with known functions.

immune response. Discussed below are some examples of VACV proteins involved in host immune modulation. For a brief description refer to Table 1-1.

All of the proteins described below contribute individually to virulence suggesting they are not functionally redundant. There are many cellular lines of attack to fight an invading pathogen and VACV has found many offensive strategies to escape, mitigate, and manipulate these host defenses.

1.6.1 Apoptosis Deterrents

The VACV B13R gene was originally investigated due to the sequence similarity of the B13R gene product with the serine protease inhibitor (serpins) superfamily of proteins. B13, also referred to as SPI-2, is an early expressed and stable intracellular protein. A B13R deletion virus grew similarly to the wild type and revertant virus controls in cell culture. Additionally, there was no attenuation observed when infecting mice with a B13R null virus in an intranasal (IN) model of infection [211]. Continued work on the B13R gene led to the finding that B13 inhibits Caspase-1, also known as ICE (Interleukin 1 Converting Enzyme), which functions in apoptosis as well as cleaves precursor forms of IL-1β into its mature form. IL-1β is an inflammatory cytokine and contributes to the febrile response in the host. In spite of the Caspase-1 inhibition, B13 did not prevent fever in infected mice, however, B13 did inhibit both Fas- and TNF-induced apoptosis [212].

VACV has also been shown to inhibit mitochondrial induced apoptosis [213], however, the virus does not encode a BCL-2 homolog which made it difficult to determine the gene responsible for this function. Initial work to find the VACV F1L protein, which provides this mechanism of inhibition, was done using the mutant

virus VV811 [214]. VV811 is a mutant of the Copenhagen (Cop) strain of VACV that happens to be missing 55 ORFs from both the left and right termini of the genome. Infection of cells with VV811 activates the apoptotic pathway, a condition not seen in cells infected with the wild type VACV Cop. To determine the gene responsible for apoptosis, ORFs carrying genes missing from the VV811 virus were transfected into VV811 infected cells. The ORF F1L was able to restore the apoptosis inhibition phenotype. Additional studies found that the F1 protein localizes to the MT and that it inhibits mitochondrial induced apoptosis by inhibiting the loss of MMP and interfering with the release of cyt c [214].

The N1L gene product is multi-functional protein and is not only involved in inhibiting apoptosis but also inhibits the NF-κB pathway (discussed below). N1 is one of the genes missing from a 17 gene deletion in the mutant VACV WR 6/2 [215]. The virus WR 6/2 grows normally in cell culture but is attenuated in vivo [216]. To determine if N1 was a contributing factor in the attenuation of VACV WR 6/2 the N1L ORF alone was disrupted in the parental WR virus and tested for virulence in follow up mouse studies. The studies revealed that N1 was a contributory factor to virulence [217-218]. Although the N1 protein has no cellular homolog the crystal structure of the protein reveals a similarity to BCL-2 proteins and it was able to co-precipitate with pro-apoptotic BCL-2 proteins: Bid, Bad, and Bax and was also shown to inhibit staurosporine-induced apoptosis [219].

1.6.2 Interruption of Pattern Recognition Receptor Signaling

VACV encodes two proteins that interfere with TLR signaling and contribute to viral virulence: A46 and A52. Both proteins were initially identified since they

share amino acid sequence identity with the TIR domain of the TLR /IL-1 receptor superfamily of receptors. Both proteins were also shown to interfere with IL-1signal transduction. A46 interfered partially with induction of NFκB while A52 effectively blocked IL-1 and TLR4 activation of NFκB [220]. Later studies of A46 showed that A46 targets multiple host cell TIR adapter molecules and inhibits intracellular signaling by many TLRs. VACV mutants lacking the A46R gene were attenuated in murine IN infections [221]. These studies demonstrate that A46 is important for VACV virulence. Additional studies concerning A52R show that the A52 protein associates with IRAK2 and TRAF6 and disrupts interactions between these two important proteins which are involved in the TLR signaling pathway. In addition, VACV mutants lacking the A52R gene were also attenuated also in a murine IN infection [222].

1.6.3 The Classical and Alternative Complement Cascade Curtailed

Initially, the VACV C21 protein was found as an abundant polypeptide secreted from infected cells and had amino acid similarity to the human C4b binding protein, which belongs to the superfamily of complement control proteins and binds the C4b complement protein [223]. Medium from cells infected with a recombinant VACV lacking the C21L gene was unable to inhibit cell lysis in a complement-mediated hemolysis assay where as the control virus was able to inhibit hemolysis. This suggested C21 was needed to inhibit cell lysis. The direct evidence that C21 had complement inhibitory activity was found when medium from RK-13 cells was chromatographed on a DEAE-Biogel column and the sample exhibiting the majority of inhibitory action (as measure by % inhibition of hemolysis) was the same sample

in which C21 was present. Additionally, C21 was found to bind to the C4b complement protein [224]. Subsequent studies determined, via a complement-enhanced neutralization assay, that the C21 protein was accountable for inhibition of the classical and alternative complement cascades in VACV infected cells possibly by interacting with both C4b and C3b. Intradermal (ID) studies with rabbits also were able to show that the C21 null virus yielded smaller lesions than the VACV WR control demonstrating that C21 is involved in viral virulence [224-225].

1.6.4 Cytokines Reined In

The B15R gene was predicted to encode a protein that had homology with the IL-1 receptor which suggested the protein may be involved in immune modulation of the host response to infection [226]. Subsequent studies demonstrated that B15 was expressed and secreted into the media of VACV infected cells and that it was able to specifically bind to IL-1β thus competitively inhibiting IL-1β from binding its natural receptor on T-cells. To resolve if B15 was an important immune modulator for VACV mice were infected with a wild type VACV WR strain and a virus lacking the B15R ORF. Mice that received the mutant virus lacking B15 had early onset of disease symptoms as compared with mice that received VACV WR suggesting B15 contributes to the virulence of the virus [227].

Another secreted VACV protein that binds cytokines is encoded by the C12L gene. When the discovery of human IL-18 binding protein (IL-18bp) was made it was noted that several poxviruses encoded a putative homologous protein [228]. IL-18bp negatively regulates the pro-inflammatory cytokine IL-18 and therefore can prevent IL-18 induced IFN-γ production from NK and T cells. Cell culture studies

found that the VACV C12L protein product is secreted from infected cells and inhibited IL-18 induced INF- γ production. Furthermore, deletion of the C12L ORF attenuated the virus in Balb/C mice [229].

The VACV Lister strain chemokine binding protein (vCKBP) was initially found while screening the medium of infected cells from many orthopoxviruses looking for a secreted viral chemokine binding protein [230]. The VACV WR A41L gene product has amino acid similarity to the Lister strain vCKBP and therefore was analyzed in further studies to determine if it was involved in immune modulation and binding to CC chemokines. Initial studies with the deletion of the ORF A41L demonstrated that A41 is dispensable for viral replication in cell culture but is required for full virus virulence in both an IN and ID model of infection. An A41L deletion virus also exhibited an altered inflammatory response to infection [231]. Additional studies made use of a purified A41 protein to screen for possible ligands. A41 was found to bind to CCL25, CCL26, CCL28, and CCL21. Interestingly, when tested, A41 did not inhibit chemokine-induced chemotaxis as it was unable to competitively inhibit the binding of the chemokines with their cellular receptors. A suggested alternative function for A43 is that it is used to block the interaction of chemokines with cell surface gags thereby changing the concentration gradient of the chemokines at the site of infection and leading to an altered immune response [232].

1.6.5 Meddling with IFN

VACV B18R gene encodes a cell surface and secreted B18 protein which is a Type I IFN binding protein. It has sequence homology with the α subunits of mouse, human and bovine Type I IFN receptors and binds human IFNα with high affinity.

Deletion of B18R attenuates the virus in an IN mouse model of infection suggesting that when present it contributes to virus virulence [233-234].

The VACV gene B8R was discovered as an immune modulator due to its sequence similarity to the extracellular binding domain of the mouse and human IFN-γ receptors. It is expressed early and is secreted from VACV infected cells. B8 has been shown to bind and inhibit INF-γ from a wide range of species but despite the sequence similarity it has with the murine IFN-γ receptor, B8 does not have an affinity for mouse INF-γ. However, the VACV protein B8 has been shown to function as a soluble IFN-γ decoy receptor and prevents antiviral effects typically seen by this cytokine [235-236]. Although there are conflicting reports on its virulence in mice, a B8 deletion virus is attenuated in rabbits [236-237].

1.6.6 Interference with IFN Induced Genes

There are two genes expressed by VACV, E3L and K3L, that interfere with IFN induced double-stranded RNA-activated protein kinase (PKR) by different mechanisms; K3L is a decoy for eIF-2α phosphorylation by PKR while E3L competitively binds and sequesters dsRNA [238].

At the outset of trying to determine proteins involved in VACV resistance to IFN, K3L was a candidate since it has 28% amino acid homology to eIF-2α. The region of homology includes the site for serine phosphorylation by PKR. Deletion of K3L from the VACV genome caused the virus to be sensitive to IFN [239]. Transfection studies found that K3 inhibited eIF-2α phosphorylation and therefore PKR activity was interrupted [240]. A follow up study showed that K3L tightly binds

inactive PKR and prevents PKR autophosphorylated activation which is the step preceding eIF-2α phosphorylation [241].

The amino acid sequence of E3 was determined to have similarity to p25, a previously found VACV dsRNA binding protein implicated in inhibition of PKR [242-243]. E3 was demonstrated to inhibit the activity of PKR. The C-terminal region of E3 also has similar homology to an RNA biding domain identified in the N-terminal region of PKR [243-244]. Further studies were able to show that E3 inhibited PKR by competitively binding to dsRNA [238]. Additionally, E3 has been shown to interfere with dsRNA and dsDNA induction of Type-1 IFN [245].

1.6.7 NF-κB Inhibition

VACV strain MVA (modified vaccinia Ankara), unlike its parental Ankara virus, is missing many immune modulator genes and activates NF-κB upon infection. Infection/transfection studies with MVA and a 5.2-kb region of Ankara DNA restored the means for MVA to inhibit NF-κB. This was determined by levels IκBα present as well as expression levels of NF- κB-transcriptionally regulated firefly luciferase. The K1L ORF is one of the genes present in the 5.2-kb region of DNA from VACV Ankara that was used in the transfection study. To test if this gene was responsible for preventing IκBα degradation it was inserted into the MVA genome and found that it did indeed prevent IκBα degradation and inhibition of NF-κB activation, although not to the same levels as a VACV WR control. Thus, the KIL gene of VACV encodes a protein that hinders activation and subsequent translocation of NF-κB into the nucleus therefore preventing the upregulation of genes involved in the immune response [246].

The observation was made that MVA can induce activation of NF-κB even when MVA infected cells are transfected with the K1L ORF. Similarly, cells infected with a mutant VACV WR lacking the K1L gene exhibit a reduction in NF-κB activation suggesting additional protein involvement in NF-κB inhibition. In addition, other pathways can be involved in activation of NF-κB in MVA infected cells; specifically MEK/ERK pathways. The M2L ORF was another gene missing from MVA but present in the 5.2kb region from VACV Ankara strain mentioned above. The M2L ORF was inserted into the MVA (MVA/M2) genome to test the ability of M2 to inhibit phosphorylation of ERK2. Compared to control virus, ERK2 phosphorylation was reduced in cells infected with MVA/M2 and subsequent mitigation of NF-κB activation [247].

The VACV B14R ORF was initially studied since computational analyses indicated B14R was highly conserved in orthopoxviruses and it was located in a region of the genome rich in immunomodulators. Deleting the gene from VACV allowed for the realization that B14 alters the inflammatory response and contributes to virus virulence in mice [248]. Subsequent studies show that B14 interacts with and inhibits the activity of the IKK subunit IKKβ and therefore prevents the downstream activation of NF-κB. By preventing activation of NF-κB infected cells may not be able to induce an appropriate immune response which is consistent with what was exhibited when infecting mice with a B14 null virus in an ID mouse model. Ensuing transfection studies demonstrated that B14 inhibits several inducers (e.g. TNFα and IL-1β) involved in NF-κB activation. Infection with a B14 null virus revealed an

increase in phosphorylated IKKα but not the IKKβ subunit suggesting B14 interacts with the IKKβ subunit of the IKK complex [249].

Many signaling cascades involved in the activation of NF-κB converge on the protein IKK. Over expression of N1 inhibited activation of NF-κB by binding IKK [250]. It is not surprising that IKK would be a target for viral immune modulators such as the N1 protein since many pathways involved in the immune response converge on the IKK complex. There is, however, disagreement that N1 is involved in this aspect to immune modulation since it was unable to co-precipitate with the IKK complex and though it was able to inhibit IL-1 induced NF-κB activation in N1 transfected cells the same was not true for infected cells [219, 249-251].

1.6.8 Virulence Proteins with Unknown Functions

In addition to the above discussed VACV proteins involved in immune modulation and virulence there are many other proteins found to be important for full virus virulence as exhibited in animal models; however their exact mechanism of action is still unknown. A summary of each of the proteins is found in Table 1-2 and includes the following proteins: A14.5 [252], A39 [253], A40 [254], A55 [255], B7 [256], C2 [257], C16 [258], and F3 [259].

Gene	Time of Expression	Localization	IN Result	ID Result	Cell Culture	Functional Motifs
A14.5L	Late	Mature Virion	Attenuated	---	No Difference	None
A35R	Early	Viral Factories	Attenuated	---	No difference	None
A39R	Late	Secreted	No Difference	Quicker Lesion Resolution	No Difference	Semaphorin
A40R	Early	Cell Surface	No Difference	Smaller Lesions	No Difference	None
A55R	Late	Intracellular	No Difference	Larger Lesions	Similar Growth, Altered CPE	BTB/Kelch Protein
B7R	Late	Endoplasmic Reticulum	No Difference	Smaller Lesions	No Difference	None
C2L	---	Intracellular	No Difference	Larger Lesions	Similar Growth, Altered CPE	BTB/Kelch Protein
C16L	Early	Intracellular/ Nuclear	Attenuated, Reduced Titers	---	Similar Growth, Smaller Plaques	None
F3L	Late	---	No Difference	Smaller Lesions	No Difference	BTB/Kelch Protein
I5L	Late	Mature Virion	Attenuated, Reduced Titers	---	No Difference	None
A43R	Late	Golgi Complex/ Cell Surface	No Difference	Smaller Lesions	No Difference	None

Table 1-2. VACV virulence genes with unknown functions. IN, ID (intradermal), and cell culture results are based on virus deletion of the specific gene. No difference refers to the comparison of a delta virus with the wild type virus.

Chapter 2: Vaccinia Virus Encodes a Small Hydrophobic Membrane Protein I5 that Enhances Replication and Virulence in Mice

2.1 Introduction

The *Poxviridae*, a family of complex DNA viruses that replicate solely in the cytoplasm of their host cells, are comprised of the *Chordopoxvirinae* and the *Entomopoxvirinae* subfamilies [1]. The orthopoxviruses, one of eight genera belonging to the chordopoxvirus subfamily, are the most intensively studied and best characterized poxviruses. VARV, a notorious member of this subfamily, has a host range that is restricted to humans and was the cause of smallpox until the disease was finally eradicated by immunization with VACV. The sequence similarity of many VACV and VARV genes is greater than 90%, accounting for the immunological cross-reactivity and vaccine efficacy. VACV has been propagated for more than 200 years; it has become the laboratory prototype poxvirus and is widely employed as an expression vector [173]. The original host of VACV is unknown although it is currently endemic in parts of Brazil [260], and a closely related virus has been isolated from Mongolian horses [261].

VACV has a linear double-stranded DNA genome with nearly 200 predicted open reading frames (ORFs). Only a few of the approximately 90 genes that are conserved in all chordopoxviruses [32] remain largely uncharacterized. One such protein, encoded by the I5L ORF, was identified as a component of the MV membrane by N-terminal sequencing of the fraction solubilized from purified MVs with NP-40 and 2-mercaptoethanol [262]. The association of I5 (the protein encoded

by the I5L ORF) with sucrose gradient-purified MVs was corroborated by mass spectroscopy [263-265]. Here, we show that I5 is expressed following viral DNA replication and is incorporated into the viral membrane at an early stage of morphogenesis. Despite its high conservation, I5 expression was not necessary or advantageous for virus replication and spread in a variety of cultured cells. Nevertheless, I5 was important for virus replication and virulence in a mouse model, suggesting a role in host interactions.

2.2 Materials and Methods

2.2.1 Cells and Viruses

BS-C-1 cells were maintained in minimum essential medium with Earle's salts supplemented with 2% fetal bovine serum, 100 units/ml of penicillin, and 100 µg/ml of streptomycin. HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum and antibiotics as described above. The VACV Western Reserve (WR) strain and recombinant viruses were propagated as previously described [266].

2.2.2 Antibodies

Rabbit antisera were raised against peptides derived from amino acids 38 to 52(FTMQSLKFNRAVTIF) of the predicted I5 sequence and from amino acids 632 to 643 (QYISARHITELF) of the A3 sequence plus a C-terminal cysteine required for coupling to keyhole limpet hemocyanin (Covance Research Products, Princeton, NJ).

2.2.3 Plasmid and Recombinant VACV Construction

To construct the recombinant vI5GFP, the flanking regions of the I5L ORF were amplified from VACV strain WR genomic DNA template using oligonucleotides ct66 (5'-CAT CAT CCA TTA GAA TTT TCA ATT CCA CTA GCG TCA AAA AAT TTC CTA CT-3'), ct68 (5'-CAT AGA AAA AAA CAA AAT GAA ATT CTT ATA TCT AAA AAT TAG ATC AAA GAA T3-'), ct69 (5'-ATG GAC GAG CTG TAC AAG TAA CGT CAA ATC CCT ATT AAT GAA AA-3'), and ct71 (5'-TCA TAC AAC TAT TTT GGT TTT AAA ACT TTG GAA AAA TCC TAC TTG TTG AAA-3'). The ORF for enhanced green fluorescent protein (GFP) under VACV promoter p11 was amplified from pA43GFF (unpublished data) using primers ct67 (5'-ATT CTT TGA TCT AAT TTT TAG ATA TAA GAA TTT CAT TTT GTT TTT TTC TAT G-3') and ct70 (5'-TTT TCA TTA ATA GGG ATT TGA CGT TAC TTG TAC AGC TCG TCC AT-3'). Primers ct67 and ct68 as well as ct69 and ct70 were designed to complement each other. The above products were used in a second recombinant PCR to yield a GFP ORF flanked by regions up- and downstream of I5L. This construct preserved the last 28 nucleotides of the I5L ORF, which functions as a promoter for I4L. The resulting PCR product was gel purified and ligated into pCR-BluntII-Topo (Invitrogen, Carlsbad, CA), resulting in pI5GFP. The endogenous I5L ORF was replaced with the GFP marker gene by homologous recombination after transfection (Lipofectamine 2000; Invitrogen) of pI5GFP into VACV WR-infected cells. Recombinant viruses expressing GFP were detected with an inverted fluorescence microscope and isolated by three rounds of plaque purification. The correct site of recombination was verified by PCR analysis.

Recombinant vI5HA-GFP (where I5HA is I5 carrying a hemagglutinin [HA] epitope tag) was made in a similar manner as vI5GFP. The primers ct81 (5'-CAT ACG ATG TTC CAG ACT ACG CTT AAG AAT TTC ATT TTG TTT TTT TCT A-3') and ct71 were used to amplify GFP under the p11 promoter from vI5GFP, and primers ct82 (5'-TTC TTA AGC GTA GTC TGG AAC ATC GTA TGG GTA ACT TTT CAT TAA TAG GGA-3') and ct66 were used to amplify I5L from VACV strain WR genomic DNA template.

The I5 revertant virus (vI5Rev) and an I5 frameshift virus (vI5Stop) were derived from vI5GFP. Primers ct66 and ct71 were used to generate a PCR product containing the I5L gene including 500 bp of up- and downstream sequence. The resulting PCR product was gel purified and ligated into pCR-BluntII-Topo, resulting in pI5Rev. Homologous recombination was used to replace the GFP marker gene with the endogenous I5L ORF after transfection of pI5Rev into cells infected with vI5GFP. Non-GFP-expressing plaques were picked and isolated by three rounds of plaque purification. The correct site of recombination was verified by PCR and sequence analysis. A stop codon was generated in the I5L sequence of pI5Rev by using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with PCR oligonucleotides containing the desired mutation. Primers ct91 (5'-GCA TAA CTG TAT TAA TGC TTT TGA TGT AAT TTC TGG TGC CGC CCT G-3') and ct92 (5'-CAG GGC GGC ACC AGA AAT TAC ATC AAA AGC ATT AAT ACA GTT ATG C-3') were used to delete nucleotide 61, resulting in an immediate stop codon. Homologous recombination was used to replace the GFP marker gene with the I5Stop sequence after transfection of pI5Stop into vI5GFP-infected cells. Again, non-

GFP-expressing plaques were picked and isolated by three rounds of plaque purification. The correct site of recombination was verified by PCR and sequence analysis.

2.2.4 SDS-PAGE

Cells were lysed in 0.2% NP-40 (10 mM Tris, pH 7.4, 10 mM $CaCl_2$, 10 mM NaCl) containing 8 µg/ml micrococcal nuclease (Worthington Biochemical Corp., Lakewood, NJ) at 4°C for 20 min. After addition of lithium dodecyl sulfate sample buffer and reducing agent (Invitrogen, Carlsbad, CA), cell lysates were heated to 70°C for 10 min. Equal volumes of lysate were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% bis-Tris-MES [2-(*N*-morpholino)ethanesulfonic acid]-SDS running buffer (Invitrogen).

2.2.5 Western Blot Analysis

Proteins separated by SDS-PAGE were electrophoretically transferred to polyvinylidene difluoride membrane (Invitrogen). Membranes were blocked in Tris-buffered saline with 5% nonfat dry milk and 0.05% Tween 20 and then incubated with antibodies for 1 h at room temperature or overnight at 4°C. Protein bands were visualized by chemiluminescence using West-Pico or Dura kits (Pierce Biotechnology Inc., Rockford, IL).

2.2.6 Analysis of Virion Extracts

VACV MVs, purified by two sucrose cushions and one sucrose gradient centrifugation from cells infected with vI5HA-GFP, were incubated at 37°C for 1 h in 50 mM Tris (pH 7.4) or in 1% NP-40 in 50 mM Tris (pH 7.4) in the presence or

absence of 50 mM dithiothreitol (DTT). Soluble and insoluble fractions were separated by centrifugation at 30,000 x *g* for 30 min and resuspended to equal volumes in sample buffer containing lithium dodecyl sulfate. Equivalent amounts of each fraction were loaded on a 10% polyacrylamide gel and subjected to electrophoresis. The separated proteins were transferred to a polyvinylidene difluoride membrane and analyzed by Western blotting as described above.

2.2.7 Confocal Microscopy

HeLa cells were grown on glass coverslips in 12-well plates. Cells were infected at multiplicity of 0.5 PFU per cell. At 24 h post infection, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 7 min at room temperature, washed three times with PBS, and then permeabilized for 10 min with 0.1% Triton X-100 in PBS at room temperature. Cells were blocked for 1 h with 10% fetal bovine serum in PBS, followed by incubation with primary antibody at room temperature. Cells were washed three times in PBS, followed by incubation with Alexa Fluor 594-conjugated secondary antibody (Invitrogen) at room temperature. After cells were washed three times with PBS, DNA was stained with 4'-6'-diamidino-2-phenylindole (DAPI), and coverslips were mounted on slides with Mowiol. Images were collected with a Leica TCS-NT/SP2 inverted confocal microscope system.

2.2.8 Electron Microscopy

BS-C-1 cells were grown in dishes of 60-mm diameter and infected with 5 PFU of virus per cell for 20 h. Cells were prepared for conventional transmission EM

by fixing with 2% glutaraldehyde and embedding in EmBed-182 resin (Electron Microscopy Sciences, Hatfield, PA). Alternatively, cells were fixed with 4% paraformaldehyde-0.05% glutaraldehyde in 0.1 M phosphate buffer for 1 h at room temperature and incubated in 10% gelatin at 37°C. The cell pellet was collected by centrifugation, solidified on ice, cut at 4°C into small cubes infiltrated with 2.3 M sucrose in 0.1 M phosphate buffer, frozen on pins in liquid nitrogen, and cut into 70-nm sections on a Leica Ultracut FCS microtome (Wetzlar, Germany). Cryosections were picked up on grids, thawed, washed free of sucrose, and stained with a monoclonal antibody to a HA epitope tag (Invitrogen) followed by rabbit anti-mouse immunoglobulin G (IgG) and protein A conjugated to 10-nm gold spheres. Specimens were viewed with an FEI-CM100 transmission electron microscope (Hillsboro, OR).

2.2.9 Determination of Virulence in Mice

Female BALB/c mice were purchased from Taconic (Germantown, NY) and maintained in a pathogen-free environment in sterile microisolator cages. Groups (n = 10) of 7-week-old mice were anesthetized by inhalation of isoflurane and inoculated via the IN route with a 20-µl suspension of purified VACV into one nostril. Mice were weighed daily for 2 weeks following challenge and were euthanized when they lost 30% of their initial body weight, according to a protocol approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee. Inoculum titers were determined in order to confirm the dose administered.

2.2.10 Titration of Virus from Lung

Lungs were removed from mice that had been infected IN with 1 x 10^4 PFU of

vI5Stop or vI5Rev; lungs were placed in 2 ml of PBS with 0.05% bovine serum albumin and kept at –80°C until use. Lungs were thawed and ground until a uniform homogenate was formed, frozen and thawed three times, and sonicated three times for 30 s. Viral titers were determined by plaque assay on BS-C-1 cells.

2.2.11 Histological Analysis

A total of 17 mice were infected IN with 1 x 10^4 PFU of vI5Stop or vI5Rev and necropsied on days 3, 5, 7, and 10. Lungs were inflated with 10% neutral buffered formalin, and other tissues were also fixed in formalin and embedded in paraffin, and sections were stained with hematoxylin and eosin. Whole lung sections were prepared from each mouse. Histopathological changes in the nasal cavity were graded in a random, blinded fashion as to extent of tissue involvement and severity, with grades of 1 to 4 as indicated in the legend of Fig. 10. For immunohistochemistry, a rabbit polyclonal antibody [267] was used at 1:2,000, followed by the Mach 4 horseradish peroxidase polymer (Biocare Medical, Concord, CA) and diaminobenzidine.

2.3 Results

2.3.1 Conservation of the I5L ORF in Chordopoxviruses

The I5L ORF (VACV WR074) is predicted to encode a 79-amino-acid protein lacking cysteine, histidine, and tryptophan residues with a mass of approximately 8.7 kDa. A hydrophilicity plot showed two putative transmembrane domains with very short N- and C-terminal sequences and only 18 amino acids between the two helices (Fig. 2-1A). The N-terminal sequence data of Takahashi and coworkers [262] indicated the absence of a cleavable signal peptide. No nonpoxvirus homologs were

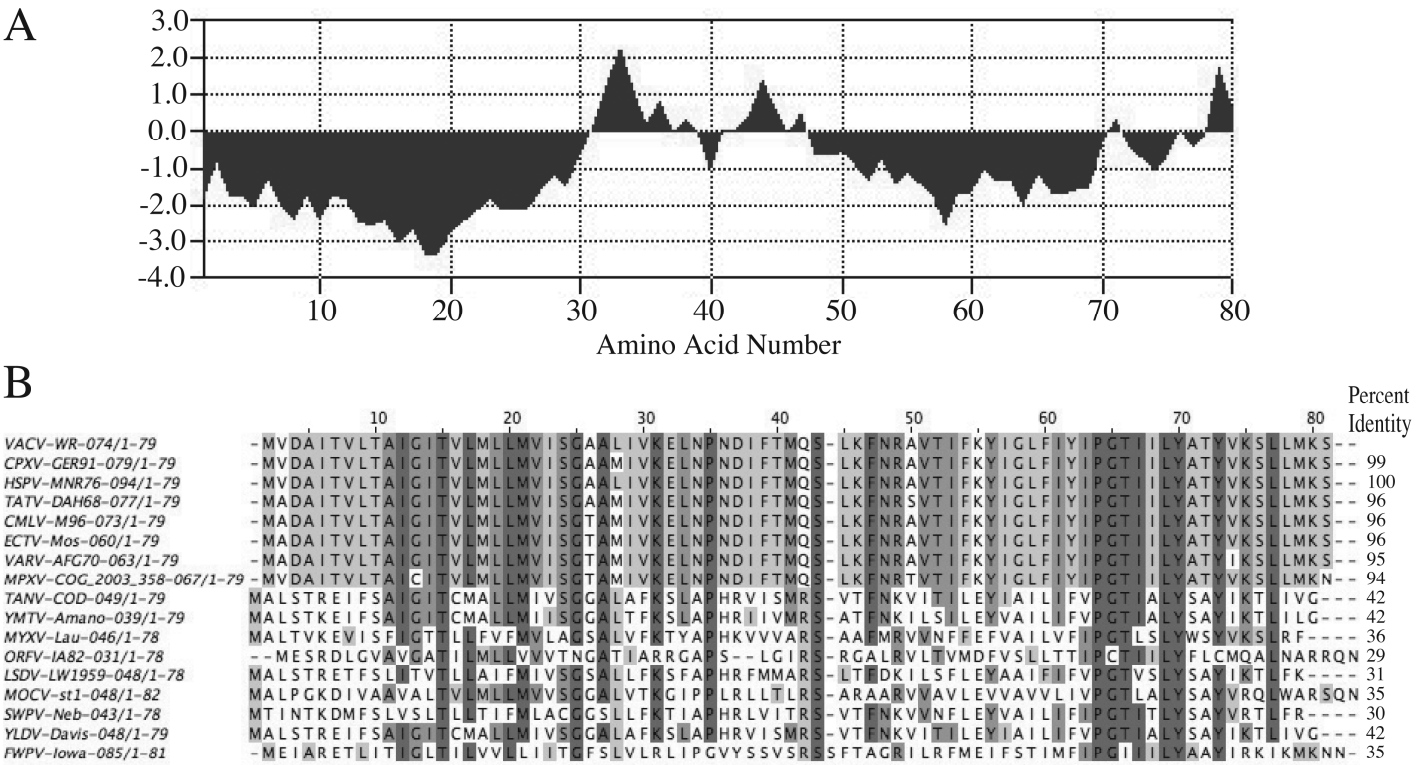

Figure 2-1. Hydrophilicity of I5 and multiple sequence alignment of orthologs. (A) Hydrophilicity plot [268] of VACV I5. (B) A multiple sequence alignment was constructed using Jalview [269]. Sequences from eight orthopoxvirus species and one or more representatives of other chordopoxvirus genera were included in the alignment. Shading increases with the degree of amino acid conservation. The percent identities between VACV WR074 and orthologs are listed on the right. Abbreviations: CPXV, cowpox virus; HSPV, horsepox virus; TATV, taterapox virus; CMLV, camelpox virus; ECTV, ectromelia virus; VARV, variola virus; MPXV monkeypox virus; TANV, tanapox virus; YMTV, yaba monkey tumor virus; ORFV, orf virus; LSCV, lumpyskin disease virus; MOCV, molluscum contagiosum virus; SWPV, swinepox virus; YLDV, yaba-like disease virus; FWPV, fowlpox virus.

found, nor were any functional sequence motifs predicted. However, all chordopoxvirus genomes sequenced to date contain an I5L ortholog (www.poxvirus.org). The amino acid sequence identities between I5 orthologs range from 94 to 100% among orthopoxviruses and 30 to 49% between members of different genera (Fig. 2-1B).

2.3.2 The I5 Protein is Synthesized at Late Times During VACV Infection and Incorporated into Virions

In order to specifically detect the I5 protein with antibody, we constructed a recombinant virus with an influenza virus HA epitope tag at the C terminus of I5 without modifying the promoter, which contains a late consensus TAAATG motif [91]. To facilitate the isolation of the recombinant virus, we also inserted an ORF encoding GFP regulated by the VACV late p11 promoter between the I5 and I4 ORFs. The resulting virus, vI5HA-GFP, formed normal-size green plaques when viewed by fluorescence microscopy. At sequential times post-infection with vI5HA-GFP, whole-cell extracts were analyzed by SDS-PAGE. The proteins were blotted to a membrane and probed with an antibody to the HA epitope tag. At 6 h after infection, a band that migrated with an estimated mass of approximately 9 kDa was detected. This band was increased in intensity from 8 to 24 h (Fig. 2-2A). A similar time course was found for the product of the A3L ORF (Fig.2-2A), a well-characterized late protein that appears as a doublet because of proteolytic processing [270]. Neither I5HA nor A3 was detected when cells were infected in the presence of cytosine arabinoside, indicating a requirement for viral DNA replication that was consistent with late stage expression. MVs were purified by sucrose gradient sedimentation from

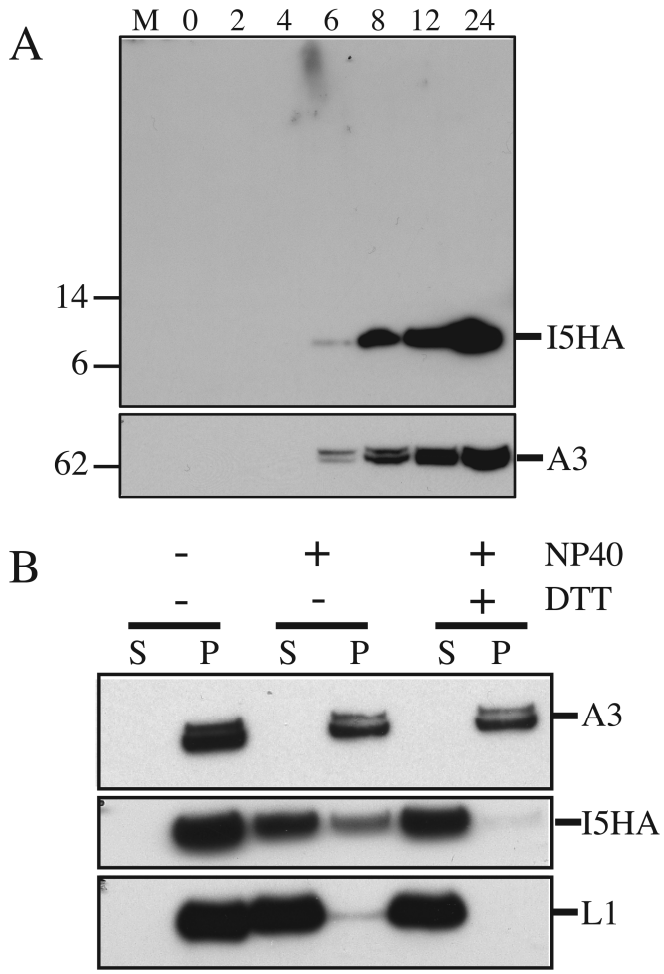

Figure 2-2. Synthesis of I5 and MV membrane localization. (A) I5 expression kinetics. BS-C-1 cells were infected with vI5HA-GFP at a multiplicity of 15 PFU per cell. At the indicated hours postinfection, cell extracts were prepared and analyzed by SDS-PAGE and Western blotting with antibody to the HA epitope tag. Masses (in kDa) of marker proteins are on the left. The blot was stripped and reprobed with antibody to the A3 protein, which appears as a doublet due to processing during virus maturation. M, mock infected. (B) Extraction of I5 from MVs. Sucrose gradient-purified vI5HA-GFP MVs were treated with NP-40 or NP-40 and DTT or mock treated and separated into soluble (S) and pellet (P) fractions. Proteins in both fractions were resolved by SDS-PAGE, followed by Western blotting with antibody to HA, A3, or L1 as indicated.

cells infected with vI5HA-GFP, and the presence of I5HA was demonstrated by Western blotting with antibody to the epitope tag. I5HA was mostly extracted with 1% NP-40 and completely solubilized when DTT was added, similar to the well-characterized L1 MV membrane protein (Fig. 2-2B). This result supported previous data indicating that I5 is a component of the MV membrane.

2.3.3 Localization of I5 to Viral Factories and Assembling Virions

Confocal microscopy was performed to determine whether I5 associates with cellular membranes in addition to virions. Following infection with vI5HA-GFP, cells were fixed, permeabilized, and stained with antibody to the HA tag, followed by a fluorescently labeled secondary antibody. Cytoplasmic factories, the site of viral DNA replication and virion assembly, were visualized by staining with DAPI, which forms fluorescent complexes with double-stranded DNA. At late times after infection, factories may appear pleomorphic but are typically located adjacent to the nucleus, which also stains with DAPI. The I5 protein colocalized with viral factories (Fig. 3), consistent with incorporation into virus particles. As a control, no specific antibody staining was found when cells were infected with an I5L deletion mutant (vI5GFP) to be described below (Fig. 2-3). Further experiments were carried out to determine the developmental stage at which I5 associates with viral membranes. Thawed cryosections of cells infected with vI5HA-GFP were stained with antibody to HA, followed successively by a secondary antibody and gold spheres conjugated to protein A. The grids were then examined by transmission EM. Gold grains were associated with IVs and MVs as well as later-stage wrapped forms and extracellular enveloped virions (Fig. 2-4). Many of the gold spheres were on or close to the viral membrane,

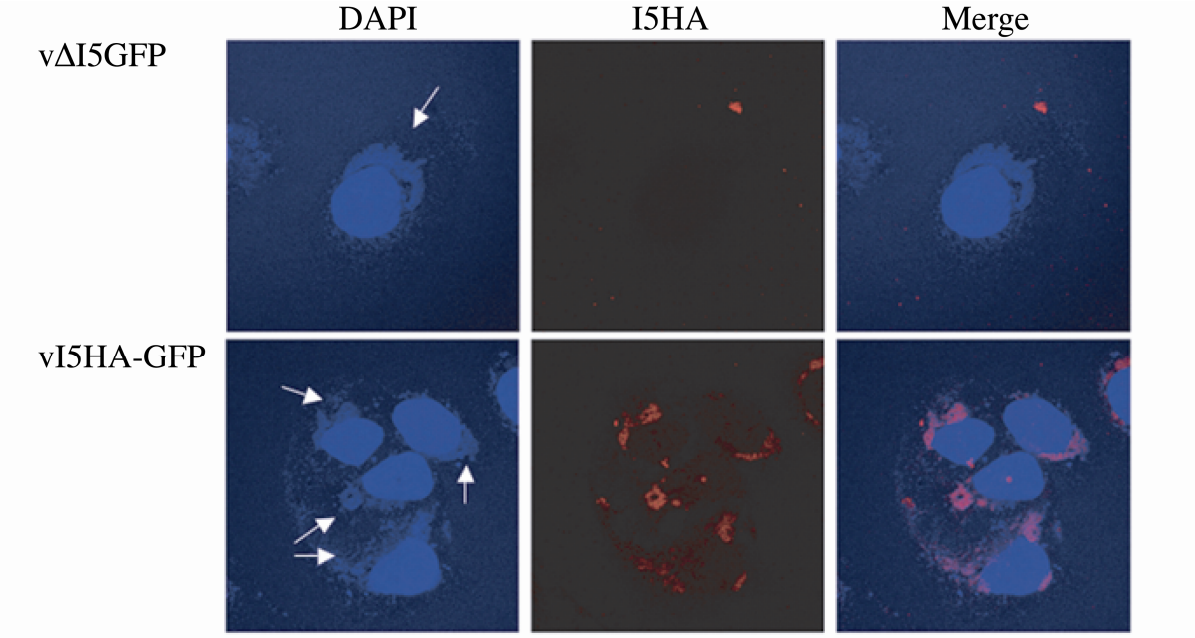

Figure 2-3. Localization of I5 in cytoplasmic viral factories. HeLa cells were infected with vI5HA-GFP or vΔI5GFP at a multiplicity of 0.5 PFU per cell. After 24 h, cells were fixed, permeabilized, and stained with anti-HA monoclonal antibody, followed by Alexa Fluor 594-conjugated anti-mouse antibody (red). DNA was stained with DAPI (blue). Images were viewed by confocal microscopy. Arrows indicate viral factories.

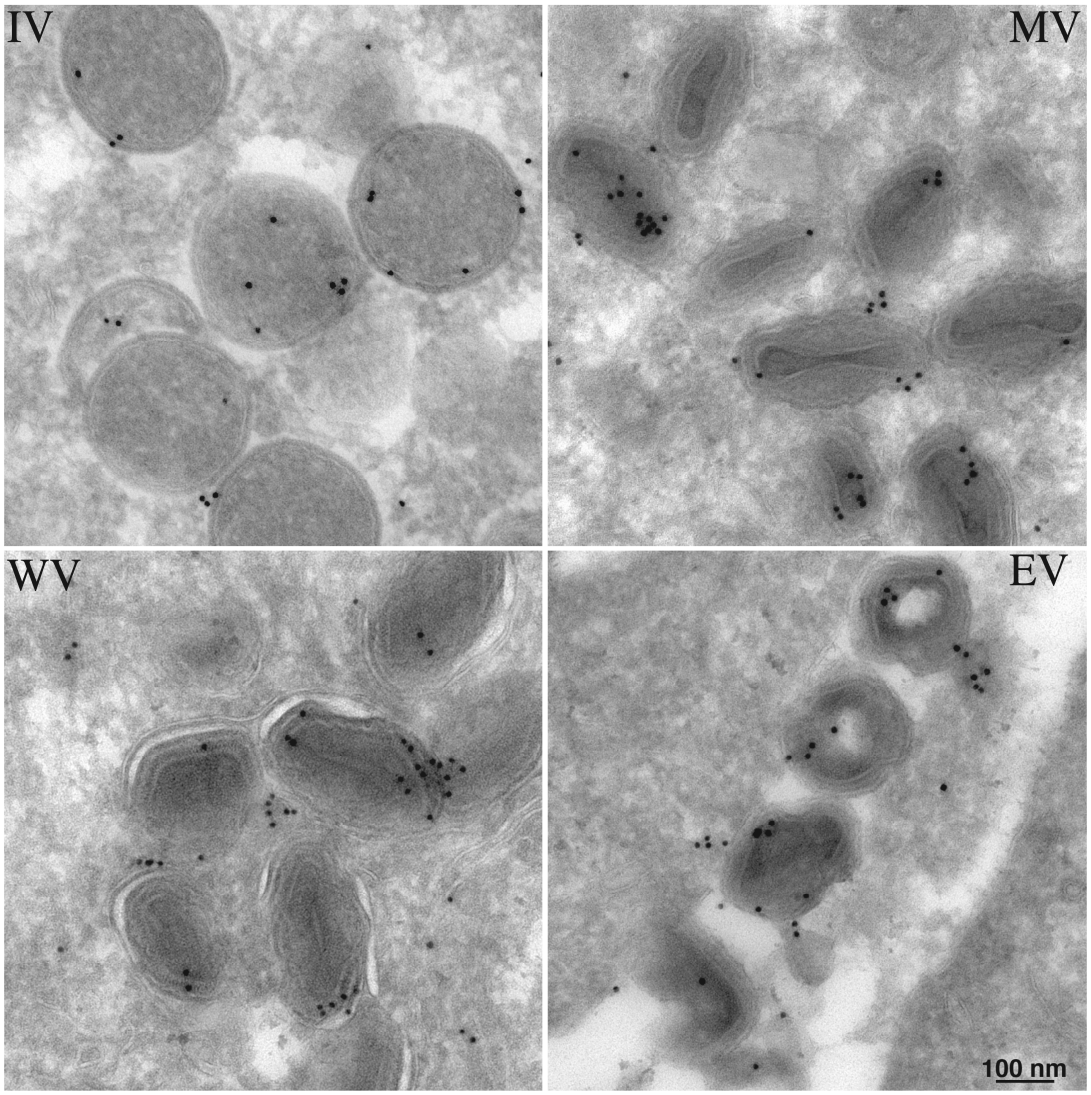

Figure 2-4. Immunogold labeling of I5 associated with immature virions and MVs. BS-C-1 cells were infected with vI5HA-GFP at a multiplicity of 5 PFU per cell. After 18 h, the cells were fixed and frozen. Thawed cryosections were incubated with mouse monoclonal antibody to HA, rabbit IgG to mouse IgG, and then with 10-nm diameter gold particles conjugated to protein A. Electron microscopic images are shown with a 100-nm scale bar. IV, immature virions; WV, wrapped virions; EV, enveloped virions.

though some seemed to be more internal. An internal appearance can result from surface immunostaining of wedge-shaped sections [124].

2.3.4 I5 is Nonessential for Virus Replication in Cultured Cells

In view of the conservation of I5, we suspected that the gene would be essential for virus replication. To confirm or refute this expectation, we transfected a plasmid containing the GFP ORF regulated by a late promoter between I5L flanking sequences into cells infected with VACV WR. An inability to isolate green fluorescent plaques would suggest that a deletion mutant was not viable. However, green plaques were readily isolated on BS-C-1 cells, and deletion of the I5L ORF was confirmed by PCR. Further characterization indicated no appreciable difference in plaques formed by vI5GFP and VACV WR on BSC1, BHK, CV-1, HeLa, HuTK$^-$, and RK13 cells and on primary human epidermal keratinocytes, indicating normal virus replication and spread (data not shown). Moreover, the yields of vI5GFP and VACV WR in BS-C-1 cells were similar (data not shown). In addition, the morphologies of cells infected with the deletion mutant and wild-type virus were similar, without signs of nuclear fragmentation or cytoplasmic blebbing.

Depending on the site of insertion, expression of GFP from a strong promoter can have subtle effects on virus replication, and this is particularly important if in vivo studies are planned. Therefore, we derived two additional viruses. Homologous recombination was used to replace the GFP ORF of vI5GFP with either the wild-type I5L ORF to generate the control vI5Rev or with the I5L ORF containing a deletion of nucleotide 61, resulting in an immediate stop codon, to generate vI5Stop. In both cases recombinant virus plaques were recognized by the absence of green

fluorescence and clonally purified. PCR and DNA sequencing confirmed the expected genome alterations. As expected, I5 could not be detected by Western blotting of lysates of cells infected with either vI5GFP or vI5Stop, indicating premature translational termination in the latter case (Fig. 2-5C). The plaque sizes and virus yields of vI5Stop and vI5Rev were indistinguishable (Fig. 2-5A & 2-5B). Furthermore, all stages of morphogenesis appeared normal as determined by transmission EM (Fig.2-6).

2.3.5 I5 Contributes to Virulence in Mice

The conservation of I5 suggested an important function despite the ability of vI5GFP and vI5Stop to replicate in cultured cells. We therefore considered the possibility that I5 has a role in host interactions that might be discerned only in vivo. An IN mouse model of infection [271] was used to determine if expression of I5 is important for virulence. Groups of 10 mice received 10^4, 10^5, or 10^6 PFU of vI5Rev or vI5Stop IN under anesthesia. Weight change and survival were recorded daily and compared to an uninfected control group. All mice that received 1 x 10^4 PFU of vI5Stop survived and exhibited less severe weight loss (P = 0.0003, day 7; Mann Whitney test) than mice that received the same amount of vI5Rev (Fig.2-7). More decisively, mice that received 1 x 10^5 PFU of vI5Stop had a 90% survival rate while mice that received 1 x 10^5 PFU of vI5Rev had a 0% survival rate (Fig. 2-7A). However, with a challenge dose of 1 x 10^6 PFU, there were no survivors in any of the groups (data not shown). Thus, I5 is important for virulence although vI5Stop was still lethal at a high dose.

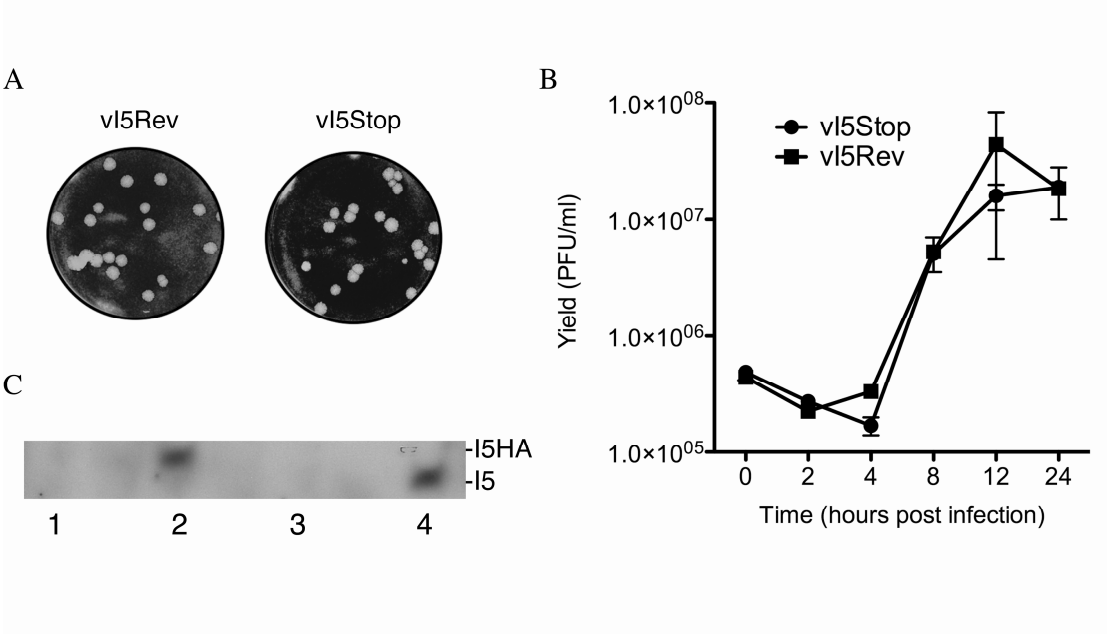

Figure 2-5. Comparison of vI5Stop and vI5Rev replication. (A) Plaque phenotypes of vI5Rev and vI5Stop. Monolayers of BS-C-1 cells were infected with vI5Rev or vI5Stop. After 48 h, cells were fixed and stained with crystal violet. (B) One-step growth curves of vI5Rev and vI5Stop. BS-C-1 cells were infected with vI5Rev or vI5Stop at a multiplicity of 10 PFU per cell. Virus yields were determined from 2 to 24 h postinfection by plaque assay. (C) Western blots. Proteins in lysates of cells that were infected with vΔI5GFP (lane 1), vI5HA-GFP (lane 2), vI5Stop (lane 3), or vI5Rev (lane 4) were resolved by SDS-PAGE and analyzed by Western blotting with rabbit polyclonal antibody to I5. The bars indicate the positions of I5HA and unmodified I5.

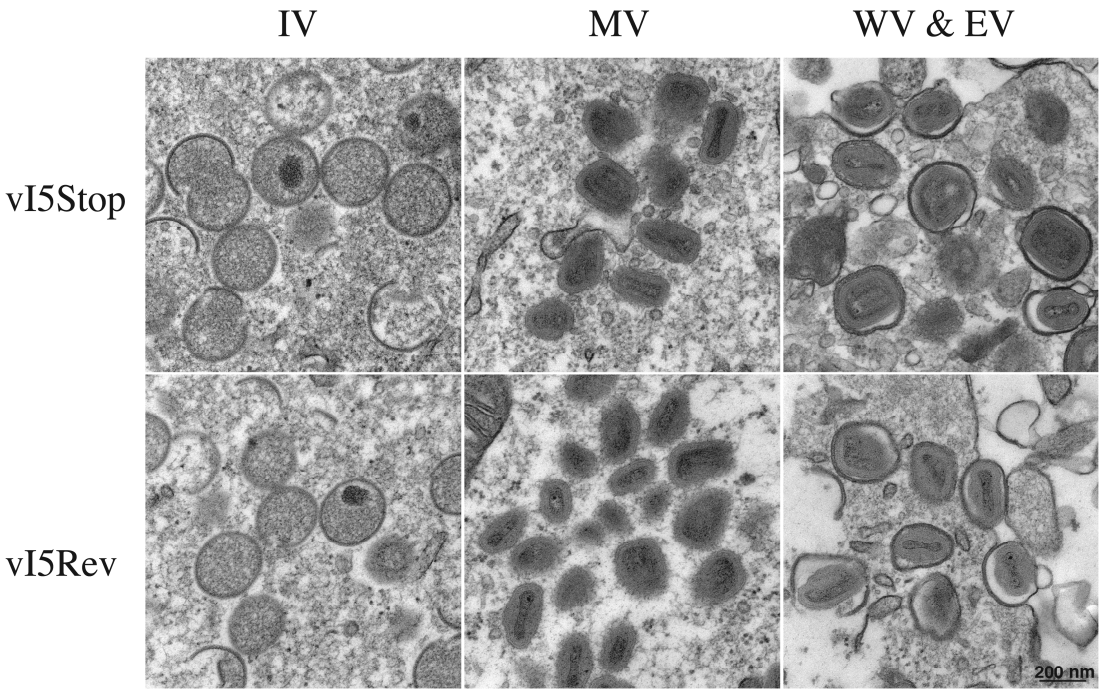

Figure 2-6. Transmission electron microscopy of cells infected with vI5Stop and vI5Rev. BS-C-1 cells were infected with vI5Stop or vI5Rev at a multiplicity of 5 PFU per cell. At 20 h after infection, the cells were fixed and prepared for EM. Electron microscopic images are shown with a 200-nm scale bar. IV, immature virions; WV, wrapped virions; EV, enveloped virions.

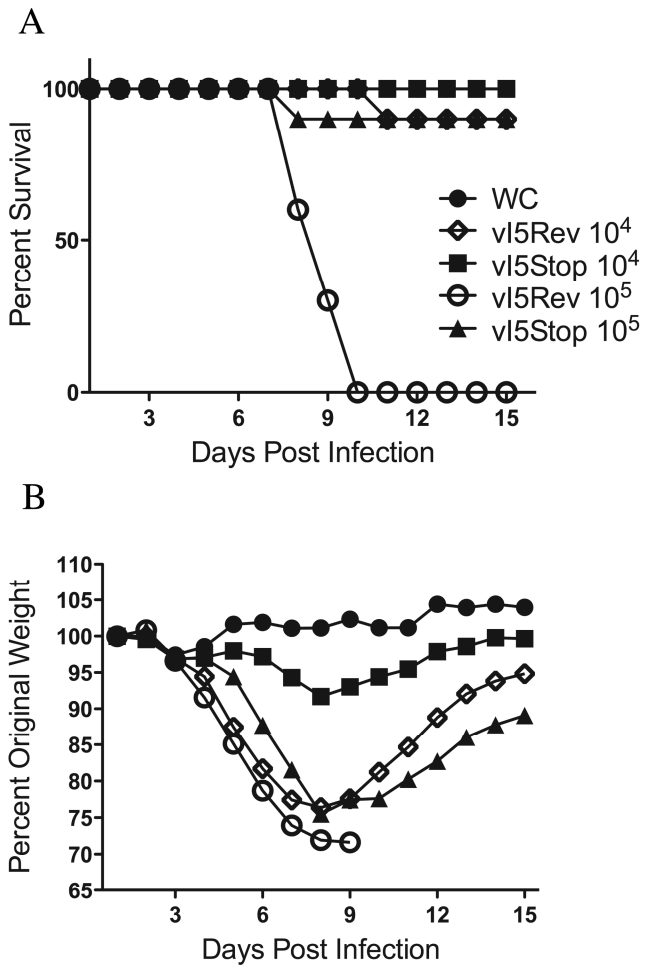

Figure 2-7. Virulence of vI5Stop and vI5Rev in mouse IN infection model. Groups of 10 BALB/c mice were inoculated intranasally with 10^4or 10^5PFU of purified vI5Stop or vI5Rev. (A) Percent survival of mice. (B) Percentage of original weight of mice. WC, untreated and uninfected weight control. Symbols in panels A and B are the same.

2.3.6 I5 Enhances VACV Replication in the Lung

Previous studies have shown that high titers of VACV WR occur in the lungs following IN inoculation and that this correlates with morbidity and death [272-275]. In order to compare the spread of vI5Stop and vI5Rev in the lower respiratory tract, we inoculated a sublethal dose of 1 x 10^4 PFU of vI5Stop and vI5Rev IN and quantified viral titers in the lungs on days 3, 5, and 7. The titers of vI5Rev and vI5Stop on day 3 were similar, suggesting that I5 was not required to establish an initial infection (Fig.2-8A). However, on day 5 the amount of vI5Stop began to decline, whereas the amount of vI5Rev increased (Fig. 2-8A). This trend continued on day 7, indicating that the vI5Stop virus was cleared rapidly. To confirm these results, the experiment was repeated with a large number of mice (n = 10) in each group, and the day 7 lung titers were determined. Although the weights of the lungs from the mice infected with vI5Stop were slightly higher than those of vI5Rev, the virus lung titers were significantly lower (P = 0.0002) (Fig. 2-8B). These results confirmed the lower progression and more rapid clearance of vI5Stop from the host.

2.3.7 Pathology Induced by vI5Stop and vI5Rev

Mice were infected IN with 1 x 10^4 PFU of vI5Stop or vI5Rev as described in the preceding section. Upper and lower respiratory tract tissue sections were examined by staining with hematoxylin and eosin and antibodies to VACV in order to discern differences in virulence. Both viruses infected the nasal epithelium and underlying glandular tissues. Destruction of the nasal epithelium occurred without inducing much of an inflammatory infiltrate. However, the lesions produced by

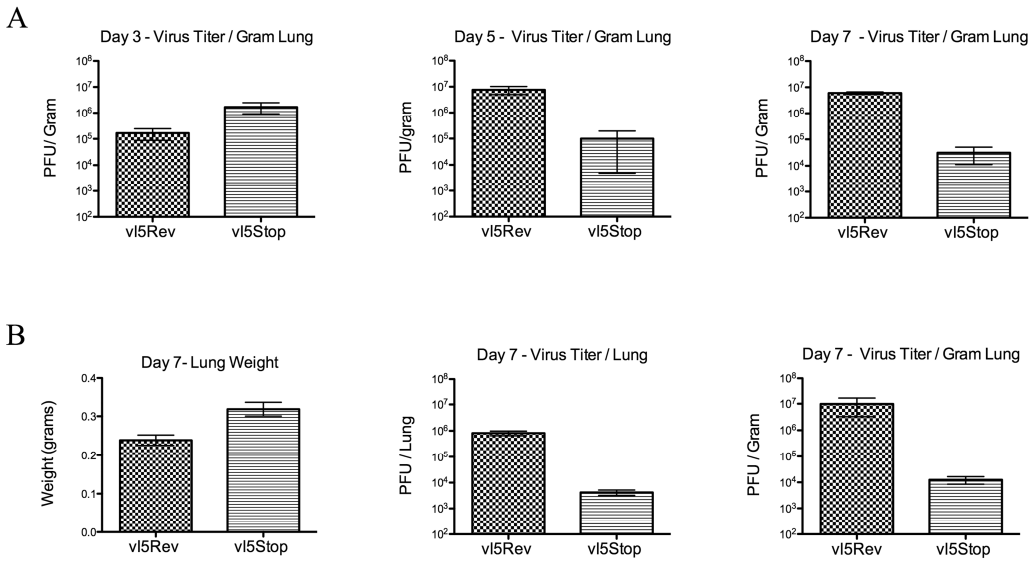

Figure 2-8. Virus titers in the lungs of infected mice. BALB/c mice were inoculated IN with 1 x 10^4 PFU of purified vI5Stop or vI5Rev. Lungs were excised, weighed, and then titers were determined by plaque assay to determine virus load. (A) Viral titers per gram of lung tissue obtained on days 3, 5, and 7. Titers were determined on lungs from three mice infected with each virus, and standard errors of the mean are indicated. (B) Lung weights and viral titers on day 7. Lung weights, viral titer/lung, and viral titer/gram of lung are plotted. Titers were determined on lungs from 10 mice infected with each virus, and standard errors of the mean are shown. A Mann Whitney *t* test yielded a *P* value of 0.005 in a comparison of the lung weights from mice infected with the two viruses and a *P* value of 0.0002 in a comparison of the viral titer/gram of lung from the two viruses.

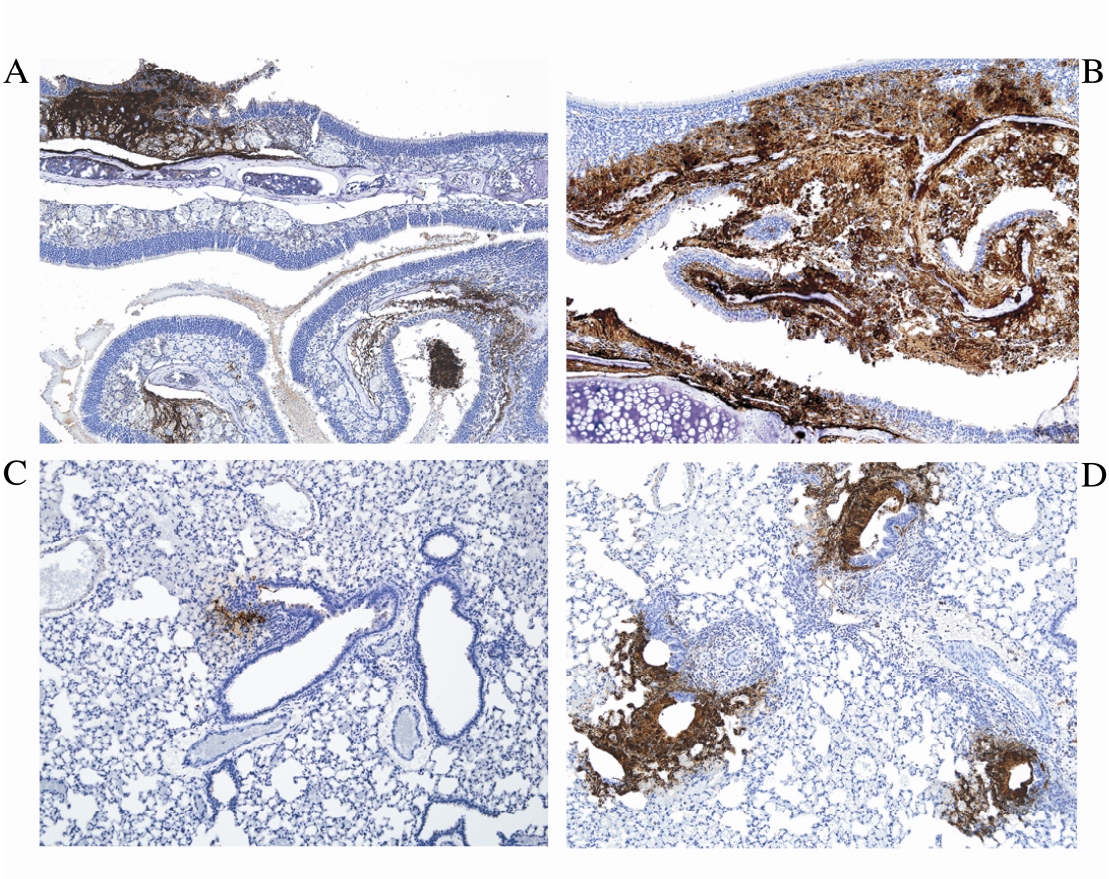

Figure 2-9. Immunostained sections of nasal epithelium and lung from infected mice. Mice were infected IN with 1 x 10^4 PFU of vI5Stop or vI5Rev. Nasal sections and lung tissue were fixed in formalin and paraffin embedded. Histochemistry was performed with an antibody to VACV proteins. Images are at a magnification of x100. (A) Nasal epithelium infected with vI5Stop at day 7. Note the focus of infection stained brown. (B) Nasal epithelium infected with vI5Rev at day 7. Note the extent of the lesions and abundant antigen. (C) Lung infected with vI5Stop at day 5 showing one bronchiole infected with associated necrosis and inflammation. (D) Lung infected with vI5Rev at day 5 showing three bronchioles infected.

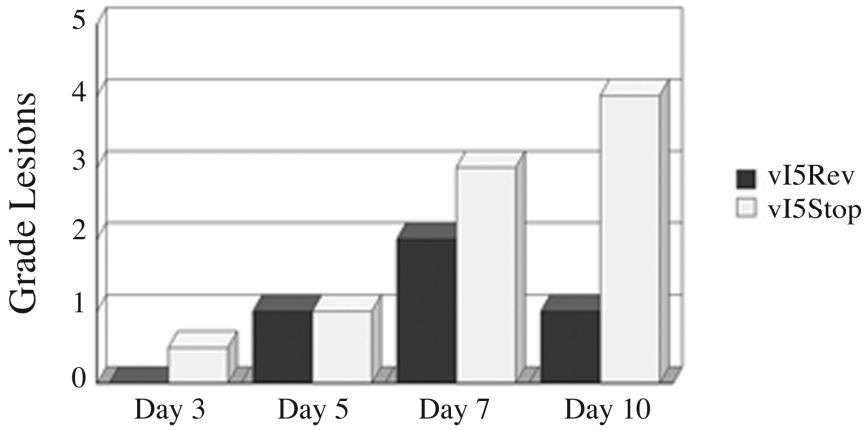

Figure 2-10. Graded lesions of nasal epithelium on indicated days after infection with vI5Stop and vI5Rev. Two to three sections of the nasal cavity (including the squamous, respiratory, and olfactory regions of the epithelium) from each of the 17 mice in this study were stained with antibody to VACV. The entire slide was scanned to make a virtual computerized slide and a section representing average antigen expression for each mouse was selected. After an initial examination, grading was done in a random blinded fashion as follows: 0, no stained foci seen; 1, few positive focal areas; 2 several positive foci or large positive areas; 3, many positive foci or larger positive areas; 4, numerous coalescing positive areas.

vI5Stop were less severe and more focal than those caused by vI5Rev (Fig. 2-9A and 2-9B). Furthermore, the severity and extent of the lesions produced by vI5Stop decreased after day 7 whereas those produced by vI5Rev continued to increase (Fig. 2-10). With both viruses, infection of the lungs occurred focally in the bronchiolar epithelial cells and around bronchioles. The lesions and viral infection appeared less severe in the mice receiving vI5Stop than in vI5Rev-infected mice, and in both cases the pathology was less extensive than in the nasal cavities (Fig. 2-9C and 2-9D).

2.4 Discussion

We have provided the first detailed characterization of the product of the I5L gene of VACV. Although previous studies had suggested that I5 is a component of the MV membrane [276], its function was not predicted because of the absence of any recognizable functional motif or nonpoxvirus homolog. We confirmed the association of I5 with the MV membrane by biochemical and electron microscopic methods. Expression of I5 was dependent on viral DNA replication, and the protein was associated with the membranes of both immature and MVs in viral factories. I5 was not detected in association with cell membranes, at first suggesting that it functioned in some aspect of the virus life cycle such as virion assembly or spread. Nevertheless, the I5L gene was readily deleted, and the mutant virus replicated and spread normally in a variety of host cells. This result was surprising because I5L is conserved in all chordopoxviruses, implying an important function. Thus, it was interesting to find that the deletion and frameshift mutants that did not express I5 were attenuated in a murine IN infection model. Thus, with an inoculum of 1×10^5 PFU (approximately five times the 50% lethal dose), there was a 90% survival rate. However, with a

challenge dose 10 times higher, there were no survivors.

In anesthetized mice, the IN route results in primary infections in the upper and lower respiratory tracts, and the latter has been associated with morbidity and death [272-275, 277]. With a nonlethal dose of 10^4 PFU, on day 3 the lung titers of the I5L frameshift mutant and control VACV were similar, suggesting that the mutant virus was able to initially infect and replicate there. However, by day 7 the titer in mouse lungs of the mutant was approximately 3 logs lower than the control, indicating less progression or more rapid clearance of the mutant. Examination of histological sections also indicated necrosis of the epithelium and underlying glandular tissue in the nasal passages of both the I5L mutant and the control virus at early times. However, there was less progression of the infection with the mutant virus than with the control virus and considerable recovery between days 7 and 10 relative to the control virus.

Our data suggest that I5 is involved in repelling the host antiviral defense though we cannot rule out some differences in cell tropism in vivo. Indeed, the latter would make sense in view of the location of I5 in the MV membrane. Further studies with a variety of primary mouse cells may help to evaluate this possibility. There have been a number of reports indicating that VACV triggers signaling pathways during the attachment or entry stage of infection [45, 278-279], which could be mediated or partially suppressed by an MV membrane protein. Some information regarding these possibilities might be obtained in follow-up studies by analysis of inflammatory cells and cytokines in mouse lung washes as well as in in vitro studies. The I5 protein is largely composed of two hydrophobic domains that presumably serve as

transmembrane segments. There is a highly conserved 18-amino-acid sequence located between the two helices that could be an interaction domain for some cellular protein, and efforts to test this hypothesis are planned. A14.5, an even smaller conserved MV membrane protein of only 53 amino acids with a similar predicted topology, is also nonessential in cell culture but is required for virulence [280]. It will be interesting to see if these two proteins have related roles.

Chapter 3: Vaccinia Virus A43R Gene Encodes an Orthopoxvirus-Specific Late Non-Virion Type-1 Membrane Protein that is Dispensable for Replication but Enhances Intradermal Lesion Formation

3.1 Introduction

The poxviruses are a group of large and complex DNA viruses that replicate exclusively in the cytoplasm of their host cells. The *Poxviridae* are divided into two subfamilies, the chordopoxviruses, which infect vertebrates, and the entomopoxviruses, which infect invertebrates [1]. The orthopoxviruses are one of the eight genera belonging to the Chordopoxviruses. The two most well known poxviruses belonging to the orthopoxvirus genus are: VARV, the causative agent of smallpox and VACV the vaccinating agent used to eradicate smallpox. VACV is the prototype poxvirus. It has a linear double stranded DNA genome that is approximately 190kbs with covalently closed hairpin termini. VACV encodes ~ 200 genes of which almost half are completely conserved within the chordopoxvirus subfamily [32]. The evolutionarily conserved genes tend to be centrally located within the genome and are mostly involved in essential replication functions, while the genus- and species-specific genes are nearer the ends of the genome and typically involved in host interactions.

Many of the genes found within the variable region of the genome encode for proteins involved in virus virulence. Virulence proteins can be implicated in various functions. They aid in viral replication, act as toxins to the host, or are involved in

host immune modulation. Viral immune modulators are a diverse group of proteins that function in many of ways to fight and/or modulate the host immune system. There are viral regulators of apoptosis [281] and inflammation [246], proteins to interfere with the complement cascade [224], as well as ones that inhibit IFN induced genes [240] . VACV encodes for many immune modulators such as the VACV B15R gene product, a secreted IL-1beta binding protein [227], the B18 gene product, a Type I IFN binding protein [234], and the E3L gene product which blocks PKR activation by binding dsRNA [243, 282]. Since not all VACV genes have been characterized, it seems likely that additional proteins involved in host interactions will be discovered.

The A43R open reading frame (ORF) is a candidate as it is embedded within the variable region of the genome. For example, the A41L ORF encodes a chemokine binding protein [283] and the protein encoded by A46R inhibits TLR signaling [221]. This study provides the initial characterization of A43, the product of the A43R ORF, which was expressed after viral DNA replication as a glycosylated protein that associated with Golgi and plasma membranes but was not appreciably incorporated into virus particles. The gene was found to be dispensable for VACV replication in cell culture but caused smaller than normal intradermal lesions in mice.

3.2 Materials and Methods

3.2.1 Cells and Viruses

BS-C-1 cells were maintained in minimum essential medium with Earle's salts supplemented with 2% fetal bovine serum, 100 units/ml of penicillin, and 100 µg/ml of streptomycin. HeLa cells were maintained in Dulbecco's modified Eagle's medium

supplemented with 2% fetal bovine serum and antibiotics as described above. The VACV Western Reserve (WR) strain and recombinant viruses were propagated as previously described [266].

3.2.2 Plasmid and Recombinant VACV Construction

To construct the recombinant vΔA43GFP, the flanking regions of the A43R ORF were amplified from VACV strain WR genomic DNA template using oligonucleotides ct49 (5'- ATG CTA ATG TCA AGT TTA TTC CAA TAG ATG TCT TAT TAA AAA ACA TAT AT-3'), ct51 (5'-CAT AGA AAA AAA CAA AAT GAA ATT CTT AAA ATT GAC ACT ACA TAT GAA TAT-3'), ct52 (5'- GCA TGG ACG AGC TGT ACA AGT AAT TAT GCT ATG CTA TTA GAA ATG -3'), and ct54 (5'- CTA TTT AGA AAA CCA TCA CTA CTC AAC AAC TAT ACG TTG AAG ATA TCC AAC -3'). The ORF for enhanced green fluorescent protein (GFP) under VACV promoter p11 was amplified using primers ct50 (5'- ATA TTC ATA TGT AGT GTC AAT TTT AAG AAT TTC ATT TTG TTT TTT TCT ATG 3') and ct53 (5'- CAT TTC TAA TAG CAT AGC ATA ATT ACT TGT ACA GCT CGT CCA TGC -3'). Primers ct50 and ct51 as well as ct52 and ct53 were designed to complement each other. The above products were used in a second recombinant PCR to yield a GFP ORF flanked by regions up- and downstream of A43R. The resulting PCR product was gel purified and ligated into pCR-BluntII-Topo (Invitrogen, Carlsbad, CA), resulting in pΔA43GFP. The endogenous A43R ORF was replaced with the GFP marker gene by homologous recombination after transfection (Lipofectamine 2000; Invitrogen) of pΔA43GFP into VACV WR-infected cells. Recombinant viruses expressing GFP were detected with an inverted fluorescence

microscope and isolated by three rounds of plaque purification. The correct site of recombination was verified by PCR analysis. A similar procedure was used to delete the A43R ORF from the IHD-J strain of VACV to form IHDΔA43GFP.

Recombinant vA43V5 was derived from vΔA43GFP. The primers ct61 (5'- AAT GCT AAT GTC AAG TTT ATT CCA ATA GAT GTC TTA TTA AAA ACA TAT AT -3'), ct62 (5'- ACC GAG GAG AGG GTT AGG GAT AGG CTT ACC AGA GTT CAT TTT TAT TTT TTT -3'), ct63 (5'- TAT CCC TAA CCC TCT CCT CGG TCT CGA TTC TAC GTA ATT ATG CTA TGC TAT TA -3'), and ct54 were used to amplify A43R from genomic DNA and incorporate a C-terminal V5 tag. Non-fluorescent plaques were picked and clonally purified by repeated isolations.

The A43 revertant virus (vA43Rev) was derived from vΔA43GFP. Primers ct61 and ct54 were used to generate a PCR product containing the A43R gene including 500 bp up- and downstream sequence. The PCR product was gel purified and ligated into pCR-BluntII-Topo (pA43Rev). Homologous recombination was used to replace the GFP marker gene with the endogenous A43R ORF after transfection of pA43Rev into cells infected with vΔA43GFP. Non-GFP-expressing plaques were picked and isolated by three rounds of plaque purification. The correct site of recombination was verified by PCR and sequence analysis. An A43V5$^-$ virus (vA43V5Stop) was also derived from vΔA43GFP. A stop codon was generated in the A43R sequence of pA43V5 by using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with PCR oligonucleotides containing the desired mutation. Primers ct79 (5'- CCG GTA TTG GCA TAC AGC TAA TCG ATT TTT AGA TTT CAT TCA GAG -3') and ct80 (5'- CTC TGA ATG AAA TCT AAA AAT

CGA TTA GCT GTA TGC CAA TAC CGG -3') were used to change nucleotide 68, resulting in an immediate stop codon. Homologous recombination was used to replace the GFP marker gene with the A43V5Stop sequence after transfection of pA43V5Stop into vΔA43GFP-infected cells. Again, non-GFP-expressing plaques were picked and isolated by three rounds of plaque purification. The correct site of recombination was verified by PCR and sequence analysis.

Primers ct61 (5'- AAT GCT AAT GTC AAG TTT ATT CCA ATA GAT GTC TTA TTA AAA ACA TAT AT -3'), ct88 (5'- TAC CCA TAC GAT GTT CCA GAC TAC GCT TAA TTA TGC TAT GCT ATT AGA AAT -3'), ct89 (5'- TAA TTA AGC GTA GTC TGG AAC ATC GTA TGG GTA AGA GTT CAT TTT TAT TTT TTT -3'), and ct54 were used to amplify A43R from genomic DNA and incorporate a C-terminal HA tag. The PCR product was gel purified and ligated into pCR-BluntII-Topo (pA43HA). pN65Q, pN93Q, and N114Q were generated in the A43R sequence of pA43HA by using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with PCR oligonucleotides containing the desired mutation. Primers ct101 (5'- CCA TAT AGA TAT AAT TTT ATT CAG CGC ACG TTA ACC GTA GAT GAA C -3') and ct102 (5'- GTT CAT CTA CGG TTA ACG TGC GCT GAA TAA AAT TAT ATC TAT ATG G -3') were used for pN65Q. Primers ct103 (5'- CAC AAA TAT GGT TCA CTT CAG CCT AGT TTG ATT GTC TCA TTA TC -3') and ct104 (5'- GAT AAT GAG ACA ATC AAA CTA GGC TGA AGT GAA CCA TAT TTG TG -3') were used for pN93Q. Primers ct105 (5'- CAA TGC TCA GTA CAG GTA TCG TGT CTC ATT AAA AAT TTG GC -3') and ct106 (5'- GCC AAA TTT TTA ATG AGA CAC GAT ACC TGT ACT GAG CAT TG -3')

were used for pN114Q. pA43HAΔTM was constructed from WR genomic DNA using primers ct64 (5'-TAA TTA AAA TAA AAA GTA ATA TTC ATA TGT AGT GTC AAT TTT AAA TGA TGA -3') and ct90 (5'- TTA AGC GTA GTC TGG AAC ATC GTA TGG GTA ATT ATA CTT GTC ATT TAT ATC TTT AT -3') in a PCR reaction to amplify the A43 coding region under the natural promoter, delete the transmembrane domain and maintain the HA tag sequence. The PCR product was gel purified and ligated into pCR-BluntII-Topo (pA43HAΔTM). All constructed plasmid sequences and mutations were verified by sequence analysis.

The plasmid pcDNAA43V5 was constructed by amplifying A43V5 from pA43V5 using primers ct85 (5'- CAC CAT GAT GAT GAT GAA ATG GAT AAT ATC CAT ATT-3') and ct86 (5'- TTA CGT AGA ATC GAG ACC GAG GAG AGG -3'). The PCR product was then gel purified and ligated into vector pcDNA3.1 (Invitrogen) forming pcDNAA43V5.

3.2.3 Endo H and PNGase Treatment of Cell Lysate

BSC-1 cells were infected with vA43V5 and harvested 24hours post infection. Cell lysates were treated with either Peptide: N-Glycosidase F (New England BioLabs) or Endoglycosidase H (New England BioLabs) for 2-24 hours. Samples were analyzed by SDS-Page and Western blotting.

3.2.4 EV Purification

RK-13 cells were infected with either WR or vA43V5. 48 h post-infection, the media supernatant was harvested and clarified by low-speed centrifugation for 20 minutes. EVs were pelleted by centrifugation at 14,000 x g for 1 h at 4°C. The EVs

were gently resuspended and further purified by layering over a preformed CsCl gradient as described previously [165] and centrifuged using a SW-41 rotor at 32,000 rpm for 4 h at 20°C. Fractions collected starting from the top of the tube were loaded on a 10% polyacrylamide gel and subjected to electrophoresis. The separated proteins were transferred to a polyvinylidene difluoride membrane and analyzed by Western blotting.

3.2.5 MV Purification

WR or vA43V5 infected RK-13 cells were scraped and collected by low-speed centrifugation. Cells were then resuspended and disrupted by Dounce homogenization. Cell suspensions were clarified by low-speed centrifugation and MV purified by centrifugation through two 36% sucrose cushions and one 25-40% sucrose gradient.

3.2.6 SDS-PAGE

Cells were lysed in 0.2% NP-40 (10 mM Tris, pH 7.4, 10 mM $CaCl_2$, 10 mM NaCl) containing 8 µg/ml micrococcal nuclease (Worthington Biochemical Corp., Lakewood, NJ) at 4°C for 20 min. After addition of lithium dodecyl sulfate sample buffer and reducing agent (Invitrogen, Carlsbad, CA), cell lysates were heated to 70°C for 10 min. Equal volumes of lysate were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% bis-Tris-MES [2-(N-morpholino)ethanesulfonic acid]-SDS running buffer (Invitrogen).

3.2.7 Western Blot Analysis

Proteins separated by SDS-PAGE were electrophoretically transferred to polyvinylidene difluoride membrane (Invitrogen). Membranes were blocked in Tris-buffered saline with 5% nonfat dry milk and 0.05% Tween 20 and then incubated with antibodies for 1 h at room temperature or overnight at 4°C. Protein bands were visualized by chemiluminescence using West-Pico or Dura kits (Pierce Biotechnology Inc., Rockford, IL).

3.2.8 Confocal Microscopy

HeLa cells were grown on glass cover slips in 12-well plates. Cells were infected at multiplicity of 0.5 PFU per cell. At 24 h post infection, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 7 min at room temperature, washed three times with PBS, and then permeabilized for 10 min with 0.1% Triton X-100 in PBS at room temperature. Cells were blocked for 1 h with 10% fetal bovine serum in PBS, followed by incubation with primary antibody at room temperature. Cells were washed three times in PBS, followed by incubation with Alexa Fluor-conjugated secondary antibody (Invitrogen) at room temperature. After cells were washed three times with PBS, DNA was stained with 4'-6'-diamidino-2-phenylindole (DAPI), and coverslips were mounted on slides with Mowiol. Images were collected with a Leica TCS-NT/SP2 inverted confocal microscope system.

3.2.9 Cell Surface Biotinylation

HeLa cells were infected with vA43V5. At 18 h post infection, cells were washed twice with PBS containing Mg^{2+} and Ca^{2+} and then incubated with 0.5 mg/ml

of the membrane-impermeable EZ-Link Sulfo-NHS-LC-Biotin (sulfosuccinimidyl-6-[biotinamido] hexanoate) (Pierce) dissolved in PBS on ice for 20 min. Cells were washed with PBS and quenched with 10% fetal bovine serum for 20 min on ice and then washed twice again. Cells were then lysed and the biotinylated proteins were affinity purified on a NeutrAvidin gel (Pierce).

3.2.10 IN Infection Model

Female BALB/c mice were purchased from Taconic (Germantown, NY) and maintained in a pathogen-free environment in sterile microisolator cages. Groups of 7-week-old mice were anesthetized by inhalation of isoflurane and inoculated via the IN route with a 20-µl suspension of purified VACV into one nostril. Mice were weighed daily for 2 weeks following challenge and were euthanized when they lost 30% of their initial body weight, according to a protocol approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee. The inocula were titered in order to confirm the dose administered.

3.2.11 Ear Pinna Infection Model

Female BALB/c mice were anaesthetized with avertin and inoculated intradermally with 10 PFU of VACV in a 10-µl suspension. Lesions were measured daily with a micrometer. Ears were removed and placed in 2 ml of PBS with 0.05% bovine serum albumin and kept at –80°C until use. Ears were thawed, cut into 1 mm pieces and treated with collagenase for 3-4 h at 37°C, frozen and thawed three times, and sonicated three times for 30 s. Viral titers were determined by plaque assay on BS-C-1 cells.

3.3 Results

3.3.1 A43R is Conserved Among Orthopoxviruses

The A43R ORF (VACV WR168) is predicted to encode a 194-amino-acid protein with an N-terminal hydrophobic domain, two N-linked glycosylation sites, and a C-terminal transmembrane domain. The SignalP program [284] predicted that the N-terminal hydrophobic region is a cleaved signal sequence with the cleavage occurring between S22 and S23. A43R is highly conserved within all orthopoxviruses; the sequence identity is >94% except for ectromelia virus which has a 78% identity (Fig. 3-1). However, there are no recognized homologs in any other poxvirus genus, nor are there non-poxvirus homologs or functional motifs to help predict the function of A43.

3.3.2 A43 is a Glycosylated Protein Expressed at the Late Stage of VACV Replication

A recombinant virus, in which a V5 epitope tag was added to the C-terminus of A43, was constructed to assist in protein characterization. The region upstream containing the promoter sequence was unaltered so as not to perturb expression. The growth kinetics and plaque phenotype of vA43V5 were similar to that of the parental virus (not shown).

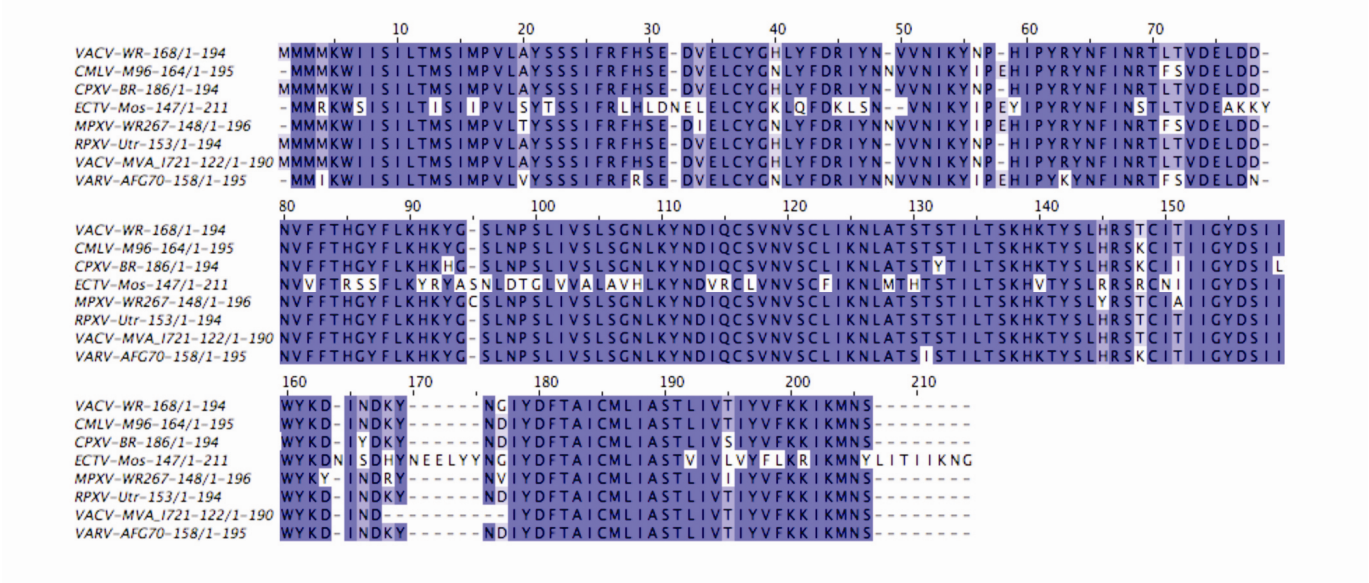

Figure 3-1. Multiple sequence alignment of A43 orthologs. Jalview [269] was used to construct a multiple sequence alignment of A43 from eight orthopoxvirus species. Abbreviations: VACV-WR, VACV strain WR, CMLV-M96, camelpox virus strain M96; CPXV-BR, cowpox virus strain Brighton; ECTV-Mos, ectromelia virus strain Moscow; MPXV-WR267, monkeypox virus strain Walter Reed 267; RPXV, rabbitpox virus strain Utrecht; VACV-MVA, VACV strain Modified VACV Ankara; VARV-AFG70, variola virus strain Afghanistan 1970.

The A43R promoter contains the late consensus sequence TAAATG. To examine the expression kinetics of A43V5 BSC-1 cells were infected with vA43V5 either in the presence or absence of the DNA replication inhibitor, cytosine arabinoside (Ara C). At different times post infection, whole cell lysates were analyzed by SDS-PAGE and Western blotting. A band corresponding to the size of A43V5 was detected at 6 hours post infection and continued to accumulate up through 24 hours post infection (Fig. 3-2A). These results were compared with the well characterized, late expressed, A3 protein and found similar expression kinetics (Fig. 3-2A). Additionally, A43 went undetected in cells infected in the presence of Ara C (data not shown), which is indicative of VACV proteins expressed at the late stage of infection.

Inspection of the predicted amino acid sequence of A43 revealed three potential N-glycosylation sites that could account for the relatively slow electrophoretic migration of A43. To determine the state of glycosylation of A43, a whole cell lysate from vA43V5 infected cells was divided into portions that were untreated or treated with Peptide: N-Glycosidase F (PNGase F) or Endoglycosidase H (Endo H). PNGase F is capable of removing all types of N-linked oligosaccharides, whereas Endo H removes only high mannose and some hybrid types of oligosaccharides. Both glycosidases caused an increase in the mobility of A43V5 as determined by SDS-PAGE and Western blotting (Fig. 3-2B), consistent with N-glycosylation of the protein.

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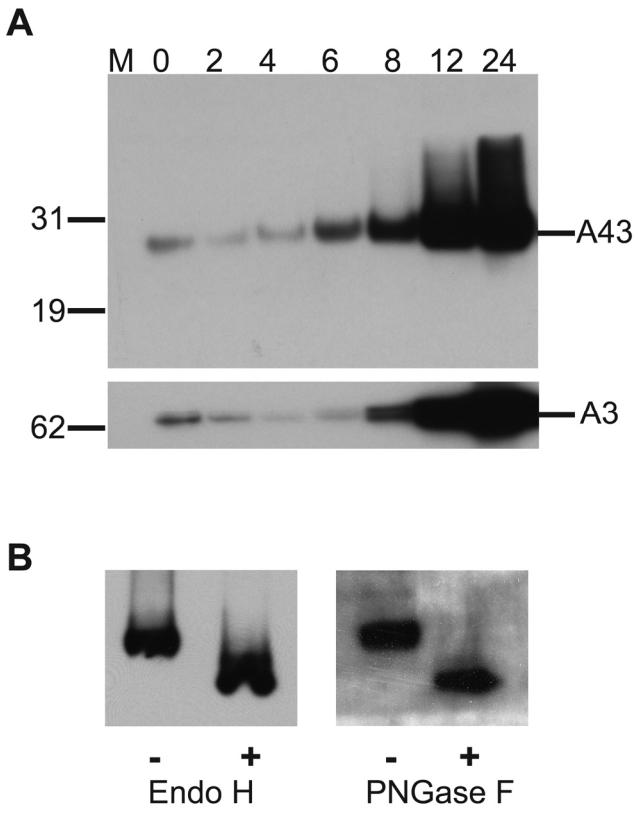

Figure 3-2. A43 synthesis and glycosylation. (A) Western blot analysis of A43 expression kinetics. BS-C-1 cells were infected at a multiplicity of 10 PFU per cell with vA43V5. At 0, 2, 4, 8 12 and 24 hours post infection whole cell lysates were analyzed by SDS-PAGE and Western blotting with an antibody to the V5 epitope tag. The blot was stripped and reprobed with an antibody to the VACV late protein A3. Position and mass in kDa of marker proteins are shown on the left. (B) vA43V5 infected cell lysates were treated with (+) or without (-) Endo H or PNGase F and analyzed by SDS-Page and Western blotting with an antibody to the V5 epitope tag.

3.3.3 A43 has Two N-linked Glycosylation Sites

To determine the number and the specific sites of glycosylation, three plasmids (pA43HAN65Q, pA43HAN93Q, pA43HAN114Q) were constructed to express the A43R gene under its natural promoter each with a C-terminal HA epitope tag and each carrying a mutation in one of the three predicted N-linked glycosylation sites. A fourth plasmid was also constructed to express A43R also under its natural promoter with an HA epitope tag but without any mutation present (pA43HA). An A43 deletion virus, vΔA43GFP (see methods) was also constructed for use in the subsequent infection/transfection experiments with the plasmids.

To confirm the expression of A43 with the mutations in each of the three N-X-S/T consensus sequences, BSC-1 cells were infected with vΔA43GFP and then transfected with pN65Q, pN93Q, pN114Q, or pA43HA. Cells were lysed and extracts were analyzed by Western blotting using an antibody to the HA epitope tag (Fig. 3-3A). If each of the N-X-S/T consensus sequences were actual sites for glycosylation then one would expect to see an increase in the mobility for each site that was disrupted by mutation as compared to the A43HA. Two of the three mutants, A43HAN65Q and A43HAN93Q, migrated faster than the intact A43HA. These data suggest A43 has two N-linked glycosylation sites. To confirm this finding, infected/transfected cell lysate was briefly treated with PNGase F and the protein migration patterns were analyzed (Fig. 3-3B). As anticipated, cleavage of 1-2 glycosylations was observed in each sample. Both A43HA and A43HAN93Q had similar patterns since neither had their glycosylation sites altered and both A43HAN63Q and A43HAN114Q had similar migration patterns to each other since

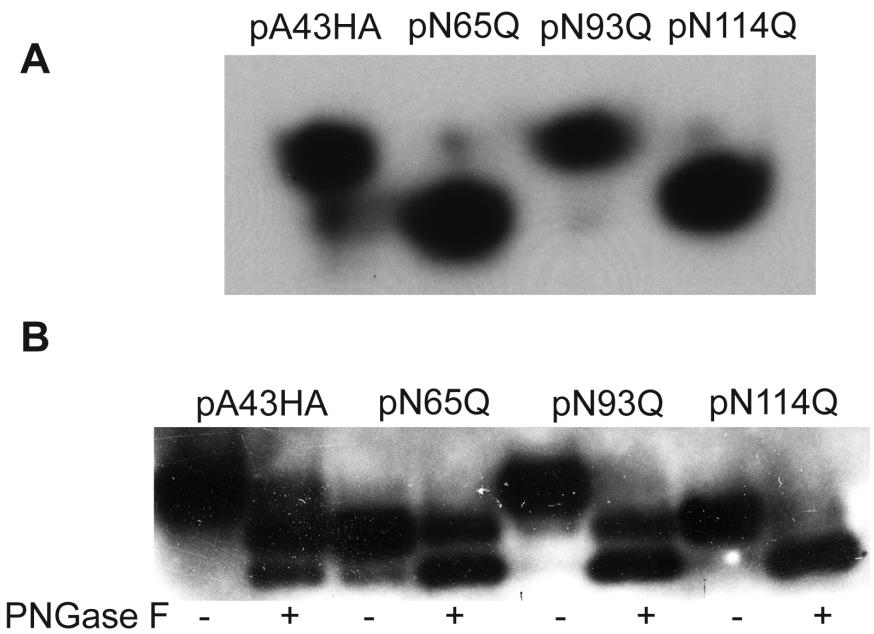

Figure 3-3. A43 has two N-linked oligosaccharides. (A) Expression of pA43HA, pN65Q, pN93Q, pN114Q. BS-C-1 cells were infected with vΔA43GFP and transfected with either one of four plasmids: pA43HA, pN65Q, pN93Q, pN114Q. Cell extracts were analyzed by SDS-PAGE and Western blotting with anti-HA antibody. (B) Partial (+) or mock (-) PNGase digestion of cell extracts 24 hours after infection with vΔA43GFP and transfection with pA43HA, pN65Q, pN93Q, or pN114Q. Digests were analyzed by Western blot with antibody to the HA epitope tag.

each had one site for glycosylation still intact. These results provide another piece of evidence to support the finding that A43 has two N-linked glycosylations.

3.3.4 A43 is Not Incorporated into the Virion

The recombinant virus, vA43V5 was utilized to determine whether A43 is a component of the MV or EV membrane. After RK-13 cells were infected with vA43V5, EVs were purified from the media supernatant on a CsCl density gradient and the MVs were purified by two sucrose cushions followed by a sucrose density gradient. Purified virions as well as whole cell extracts were analyzed by SDS-PAGE and immunoblotting. A43 was readily detected in whole cell lysate but not at all detected in EV extracts and just barely detectable in MV extracts (Fig. 3-4). Both membranes were stripped and re-probed with either the EV protein B5 or the MV core protein A3. B5 and A3 were detected in similar amounts in the EV extract and MV extract, respectively, as well as the whole cell lysate. The slight detection of A43 in MVs can be attributed to the small amount of cellular membranes acquired during virion morphogenesis or to contamination from cell debris during the purification process. Since there is a much higher detection of A43 in whole cell lysate it is presumed A43 is associated with cell membranes and not associated with the viral membranes.

3.3.5 The A43 is a Type-1 Transmembrane Protein Localizing to the Golgi Complex as well as to the Plasma Membrane

A43 was primarily detected in whole cell extract so the next step would be to determine the cellular localization of A43. Confocal microscopy was utilized to view

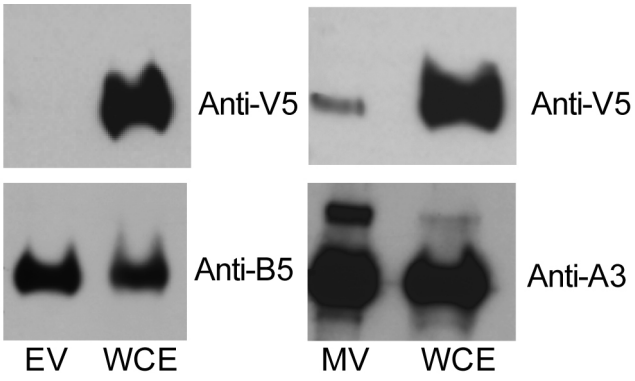

Figure 3-4. Distribution of A43 in EV and MV and whole cell extract. RK-13 cells were infected with vA43V5. EV and MV were purified from media or cell lysates, respectively (methods). Antibody to the EV protein B5 was used to compare the presence of the protein in EV with total cell extracts. Antibody to the MV core protein A3 was used to compare the presence of the MV protein with total cell extract. Antibody to V5 epitope tag on A43 was used to analyze the presence of A43 in EV, MV, and total cell extract.

cells infected with vA43V5. After infection, cells were fixed, permeabilized, and stained with an anti-V5 antibody followed by a fluorescently labeled secondary antibody. Infected cells were also stained with either DAPI or HOECHTS; both are known to form fluorescent complexes with DNA. This allowed for the visualization of cytoplasmic viral factories, the site of viral DNA replication and virion assembly, as well as for visualization of the cell nucleus. The A43V5 protein colocalized with β-cop, a specific Golgi complex protein, suggesting A43 localizes to the Golgi complex (Fig. 3-5).

Additionally, a plasmid expressing A43V5 under the cytomegalovirus promoter was transfected into uninfected cells and co-localization of A43 with a Golgi membrane marker was again visualized by confocal microscopy. These data indicate A43 has the ability to enter the secretory pathway independent of a viral infection (Fig. 3-6).

Confocal microscopy was also used to determine the topology of A43. vA43V5 infected cells were either left unpermeabilized or treated with digitonin to selectively permeabilize the plasma membrane. The V5 epitope tag on A43 was only detected in digitonin-permeabilized cells and not in unpermeabilized cells suggesting the V5 tag is cytoplasmic. The V5 tag was added to the C-terminus of the A43V5 protein therefore detection of A43 in cells permeabilized with digitonin provides evidence that A43 is a type-1 transmembrane protein. These data are consistent with the previous finding that A43 is glycosylated, as the N-X-S/T consensus sequence would have to be luminal for glycosylation modification to occur. As a control, cells were then stained with a specific antibody for the protein B5. B5 is a

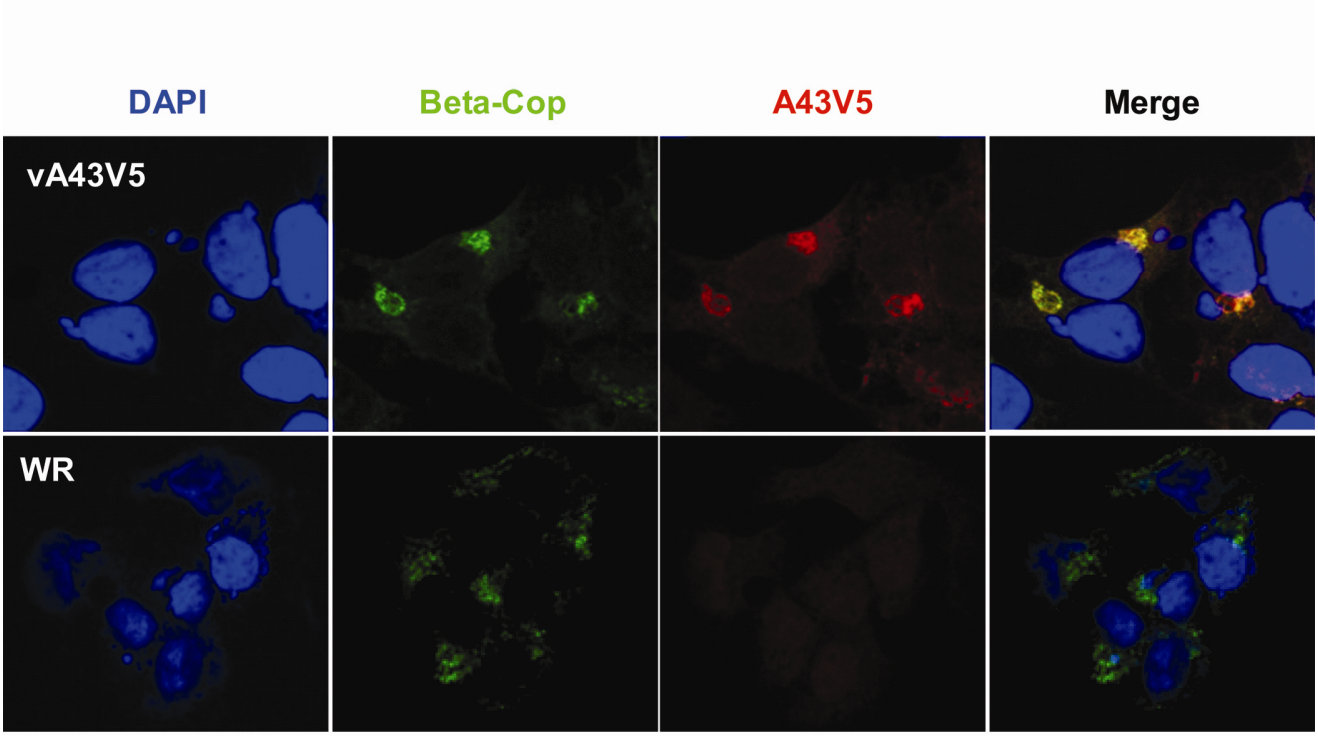

Figure 3-5. Intracellular localization of A43. HeLa cells were infected with VACV-WR or vA43V5 at a multiplicity of .05. 24 hours after infection, cells were fixed, permeabilized and stained with an anti-V5 antibody as well as an anti-Beta-Cop antibody followed by fluorescently labeled secondary antibodies (red and green respectively). DNA was stained with DAPI (blue). Confocal microscopy was used to view the samples.

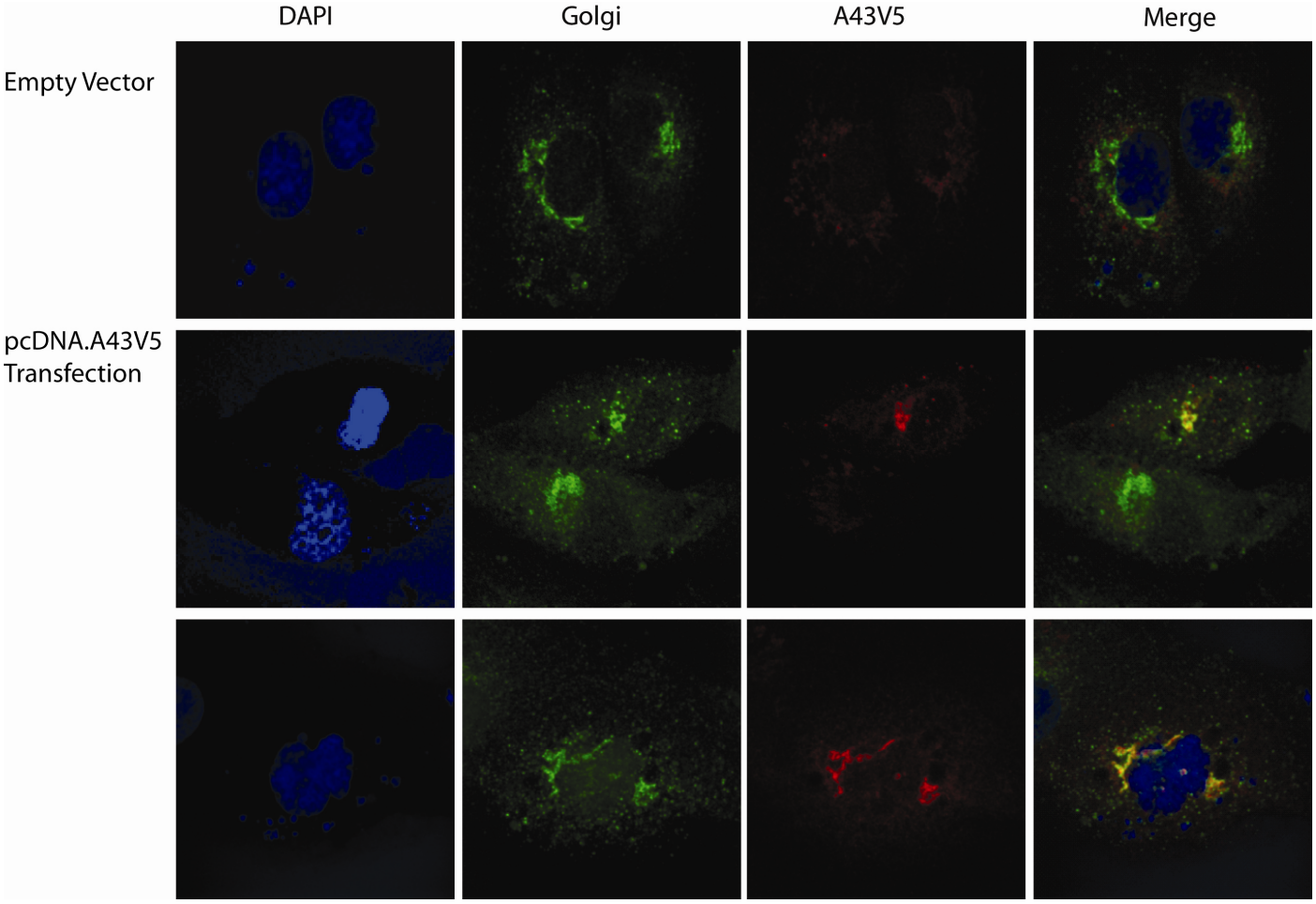

Figure 3-6. Intracellular localization of A43 in uninfected cells. BS-C-1 cells were either non-transfected or transfected with pcDNA.A43V5. 24 hours after transfection, cells were fixed, permeabilized and stained with an anti-V5 antibody as well as an anti-Beta-Cop antibody followed by fluorescently labeled secondary antibodies (red and green respectively). DNA was stained with DAPI (blue). Confocal microscopy was used to view the samples.

type-1 transmembrane EV protein that can also be found at the cell surface as well as in the Golgi complex. The antibody to B5 recognizes an epitope in the luminal, N-terminal domain of the protein [158] and is able to recognize B5 at the plasma membrane when cells are either unpermeabilized or digitonin-permeabilized (Fig. 3-7).

In addition to detecting A43V5 in the Golgi apparatus, A43V5 was also unexpectedly detected at the plasma membrane (Fig. 3-7). To confirm that A43 traffics through the secretory pathway the plasmid pA43HAΔTM was constructed in which the putative transmembrane domain of A43 was removed. BSC-1 cells were then infected with vΔA43GFP and transfected with the plasmid pA43HAΔTM. 24 hours post infection media supernatant was harvested and TCA precipitated at the same time as the infected cells were harvested and lysed. Samples were analyzed by SDS-PAGE and Western blotting. A43HA was only detected in the lysate while A43HAΔTM was detected in both the lysate and as a secreted protein in the medium (Fig. 3-8A).

In order to verify that A43 traffics to the plasma membrane during a VACV infection vA43V5 infected cells were surface labeled with biotin in the presence or absence of Brefeldin A. Brefeldin A inhibits the transport of proteins from the ER to the Golgi complex but does not interfere with production of MVs in a VACV infection [285]. An affinity pull down was then performed from the labeled cells and we found that A43V5 was indeed affinity purified from the cells that were not treated with Brefeldin A (Fig. 3-8B) suggesting A43V5 transits to the plasma membrane through the secretory pathway.

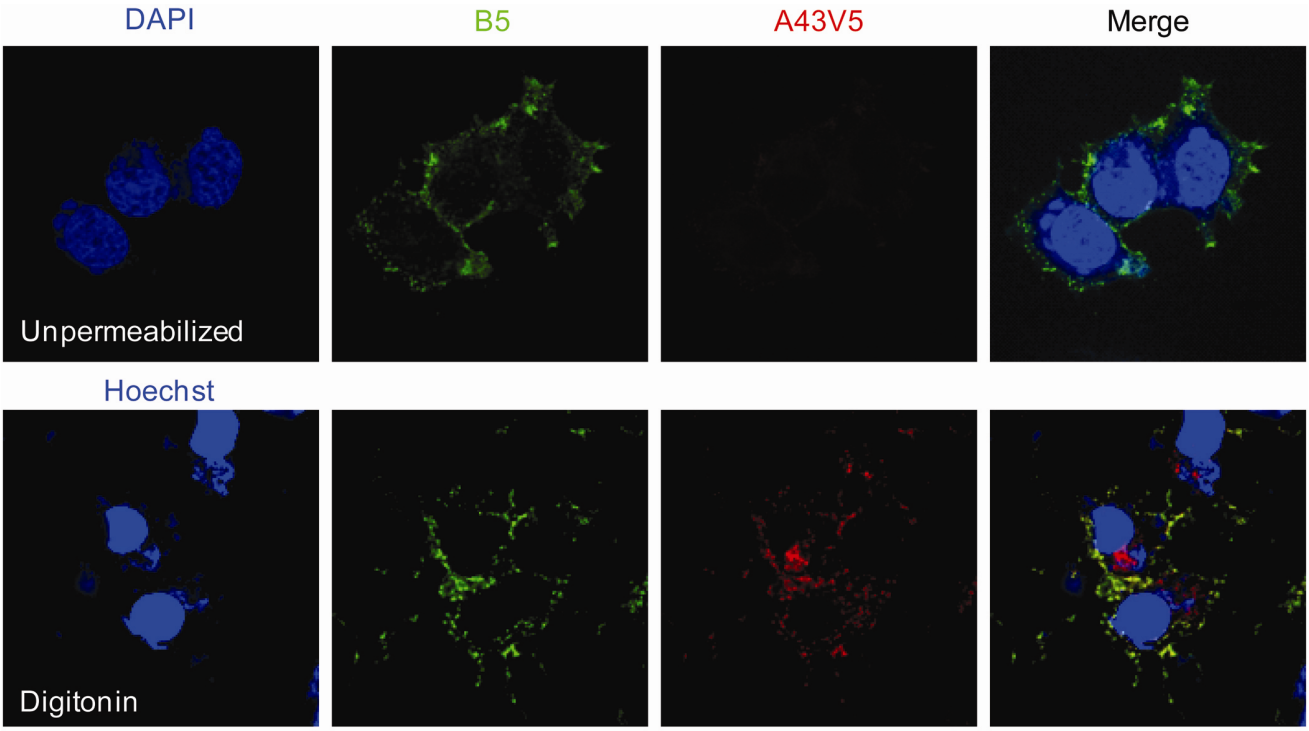

Figure 3-7. Topology of A43. vA43V5 infected HeLa cells were fixed and left unpermeabilized (top panels) or permeabilized with digitonin (bottom panels). Cells were then stained with an antibody to the EV protein B5 (green) as well as with an antibody to the V5 epitope tag (red) followed by fluorescently labeled secondary antibodies. DAPI or Hochest were used to stain DNA (blue). Confocal microscopy was used to view samples.

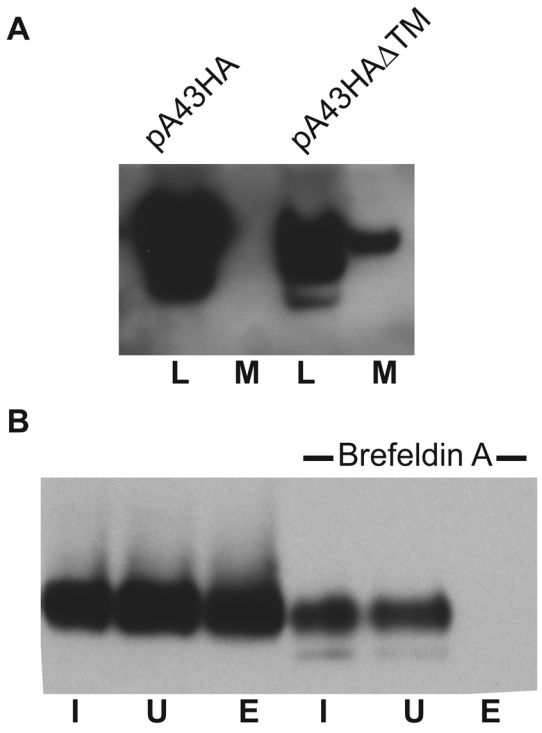

Figure 3-8. Intracellular trafficking of A43. (A) BS-C-1 cells were infected with vΔA43GFP followed by transfection with plasmids expressing full length HA-tagged A43 (pA43HA) or a truncated version missing the transmembrane domain (pA43HAΔTM). After 24 hours, cells lysates (L) and TCA precipitated media supernatant (M) were analyzed by Western blotting with an antibody to the HA tag. (B) HeLa cells were infected with vA43V5. 18 hours post infection, cells surfaces were labeled with a membrane-impermeable biotin reagent. Biotinylated proteins were affinity purified on NeutrAvidin beads and input (I), unbound (U), and bound and eluted (E) fractions were analyzed by Western blot with antibody to V5 tag.

3.3.6 A43 is a Non-Essential Protein, Dispensable for VACV Replication, Growth, and Cell-to-Cell Spread in Cultured Cells

To investigate the function of A43 in the virus life cycle an A43 deletion mutant was made from the VACV WR strain. A plasmid carrying a GFP ORF under the VACV P11 late promoter and between A43R flanking sequences was transfected into cells infected with VACV WR. GFP expressing plaques were readily detected, isolated, and purified suggesting A43 was deleted by homologous recombination and hence non-essential for viral replication. The deletion of A43R was confirmed by PCR and sequence analysis. BS-C-1 cells were infected with the parental VACV or the recombinant vΔA43GFP viruses and at different times after infection viral yields were determined and were not significantly different (Fig. 3-9A). Additionally, BS-C-1 cells were infected with the parental VACV or the recombinant vΔA43GFP viruses and 48 hours post infection cells were fixed and stained with crystal violet to view plaque formation, which appears similar between the two viruses (Fig. 3-9B). The deletion virus also had normal replication and spread in BHK, CV-1, HeLa, RK13 (Fig. 3-10), A549 cells (not shown) and primary human epidermal keratinocytes (not shown). Furthermore, all stages of morphogenesis appeared normal in vΔA43GFP infected cells as determined by transmission EM (Fig. 3-11).

An A43 deletion was also constructed in the IHD-J strain of VACV. This was done to determine if A43 has any involvement in the release of the virus from infected cells. The IHD-J strain is known for forming comet-shaped plaques due to a large number of EV released into the liquid medium [286]. The high number of EV

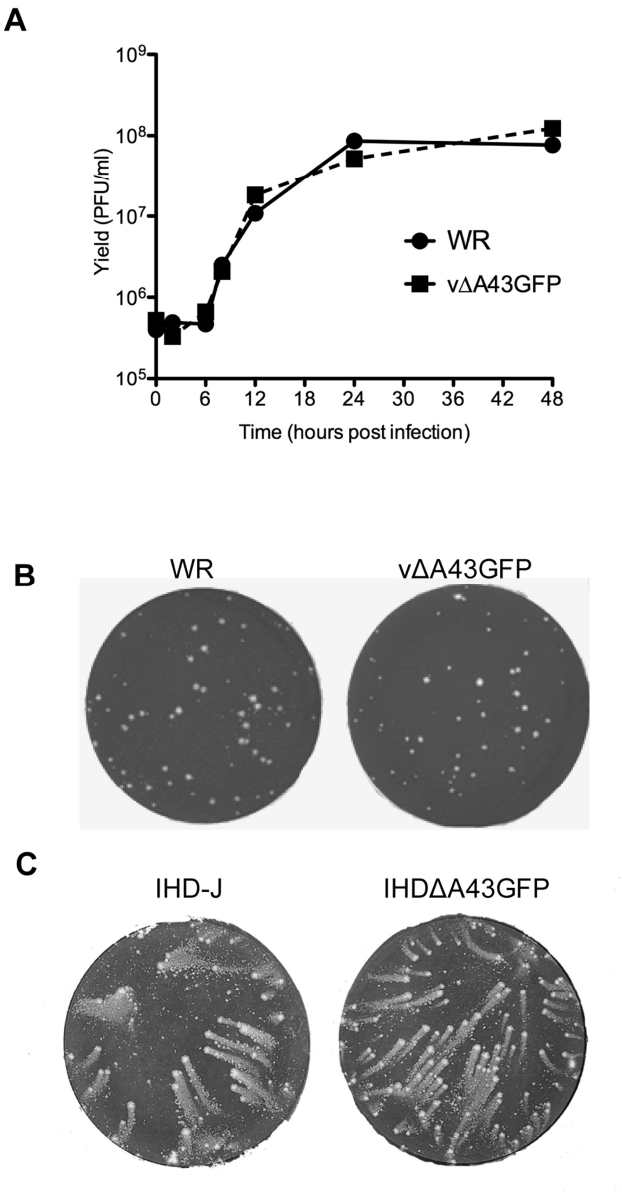

Figure 3-9. Replication of an A43R deletion mutant in tissue culture cells. (A) One-step growth curve. BS-C-1 cells were infected at a multiplicity of 10 PFU/cell with vΔA43GFP and VACV WR. Virus yields were determined at the indicated times post infection by plaque assay. (B) Formation of vΔA43GFP and VACV WR plaques. BS-C-1 cells were infected with vΔA43GFP and VACV WR. Cells were fixed and stained with crystal violet at 48 h after infection. (C) Formation of IHDΔA43GFP and VACV IHD-J plaques. BS-C-1 cells were infected with IHDΔA43GFP and VACV IHD-J. Cells were fixed and stained with crystal violet at 48 h after infection.

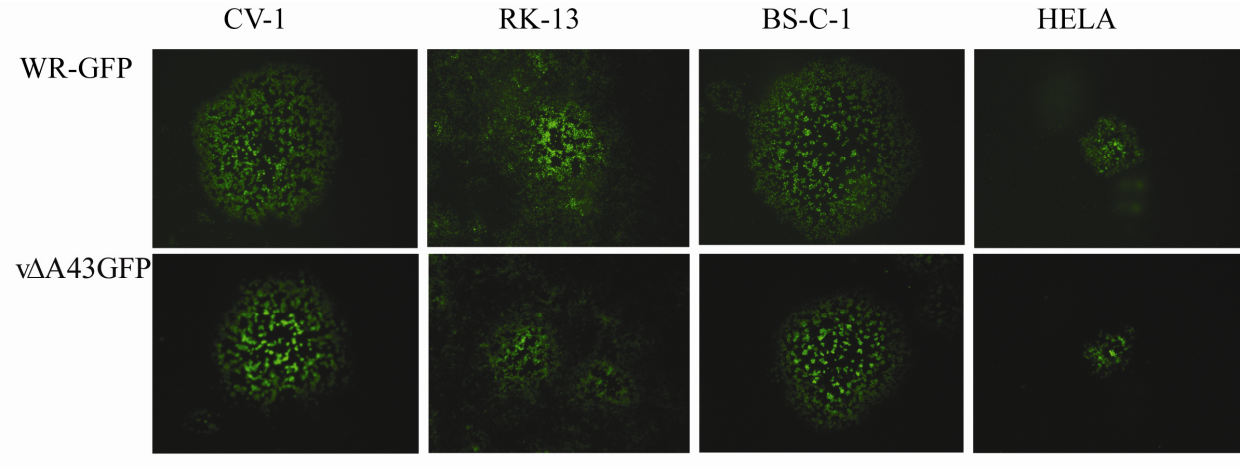

Figure 3-10. Plaque phenotype of WR-GFP and vΔA43GFP in different cell lines. Phase contrast images of individual plaques from either WR-GFP or vΔA43GFP infected cells. CV-1, (monkey); RK 13, (rabbit); BSC-1, (monkey); HeLa, (human).

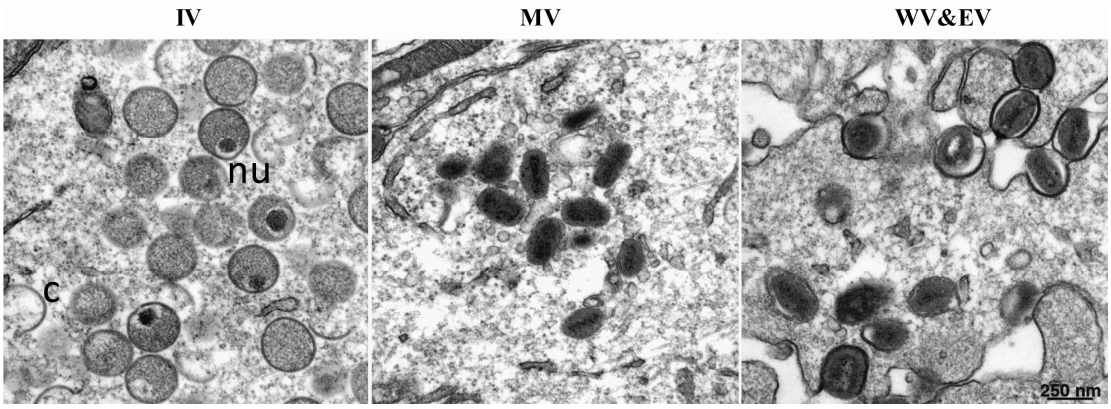

Figure 3-11. Transmission electron microscopy of cells infected with vA43Stop. BS-C-1 cells were infected with vA43Stop at a multiplicity of 5 PFU per cell. At 20 h after infection, the cells were fixed and prepared for EM. Electron microscopic images are shown with a 250-nm scale bar. C, crescents; NU, nucleoid; IV, immature virions; MV, mature virions; WV, wrapped virions; EV, enveloped virions.

released is due to a point mutation in the A34R gene [287]. However, deletion of A43 from VACV IHD-J strain had no effect on comet formation (Fig. 3-9C).

3.3.7 A43 is Required for Full Virus Virulence in a Murine Intradermal Model

In cell culture, vΔA43GFP did not exhibit a difference in virus yields as compared with WR. With this in mind and considering the placement of A43R in the viral genome, it was hypothesized that A43 is involved in virus virulence. Further characterization of vΔA43 in animals would require the construction of two additional viruses. A revertant virus was constructed replacing GFP ORF with the wild type A43R ORF (vA43Rev). This was necessary to verify that any effect seen with the deletion virus was caused by the desired gene deletion and not by some additional random mutation. The second virus needed was a non GFP-expressing A43 deletion virus where the GFP ORF was replaced by the A43V5 ORF which contained a point mutation at nucleotide 68, resulting in an immediate stop codon (vA43V5stop).

Two mouse models were used to determine the virulence of vΔA43V5 as compared to vA43Rev. Initially, the virulence in an IN respiratory model was evaluated [271]. At a dose of 10^4 PFU, weight loss between the two groups was similar (Fig. 3-12A) and all but one vA43Rev infected mouse survived (Fig. 3-12B). At a dose of 10^5 PFU, of the mice that received vA43Stop none survived and of the mice receiving vA43Rev, three of ten survived (Fig. 3-12B). None of the mice survived in either group with a dose of 10^6 PFU (data not shown). The second model used was an ID model that has been described previously [288-289]. This is an excellent model for genes that have a slight effect on viral virulence since the

infection remains localized to the ear pinna. Groups 5 BALB/c mice were anesthetized and challenged intradermally with 10^4 PFU of purified vA43V5stop or vA43Rev. Once lesions appeared, they were measured daily using digital calipers. Mice that received vA43stop had significantly smaller lesions over the entire course of the infection (p=.001; Mann Whitney test) than the mice that received vA43Rev (Fig. 3-13A). In C57BL/6 mice lesions produced by vA43V5stop were also smaller than lesions in the mice that received vA43Rev (p=.005) (Fig. 3-14). Balb/C mice had a larger difference in lesions size between the two viruses than seen with the C57BL/6 mice. Viral yields were quantified from Balb/C mice that had been intradermally infected with vA43V5 or vA43Rev. Despite the smaller lesions found in mice ID infected with vA43V5stop, there was no difference in viral yields (Fig 3-13B).

3.4 Discussion

This report is the first initial characterization of the VACV virus gene A43R. Although A43R is highly conserved amongst orthopoxvirus there are no non-poxvirus homologs or recognizable functional motifs to elucidate its function. It was found that A43 is a type-1 transmembrane protein that is expressed late in a VACV infection and is modified by the addition of two high-mannose or hybrid types of glycosylations. A43 did not associate with the virion but the protein localizes to both the Golgi complex as well as to the plasma membrane suggesting A43 may play a role in host interactions. Although A43 was not required for replication and growth in cell culture deleting A43 from VACV yielded significantly

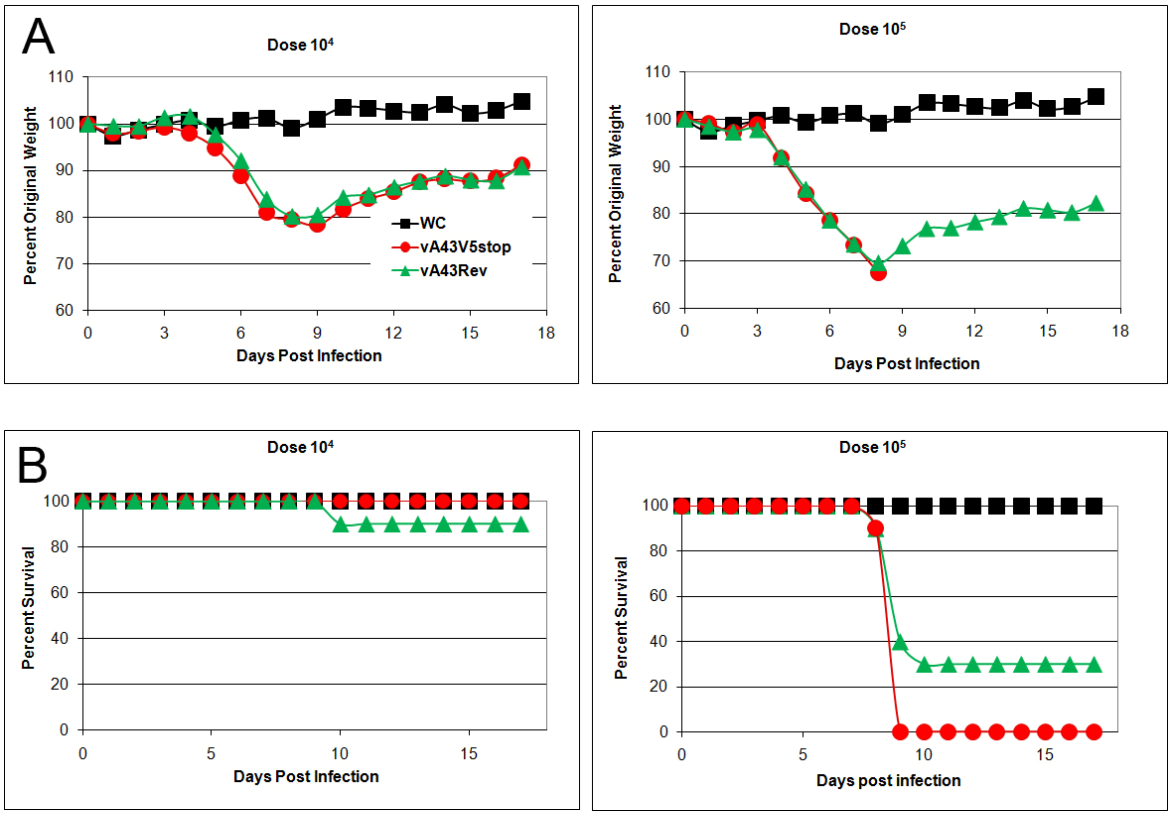

Figure 3-12. Virulence of A43R null mutant after respiratory infection. Groups (n = 10) of 7-week-old mice were inoculated via the IN route with 10^4 or 10^5 PFU of VACV. Mice were weighed daily for 2 weeks following challenge and were euthanized when they lost 30% of their initial body weight. (A) Percentage of original weight of mice. WC, untreated and uninfected weight control. Symbols in panels A and B are the same. (B) Percent survival of mice.

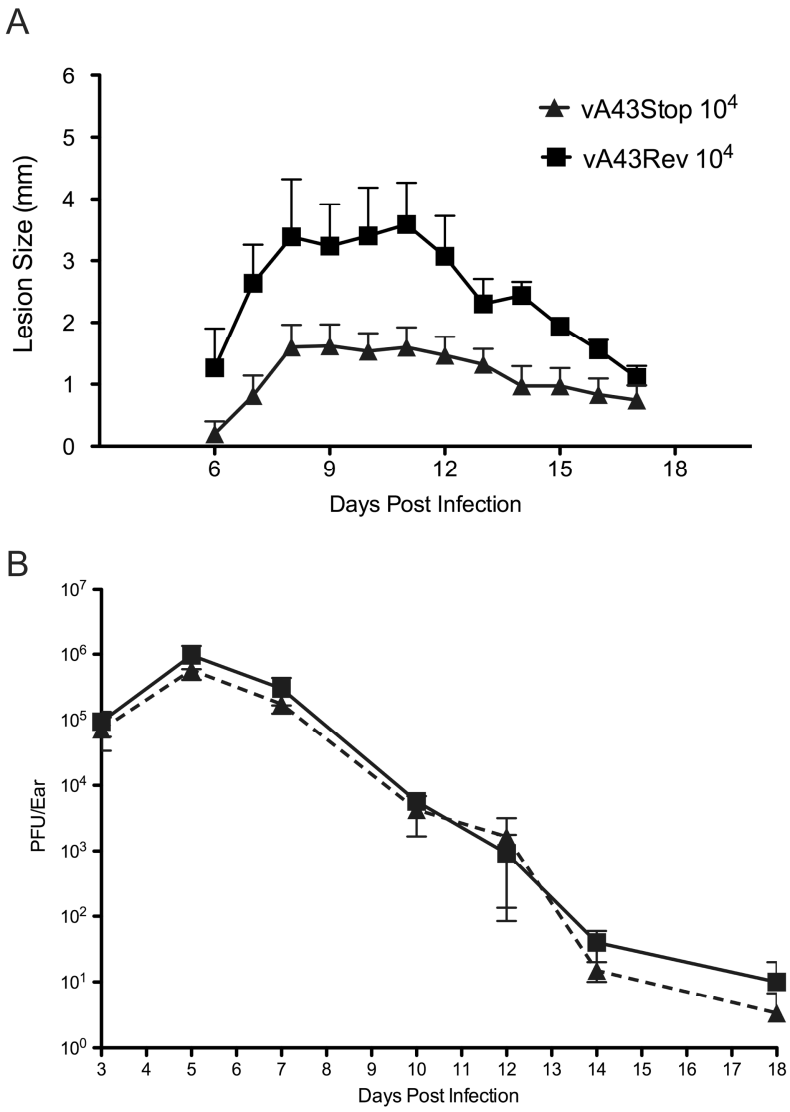

Figure 3-13. Intradermal replication and lesion formation by A43R null mutant in Balb/C mice. (A) Groups of Balb/C mice were infected intradermally in the ear pinnae with 10^4 PFU of vA43Rev or vA43V5Stop. Lesion sizes were determined daily with digital calipers to the nearest 0.5 mm. Standard errors of the mean are shown. (B) Mice were sacrificed and virus titers were determined from three individual ears by plaque assay on BS-C-1 cells. Standard errors are shown.

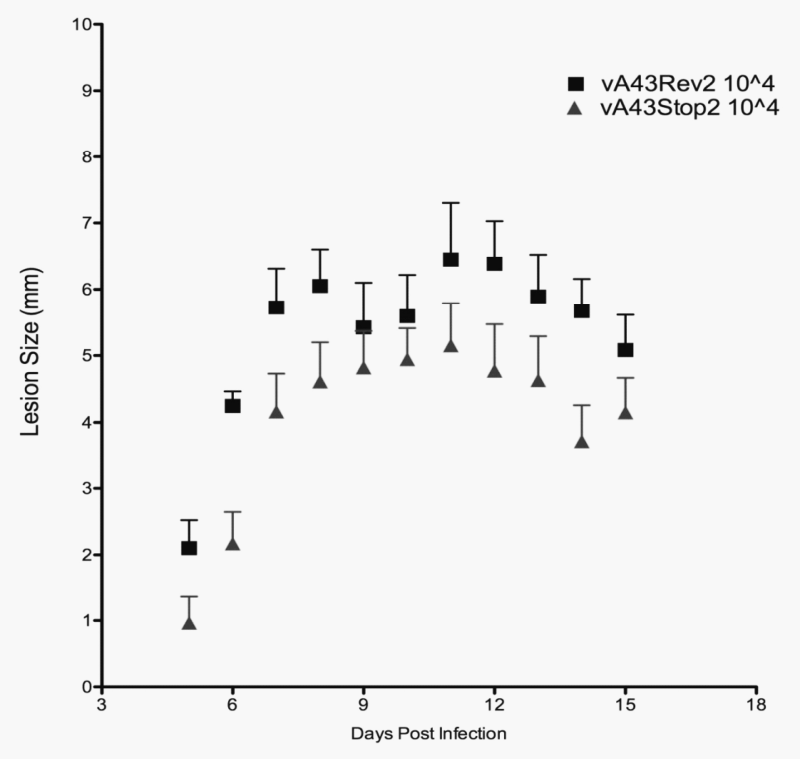

Figure 3-14. Intradermal lesion formation by A43R null mutant in C57BL/6 mice. (A) Groups of C57BL/6 mice were infected intradermally in the ear pinnae with 10^4 PFU of vA43Rev or vA43V5Stop. Lesion sizes were determined daily with digital calipers to the nearest 0.5 mm. Standard errors of the mean are shown.

smaller lesions in a murine ID infection model suggesting A43 is required for full virus virulence. A43 is not the first VACV protein that, when deleted, reduces virus virulence but not viral replication. Similar to A43 is the B7R gene which when deleted also produced smaller lesions in the ID mouse model and also had no difference in viral titer [256]. A40, also found at the plasma membrane but not in EV [254], was also reported to produce smaller lesions [290] but there are no reports indicating a reduction in viral replication.

The bulk of the A43 protein is found within the lumen of the Golgi complex as well as in the extracellular environment of the cell. It is possible that A43 is sequestering a host defense molecule within the Golgi complex preventing its secretion and/or at the same time interacting with that or another host defense protein at the cell surface and thus preventing it from inducing a signaling cascade. Although able to see a small effect on virulence after infection of mice with the vA43V5stop virus, it is possible that A43 might be more host specific. The full effects of the deletion are difficult to see since the animal model used is not the natural host for VACV. It might be interesting to try deleting A43 from the ectromelia virus and then look at the outcome of infection in mice.

Chapter 4: Conclusions

4.1 Conclusion

There are two main objectives for elucidating the strategies used by viruses to inhibit and to manipulate the host responses to infection. First, the knowledge will enhance our understanding of viral pathogenesis. Second, insight into how viruses manipulate and inhibit certain host responses can lead to further comprehension of those particular host pathways. There is, however, no precise systematic method for identifying virulence genes. The multifaceted actions of the immune response to a viral infection cannot be reproduced in tissue culture and although inspection of the viral genome may lead to suspicious candidates there are often virulence genes with no identifying markers.

Historically, attenuated viruses obtained experimentally were often found to harbor multiple mutations. It was often difficult to find the contributory factor to an attenuated phenotype considering all essential viral genes are also virulence genes and one would have to ascertain the genes specifically involved in host immune modulation and not just viral replication. Methods used now for predicting immune modulators are easier, however, once an immune modulating gene is determined, further studies must be done to in order to define its mechanism of action. This only adds to the complexity of studying such genes. Different methods have been utilized for identifying immunomodulators in the poxviruses such as, computational comparisons, which reveal sequence homology between VACV proteins and host proteins; ligands sought for predicted secreted proteins; screening for biological

activity of proteins secreted from infected cells; and a reverse genetics approach which allows for phenotypic analysis following the deletion of a gene from the viral genome.

The work presented in this dissertation includes the first account of two VACV genes that had not previously been described: I5L and A43R. Through this initial characterization, both I5 and A43 were found to be important for the virulence of VACV. My project focused on discovering the characteristics of I5 and A43 that could aid in the eventual elucidation of these proteins functions.

The major conclusions regarding I5 are that the protein (i) is conserved in all chordopoxviruses but not in non-poxviruses; (ii) contains no obvious functional motifs; (iii) is a late protein expressed after viral DNA replication; (iii) is specifically associated with the MV membrane but not with EV nor cell membranes; (iv) is dispensable for replication in tissue culture cells; (vii) null mutant are attenuated in mice by IN route.

Considering its high conservation in all chordopoxviruses and association with immature and MVs it was expected that I5 would contribute to the VACV life cycle, however, it was not essential for replication in cell culture and even more surprising it was necessary for full virus virulence in mice. Lung titer data from IN inoculation with the I5L frameshift mutant revealed that the virus was able to initially infect and replicate in the lung but progression was limited and the virus was cleared more rapidly than the control revertant virus. Given that I5 is on the surface of the MV, it is entirely possible that I5 may be involved in cell tropism since only a limited number of cell lines were tested for infectability. In addition, the 18-amino acid

sequence located between the two transmembrane domains may be important for interaction with a cellular protein and initial studies to test cellular binding of I5 have been favorable.

The major conclusions regarding A43 are that the protein (i) is conserved in all orthopoxviruses but not other poxvirus genera or non-poxviruses; (ii) contains no obvious functional motifs; (iii) is a late protein expressed after viral DNA replication; (iii) is not specifically associated with MVs or EVs; (iv) contains N-linked oligosaccharides at two sites; (iv) concentrates in Golgi membranes and traffics to plasma membrane; (v) exhibits a type 1 membrane topology; (vi) is dispensable for replication in tissue culture cells; (vii) null mutant retains virulence in mice by IN route; and (viii) null mutant produces smaller ID lesions in mice.

Because of the predicted late promoter and transmembrane domain, it was anticipated that A43 would be an EV membrane protein and have a role in virus egress. Nevertheless, this was not the case as A43 was not associated with the EV and neither plaque size nor comet formation was altered. Indeed, with regard to tissue culture studies A43R null mutants were indistinguishable in every respect from the parental WR or IHD-J strain of VACV. In view of these results, it was suspected that A43 is likely to be involved in host interactions and that the null mutant would be attenuated in animal models. It was surprising therefore to find that the A43 null mutant was as virulent as the revertant in the mouse IN model. However, the null mutant made smaller lesions than the revertant in mouse ear pinnae when injected intradermally. The increased thickness of the lesion is due to proliferation of epidermal cells and leukocyte infiltration [291]. This study confirmed previous data

that replication of VACV in the ear pinna reaches a peak on day 4-5 and the titer is declining before the lesion reaches a maximal size several days later [291]. Despite the difference in lesion size, the titers of mutant and revertant viruses were similar to each other at all time points. The similar titers may be related to the observation that the same amounts of virus are produced when the inocula vary over a 100-fold range [291]. Unreduced titers and smaller lesions were also found with the B7R deletion mutant [256].

Taken together the above characteristics differentiate A43R from other replication dispensable genes. The majority of such genes have early rather than late promoters, which would seem to be generally advantageous if the product interacts with the host. The five previously characterized replication dispensable late genes consist of A14.5L [292], A39R [293-294], A55R [295], B7R [256], I5L [296] and F3L [297]. Except for A55R, absence of the gene resulted in attenuation in either the IN (A14.5, I5L) or ID (A39, B7R, F3L) mouse model. Loss of A55 actually increased skin lesion size. As pointed out above, the B7R and A43R null mutants exhibited decreased skin lesion size. The retention of virulence in the IN mode by the B7R null mutant was also similar to that of the A43R mutant [256]. There are, however, substantial differences between B7 and A43; B7 localizes in the lumen of the ER whereas A43 is a type 1 membrane protein in Golgi and plasma membranes. It had been suggested that B7 might sequester a cell protein in the ER, though that has not yet been demonstrated. Although A43 might also act by sequestering a cell protein in the Golgi apparatus, its presence on the plasma membrane suggests that it interacts with a soluble protein, perhaps a cytokine.

The coexistence of virus and host has imposed selective pressure on both the virus defenses and the host offense. 15% of VACV expressed proteins are directly involved immune modulation and therefore add to virus virulence and pathogenesis. Some of these proteins are structurally similar and functionally similar to host proteins, some are structurally similar but function in an unexpected capacity, and some have completely novel structure and function. At the same time, the host has developed an astonishing system able to attack viruses and virally infected cells.

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