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CHARACTERIZACION OF A16L AND G9R VACCINIA VIRUS GENE PRODUCTS

Tesis

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Por

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Director de Tesis Dr. Bernard Moss

DEDICATORIA

Es cubano todo americano de nuestra América.

"No hay más goces reales en el mundo que el amor fiel de la casa, y la amistad en los pocos hombres buenos." José Martí

Gracias a mi tata, mis abuelos y mis padres por estar ahí siempre, por inculcarme los valores con los cuales hoy vivo pero sobre todo por decirme siempre se puede.

"Amor es delicadeza, esperanza fina, merecimiento, y respeto." José Martí

Gracias Mario por tu paciencia, dedicación y ayuda. Por aquellos comentarios, las discusiones científicas y el acertado consejo cuando más lo necesitaba. Gracias por todos estos años de dedicado e incondicional apoyo a mi carrera científica.

"Enseñar, que es lo más bello y honroso del mundo." José Martí

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"El mundo es fuerte y bello por los amigos." José Martí

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"Ser culto es el único modo de ser libre." José Martí

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Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la Tesis de Doctorado presentada por la candidata

SUANY OJEDA FERNÁNDEZ

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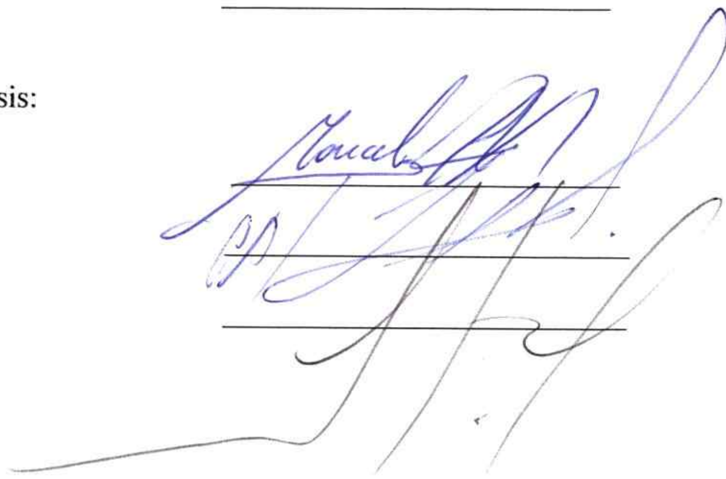

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ABSTRACT

Poxviruses comprise a large family of enveloped DNA viruses that replicate in the cytoplasm of infected cells. Among the members of this virus group are variola virus, the causative agent of smallpox and vaccinia virus, which was used as a vaccine strain against smallpox. Vaccinia virus, the best-studied member of the Poxviridae, has a double stranded-DNA genome of approximately 190 kbp encoding for nearly 200 polypeptides.

The assembly of vaccinia is a multi-step process resulting in formation of two major infectious forms: the mature virus (MV) and the extracellular virus (EV), which contains an additional outer membrane. The mechanism used by vaccinia virus to enter cells is not completely understood yet. It involves a putative entry-fusion complex comprised of eight proteins that are incorporated in the viral membrane common to both the mature and extracellular forms. Two of these proteins are G9 and A16, which have common structural characteristics such as conserved cysteines and C-terminal transmembrane domain. In order to investigate their role in virus entry, I constructed two recombinant vaccinia viruses in which the synthesis of A16 or G9 protein is regulated by an inducer. The induced A16 and G9 proteins were found exposed on the membrane of mature infectious virions. Under conditions in which the expression of either G9 or A16 was repressed, the yield of infectious virus, as well as the plaque size, was greatly reduced. Attempts to isolate a mutant virus in which the A16L or G9R genes were deleted failed, suggesting that both proteins are essential for replication and have non-redundant functions. In cells infected with the A16 and G9 mutants in the absence of inducer, all stages of virus morphogenesis appeared normal and extracellular virions were found on

the cell surface. Purified virions which were assembled in the absence of inducer, appeared morphologically normal but had very low infectivity, whereas their overall polypeptide composition including other components of the entry-fusion complex were similar to that of virions made in the presence of inducer or of wild type virions. A16 or G9-defficient virions were capable of binding to cells, however, penetration of cores into the cytoplasm and early viral RNA synthesis were barely detected. A16 and G9 proteins, as well as the other proteins comprising the complex, were required for virus-induced syncytium formation and cell-to-cell spread of extracellular virions. The requirement for so many proteins to enable poxvirus entry into cells and membrane fusion is unique among animal viruses.

RESUMEN

La familia Poxviridae la conforman virus DNA con membrana cuyo ciclo replicativo ocurre en el citoplasma de la célula. Entre los miembros más conocidos de esta familia se encuentran los virus de la viruela y el virus vacuna, el cual fue usado como cepa viral para la campaña de erradicación de la viruela. El virus vacuna, el miembro más caracterizado de esta familia, tiene un genoma de DNA de doble hebra de aproximadamente 190 kilobases y codifica cerca de 200 polipéptidos.

El ensamblaje del virus vacuna consiste en una serie de eventos que resultan en la formación de dos tipos principales de partículas infecciosas: los virus maduros (MV) y los virus extracelulares (EV) contienen una membrane externa adicional. Sin embargo, el mecanismo por el cual estas partículas entran a la célula aún se desconocen. Recientemente, un complejo de ocho proteínas fue aislado e identificado como possible complejo de entrada y fusión viral. Algunas de estas proteínas son incorporadas en la membrana viral común tanto en las formas maduras como en las extracelulares del virus vacuna. Dos de estas proteínas, identificadas como A16 y G9, comparten similares características estructurales como un dominio transmembrana en su extremo C-terminal y numerosos residuos conservados de cisteínas. Con el objetivo de investigar su papel durante la entrada del virus a la célula, se construyeron dos virus recombinantes en los cuales la síntesis de la proteína A16 o G9 está regulada por medio de un inductor. En los virus maduros (MV) purificados a partir de células infectadas con cada uno de estos virus recombinantes en condiciones permisivas, ambas proteínas fueron incorporadas en la superficie de la membrana viral con su dominio N-terminal hacia afuera de la partícula. En condiciones donde la expresión de A16 y G9 fue reprimida, se obtuvo un

menor tamaño de placa y una menor cantidad de partículas infecciosas. Esta observación nos sugiere que tanto la proteína A16 como la proteína G9 podrían no ser esenciales para la replicación viral. Sin embargo, no fue posible de aislar virus mutantes con deleción en estos genes, indicándonos que ambas proteínas son esenciales para la replicación viral y sus funciones durante el ciclo viral no son redundantes. En células infectadas con el mutante A16 o G9 en ausencia del inductor, todos los estadíos del ensamblaje viral ocurrieron normalmente y viriones extracelulares se observaron en la superficie de la célula. Las partículas virales ensambladas en ausencia del inductor presentaron una morfología normal pero resultaron ser menos infectivas. Por el contario, su composición polipeptídica incluyendo otras proteínas del complejo entrada-fusión fue similar a la de los viriones ensamblados en presencia del inductor o a la de los viriones tipo silvestre. Los viriones deficientes en A16 o G9 fueron capaces de unirse a la membrana celular pero sus cores no pudieron entrar a la célula y la síntesis de RNA viral temprano apenas fue detectada. Las proteínas A16 y G9 también fueron necesarias para la formación de sincicios inducida por el virus vacuna y para la propagación célula a célula mediada por los viriones extracelulares. Dicha correlación entre entrada viral y fusión también fue observada en algunas de las proteínas que componen el complejo entrada-fusión. La interacción específica entre componentes de dicho complejo con las proteínas A16 y G9 fue demostrada mediante ensayos de immunoprecipitación con anticuerpos específicos para proteínas del complejo. El requerimiento de tantas proteínas para permitir la entrada de los poxvirus a la célula y permitir la fusión de membrana es un evento único entre los virus animales.

INTRODUCTION

1. Virus structure.

Vaccinia virus (VACV), the prototype member of the family Poxviridae, was used extensively in the past as the smallpox vaccine. Poxviruses comprise a large family of DNA viruses that replicate and assemble in the cytoplasm of vertebrate and invertebrate cells. VACV strain Western Reserve (WR) contains approximately 200 genes packed in a double-stranded linear DNA genome of 190 kbp connected at each end by hairpin loops (Goebel et al., 1990). The virion was described as "brick-shaped" with slightly rounded edges and dimensions of approximately 300 nm long (Fenner et al., 1989). The outer surface is composed of a lipid-protein layer that surrounds the core. The lipid membrane contains more than a dozen viral proteins, none of which are glycosylated (Moss et al., 2001). The viral core is biconcave (dumbbell-shape) with two lateral bodies, and consist of viral proteins enclosing the genomic DNA. The major components of such cores are the gene products of A10L, A3L, L4R, A4L and F17R as well as enzymes required for early transcription (Jensen et al., 1996).

2. Vaccinia virus life cycle.

Vaccinia virus coordinates the processes of genome replication and virion assembly through a temporal cascade with the transcription of each gene class being dependent upon prior expression of genes of the previous class (Figure 1). Thus, three classes of genes: early, intermediate and late are expressed once the virion core is uncoated. The early class of genes represents approximately half of the virus genome and encodes enzymes required for DNA replication, initiation of intermediate transcription and host

defense (Broyles, 2003). Synthesis of early mRNAs takes place inside the core by enzymes and cofactors packed within the virion, and they are extruded through pores in an ATP-dependent manner (Munyon et al., 1967; Kates and McAuslan, 1967). Early viral messengers move away from cores and organize into discrete granular structures (Mallardo et al., 1991). Subsequently, cores are uncoated and genomic DNA is released to the cytosolic sides of ER membranes where DNA synthesis initiates (Joklik et al 1964; Van der Meer et al., 1999). Once DNA replication starts, the synthesis of intermediate messenger RNA begins. These are translated into factors that allow transcription of late genes (Keck et al., 1990). The major virion structural proteins and components of the early transcription apparatus packaged within new virions are products of late mRNA translation.

All the major events mentioned above as viral DNA replication, transcription and progeny assembly, occur in discrete areas in the infected cell called viral factories, located near the cell nucleus (Joklik and Becker, 1964). These factories are dynamic structures with a sponge-like architecture, in which specific domains become active sites for intermediate and late RNA transcription and translation (Katsafanas, G; unpublished data). Viral and host proteins as well as cellular membranes are recruited to these areas (Oh et al., 2005; Risco et al., 2002).

Vaccinia virus morphogenesis is a complex process, producing multiple distinct forms of infectious virus from each infected cell (Dales et al., 1961; Morgan et al., 1954). The first viral structure discernible by electron microscopy is a crescent-shaped membrane with an electron-dense granular viroplasm in the concavity. The membrane encloses the granular material to form a spherical immature virion (IV) (Figure 2). The IVs particles

undergo additional maturational processes such as genome condensation and proteolytic cleavage of viral core proteins, thus forming the infectious brick-shaped mature virion (MV). The MV particle is surrounded by a single lipid bilayer (Figure 2) (Carter et al., 2005) and contains approximately 75 viral proteins (Chung et al., 2006). Most of the MV remain within the cytoplasm of the intact cell and are released upon cell lyses, bud through the plasma membrane or in the case of cowpox virus, become occluded in A-type inclusion body (ATIs). ATIs are proteinaceous bodies appearing late in infection (Ichihashi et al., 1971) and are composed predominantly of a single polypeptide (Patel et al., 1986). Some MV particles are wrapped with membranes derived from the trans-Golgi network (TGN) or endosomal cisterna to form the wrapped virions (WV) (Figure 2) (Hiller and Weber, 1985; Tooze et al., 1993). The wrapping is mediated by the interaction of A27 protein on the MV surface and B5 and F13 proteins previously loaded on the TGN membranes (Rodriguez and Smith, 1990; Duncan and Smith, 1992). The number of MVs that undergo this second wrapping varies according to cell type and virus strain. The WVs are transported on microtubules to the cell periphery and exit the cells by fusing their outermost membrane with the plasma membrane (Moss and Ward, 2001; Smith et al., 2002). The released enveloped virions can either remain cell associated (CEV) (Blasco and Moss, 1992) or released to the medium (EV) (Figure 2) (Appleyard, et al., 1971). Although EVs represents less than 1% of infectious progeny, they are biologically important due to their role in virus dissemination by facilitating cell-to-cell spread to adjacent and further distal cells by actin tails (Blasco and Moss, 1992; Roper et al., 1998). The EV thus contains the underlying MV surrounded by an additional membrane with a unique set of six viral proteins. Of these, four proteins (B5,

A56, A33 and A34) are glycoproteins, and another (F13) is a nonglycosylated peripheral membrane protein (Duncan et al., 1992). Their functions during viral cycle are diverse. While F13 and B5 proteins are required for MV wrapping A33, A34 and A56 are involved in subsequent steps including actin tail formation, intracellular movement and virus spread (Smith et al., 2002).

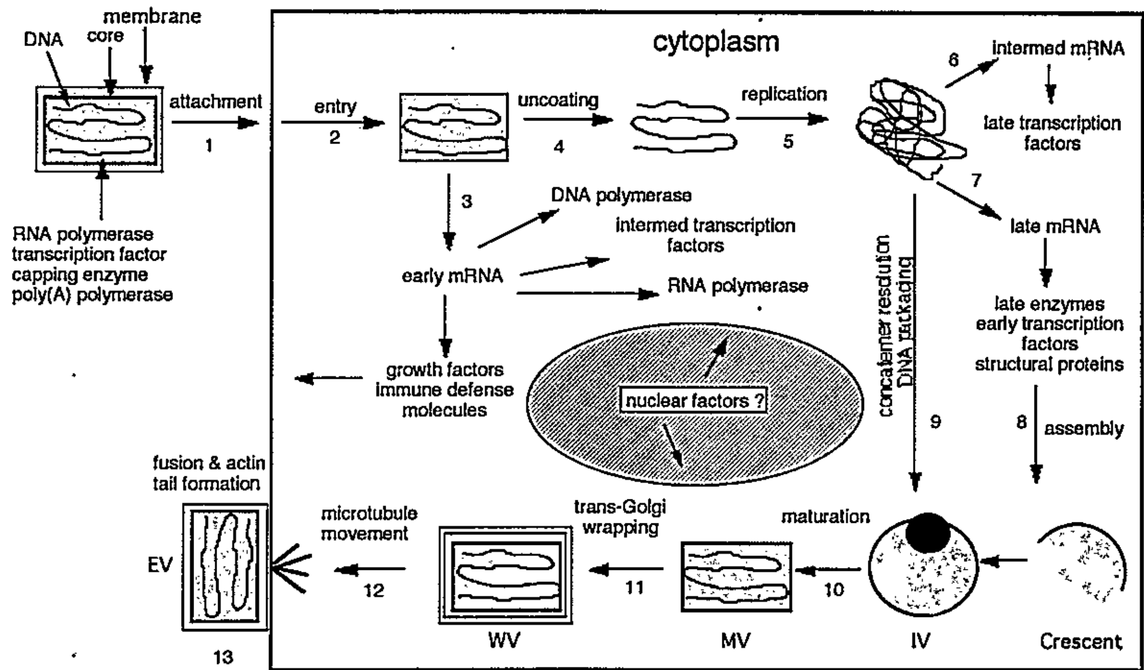


Figure 1. Vaccinia virus life cycle. Different forms of particles: crescents, immature virions (IV), mature (MV) and enveloped virions (EV). From Fields

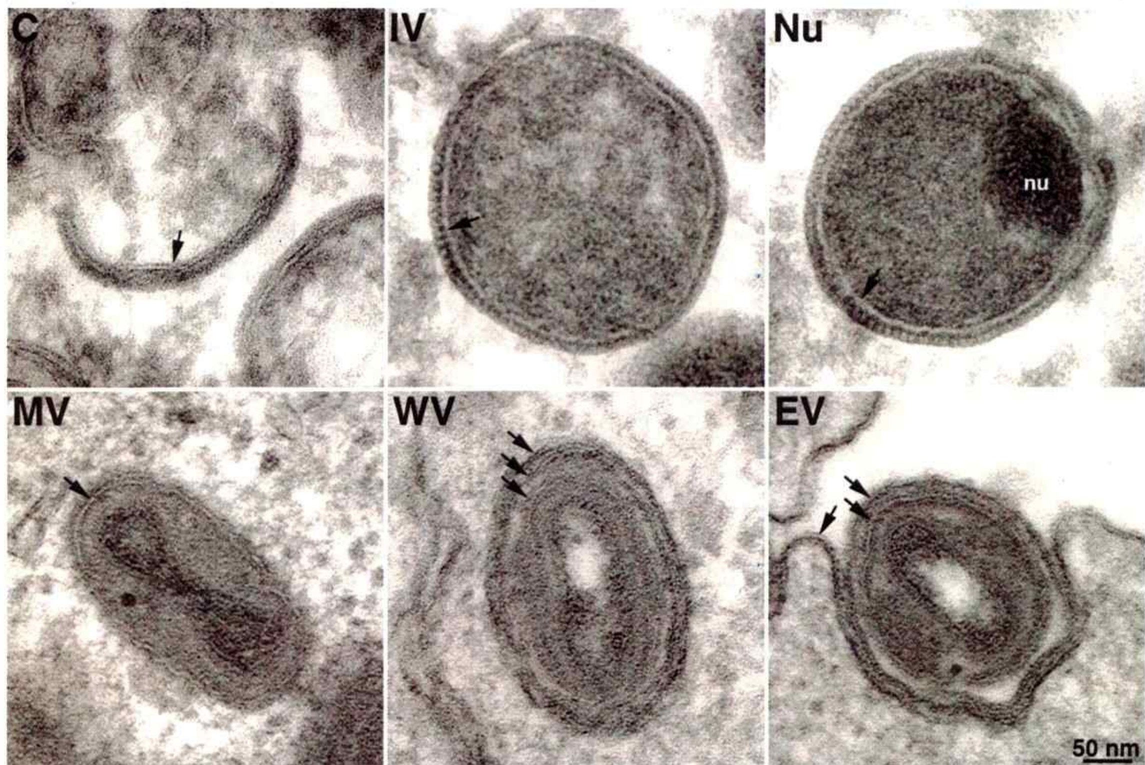


Figure 2. Transmission electron micrographs of different forms of VACV. Crescent membranes, immature particles (IV) with nucleoids (Nu), mature (MV), wrapped (WV) and enveloped virions (EV). Arrow points to: the single membrane of MV, the two outer wrapping membrane of WV, the wrapping membrane that has fused with the plasma membrane and MV membrane and remaining wrapper of EV. Electron microscopic images were kindly provided by Andrea S. Weisberg.

3. Vaccinia virus entry into cells.

As for most viruses, cell entry is the first step in the invasion of host cells. Nevertheless, controversial data in the literature and the existence of two major infectious forms (MV and EV) have complicated our understanding of the mechanism of poxviruses entry. This process comprises three major steps: attachment, activation of the fusion stage and membrane fusion.

MVs attachment appears to be mediated by binding of virion membrane proteins to the cell-surface glycosaminoglycans (GAGs). GAGs are complex structures with different carbohydrate moieties linked to core proteins through serine residues (Kjellen, L et al., 1991). Most cell types express GAGs such as condroitin sulfate or heparin sulfate to different extents and their biological role are quite diverse. They have been used as mediators of virus infections (Jackson et al., 1996; Shieh et al., 1992). In vaccinia virus infection, the binding of A27, H3 and D8 MV surface proteins to GAGs allows virion attachment. Viral envelope A27 and H3 proteins have been reported to bind to heparin sulfate (Chung et al., 1998; Vasquez et al., 1999; Lin et al., 2000) and D8 binds to chondroitin sulfate (Hsiao et al., 1999). However, the significance of this interaction for virus infectivity is uncertain. Mutant viruses defective in A27, D8 or H3 were still infectious and binding of MVs to GAGs was found cell-type specific and not essential (Carter et al., 2005). This suggests that virus binding to GAGs is the first step in the interaction of the virus with the cell, and additional viral and host molecules are then involved in virus penetration. In addition, it was found that a monoclonal antibody recognizing the L1 protein blocked MV entry at post binding step (Ichihashi et al., 1996; Wolffe et al., 1995).

The binding of EVs to cells has been more difficult to study due to their fragile outer membrane and the low number of EV made during viral infection. Recently, Smith and cols showed that dissolution of the outer EV membrane depends on interactions with cell surface GAGs and requires the specific EV glycoproteins A34 and B5 (Law et al., 2006). The ligand-induced nonfusogenic mechanism solves the topological problem associated with entry of double-enveloped forms (EV) by proposing the outer membrane disruption on the cell surface at neutral pH followed by fusion of the single MV envelope with the plasma membrane or the vesicle membrane.

After MV and EV binding, other specific interactions between viral membrane proteins and receptors located on the cell surface drive the fusion process. Although VACV MVs and EVs attach to cells differentially, the nature of these receptors remains largely unknown (Vanderplasschen et al., 1997). In addition, MVs but not EVs induce signaling cascade and the production of actin-containing cell surface projections (Locker et al., 2000). A role of lipid rafts has also been suggested due to the inhibition of MV entry by cholesterol depletion of the plasma membrane (Chung et al., 2005). Although MVs and EVs may use different receptors, the entry of such particles require a final fusion event that allows the entry of cores into the cytoplasm.

3.1 Entry of MV particles to the cell.

Many findings suggest an endosome and plasma membrane entry pathways of vaccinia virus into cells. Evidence for MVs membrane fused with the plasma membrane at neutral pH was provided by electron microscopy (Armstrong et al., 1973; Chang and Metz, 1976; Carter et al., 2005) and by the dispersal of fluorescent dye when prelabeled MVs were used (Doms et al., 1990), although nonfusion mechanism has also been

suggested (Dales and Kajioka, 1964; Payne and Norrby, 1978). Moreover, blocking of MV entry and cell fusion by methyl-ß-cyclodextrin, a raft-disrupting agent, suggested a possible requirement of cholesterol-rich membrane microdomains for fusion at the plasma membrane triggered by virus-receptor interactions at neutral pH (Chung et al., 2005). The involvement of low pH step is controversial. Vanderplaschen et al found that drugs that raised the intracellular pH did not reduce MV infectivity (Janeczko et al., 1987; Chillakuru et al., 1991; Vanderplaschen et al., 1998). On the other hand, a brief exposure to low pH was found to accelerate MV penetration (Ichihashi et al., 1996), perhaps by synchronizing the fusion between the viral and the cellular membranes (Townsley, unpublished).

The presence of one or two membranes in the MV particles has important implications with regard to the mechanism of MV entry. The two membranes model would be inconsistent with a fusion mechanism because it would result in release enveloped viral particle into the cytoplasm, instead of a naked functional core (Griffiths et al., 2001). However, an alternative nonfusion mechanism has been proposed due to the observation of MVs with disrupted membranes near the cell surface (Locker et al., 2000; Sodeik et al., 2002). In such scenario, free cores could enter the cytoplasm through putative open pores in the membrane layer. On the other hand, different microscopical techniques support a single lipid bilayer model (Hollinshead et al., 1999; Cyrklaff et al., 2005; Heuser, 2005). This model could topologically explain a one-to-one membrane fusion event for delivery of the MV core into the cytoplasm.

3.2 Entry of EV particles to the cell.

EVs particles, with one additional membrane to that of MVs, are likely to enter cells by a different mechanism. The presence of two membranes and the fragility of the outer one complicate our understanding of EV entry. Since there is a discrepancy in the scientific literature of whether EV penetration is affected by low pH, where the fusion takes place and how drugs affecting actin can prevent the entry process. Ichihashi proposed that after EV particles are endocytosed, the outer envelope is destroyed within acidified endosomes, and the exposed MV membrane fuses with the endosomal membrane thus delivering the cores into the cytosol (Ichihashi et al., 1996). This model is supported in the inhibition of EV entry by drugs that raise the pH of intracellular vesicles (Vanderplasschen et al., 1998). However, other evidences suggest that low pH is not required for EV entry (Doms et al., 1990; Krijnse et al., 2000; Payne et al., 1978). Moreover, electron microscopy observations have shown that MVs fused with the plasma membrane beneath a disrupted EV wrapper (G.L, Smith personal communication; J. Heuser, personal communication). In addition, a recent report by Smith et al showed that the outer viral membrane is disrupted by a ligand-induced nonfusogenic reaction, followed by the fusion of the inner viral membrane with the plasma membrane and the subsequent delivery of viral cores into the cytoplasm (Law et al., 2006). However, due to the fragility of the outer EV membrane, the method used to obtain the EV preparation should be considered.

4. Features of vaccinia virus A16L and G9R open reading frames.

The availability of the complete genome sequences of several distantly poxviruses species allows the identification of genes that are conserved and probably have an essential role in virus replication. Two of these are A16L (WR 136) and G9R (WR 089)

open reading frames, which are conserved but their function is still unknown. Orthologs of these proteins are present in all poxviruses sequenced to date, but no nonpoxvirus homologs were detected by a position-specific iterative BLAST search. The A16L and G9R were predicted to encode 43.4 and 38.9 kDa proteins respectively. A typical vaccinia virus late promoter precedes their N-terminal sequences and a TAAATG transcription initiation element is overlapping the translation initiation codon (Senkevich et al., 1997). Both ORF have similar structural features that are conserved in all poxviruses: fourteen (G9R) or twenty (A16L) cysteine residues, a site for N-terminal myristoylation and a C-terminal transmembrane domain. Secondary structure analysis showed that they have a predicted N-terminal region on the MV surface, and a short C-terminal within the virion. A16 and G9 are structurally related to J5, although their low sequence similarity (Senkevich et al., 1997). Furthermore, both G9 and A16 are myristoylated (Martin et al., 1997). The cysteine-rich domain of A16, G9 and J5 are homologous, suggesting that they evolved by duplications in the ancestral poxvirus genome (Senkevich et al., 1997). The conservation of so many cysteins residues through the poxvirus family suggests that they may have a role in the function and/or stability of the protein. Nevertheless, the questions of whether they form intramolecular disulfide bonds and if so are formed through the vaccinia virus-encoded cytoplasmic redox pathway remains unknown (Senkevich et al., 2000).

HYPOTHESIS AND SPECIFIC AIMS

Hypothesis: Vaccinia proteins A16 and G9 are essential for virus replication.

Main goal: Characterization of the proteins encoded by G9 and A16 vaccinia genes and determining their role during viral replication.

Specific aims:

1- Determine whether A16L and G9R open reading frames are expressed during viral infection.

- Analysis of protein synthesis
- Kinetics of expression and requirement for prior DNA synthesis.

2- Characterization of the A16L and G9R ORF protein products.

- Determine the size of the proteins
- Investigate the presence of intra- or intermolecular disulfide bonds
- Study mode of formation of disulfide bonds
- Locate the A16 and G9 proteins within virus particle and in the infected cell.

3- Study role of A16 and G9 protein in viral replication.

- Determine whether A16L and G9R gene can be deleted
- Construct and analyze the phenotype of an inducible A16 and G9 virus mutant
- Follow interactions of the above proteins with other viral or cellular proteins

MATERIALS AND METHODS

1. Cells and viruses.

BS-C-1 cells (ATCC CCL6), grown in Eagle's minimum essential medium (Quality Biological Inc) supplemented with 2.5% fetal bovine serum, were used for all experiments unless otherwise indicated. The VACV WR strain and the recombinant viruses vT7acOI (Alexander et al., 1992), vA16i and vG9i were propagated in HeLa S3 cells as described previously (Brodder CC and Earl, PL, 1997) and stored at -80°C.

2. Generation of recombinant virus vA16i and vG9i.

vT7lacOI, used as parental virus for both recombinants, contains the E. coli lac repressor gene and the bacteriophage T7 RNA polymerase gene regulated by a VACV late promoter and the Escherichia coli lac operator (1). DNA containing (i) the left and right flanking regions of the each ORF, (ii) a copy of the A16L or G9R gene regulated by a T7 promoter and lac operator and (iii) the gene encoding ß-glucuronidase or enhanced green fluorescent protein (GFP) under a synthetic early late VACV promoter was prepared by overlapping PCR. The inducible G9R ORF was inserted in its natural site but in the opposite "L" orientation to avoid RNA polymerase read through from neighboring genes. The PCR product was transfected into cells infected with vT7lacOI and recombinant virus forming green fluorescent plaques was clonally purified. Relevant segments of the viral genome were amplified by PCR and sequenced to verify the construction.

3. Antibodies.

Mouse monoclonal antibody (MAb) to L1 (Wolffe et al., 1995) was prepared from a hybridoma kindly provided by A. Schmaljohn. Rat monoclonal antibody HA- HRP

conjugated (3F10) from LaRoche. Rabbit polyclonal antibodies to the following VACV proteins were used: anti-A4 (Demkowicz et al., 1992), anti-A14 (Betakova et al.,1999), anti p4b/4b (R. Doms and B Moss, unpublished), anti-A21 and -L5 (Townsley et al., 2005; Townsley et al., 2005), anti-H3 (da Fonseca et al., 2000). Gretchen Nelson provided rabbit antiserum to A28. Rabbit anti-A16 serum was produced by immunizing a rabbit with a synthetic peptide corresponding to amino acids 364 to 378 (SRPKIKTNDINVRRR) of the predicted A16L ORF with an additional cysteine residue for protein conjugation (Covance Research Products Inc., Denver, Pa). Proliferating cell nuclear antigen (PCNA) (rabbit, Santa Cruz SC-7907).

4. Western blot analysis.

Cells were lysed in SDS gel loading buffer (Invitrogen) containing reducing agent unless otherwise specified and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to a polyvinylidene difluoride membrane (Invitrogen) and the membrane was blocked overnight in 5% non-fat dry milk in phosphate buffered saline-T [9 g/l of NaCl and 0.01% (v/v) Tween 20]. The membranes were incubated with a 1:1000 dilution of anti-A16 serum and proteins were detected using a chemiluminescence kit (West-Pico, Dura or Femto; Pierce).

5. Confocal microscopy.

Infected HeLa cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), washed and then permeabilized with 0.1% Triton X-100 for 10 min at room temperature. The cells were incubated with the primary antibody or Alexa Fluor 594 phalloidin diluted in 10% complement-inactivated fetal bovine serum and PBS for 1 h at

37^0C. The cells were washed and incubated with the fluorophore-conjugated secondary antibody for 1 h at 37^0C. DNA was stained with diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen). Images were collected using a Leica TCS-NT SP2 inverted confocal microscope.

6. Detergent extraction of purified virus.

Purified virions were extracted with 50 mM Tris pH 7.4 containing 1% Nonidet P-40 detergent and 150 mM NaCl in the presence or absence of 50 mM dithiothreitol (DTT). The mixture was incubated for 1 h at 37^0C and insoluble and soluble material were separated by centrifugation at 12,000 x g for 30 min.

7. Partial trypsin digestion of virions.

Purified virions were incubated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-treated trypsin (Sigma) in 10 mM Tris-HCl pH 9 without or with 1% NP-40 and 140 mM NaCl. Samples were incubated for 1 h at 37^0C and then 1mM phenylmethylsulfonyl fluoride was added to stop the reaction. The supernatant and pellet fractions were separated by centrifugation 30 min at 4^0C and immediately boiled. Proteins were analyzed by western blotting as described above.

8. Disulfide bond analysis.

Cells were collected by centrifugation, solubilized in non-reducing SDS gel loading buffer (Invitrogen) containing 20 mM 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS; Molecular Probes) or N-ethylmaleimide (NEM). In some cases, the proteins were reduced with Tris-(2-carboxyethyl) phosphine (Invitrogen). Lysates were sonicated, heated to 100°C and analyzed by SDS-PAGE (10% polyacrylamide) in Tris-glycine buffer (Invitrogen). The proteins were transferred to a nitrocellulose membrane,

incubated with mouse anti-V5 IgG conjugated to horseradish peroxidase (Invitrogen) and detected by chemiluminescence.

9. Electron microscopy.

BS-C-1 cells were infected with 10 PFU of virus per cell for 1 h at 37°C and incubated in the absence or presence of 100 μM IPTG. The infected cells were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, washed in 0.1 M sodium cacodylate buffer, postfixed with reduced osmium tetroxide, and washed in buffer. Cells were dehydrated in a series of ethyl alcohol dilutions, 50, 70, and 100%, followed by incubation in propylene oxide. The cells were then embedded in EMbed 812. Sections were obtained using the Leica Ultracut S ultramicrotome. Thin sections were stained with 7% uranyl acetate in 50% ethanol and then with 0.01% lead citrate and analyzed on the Philips CM100 transmission electron microscope.

10. Northern blot analysis.

BS-C-1 cells were infected with 5 PFU per cell of VACV and harvested after 3 h. RNA was extracted with the RNeasy Mini kit (Quiagen) and 3 μg of denatured RNA was separated by electrophoresis on a 1.2% glyoxal agarose gel (Ambion). The RNA was transferred to a nylon membrane (Amersham) and the blots were hybridized with an [α-^{32}P]dCTP probe that had been labeled using the DECA prime kit (Ambion), washed and subjected to autoradiography.

11. Fusion from within.

BS-C-1 cells were infected with 2 PFU of vA16i or vG9i viruses for 18 h in the presence or absence of 50 μM IPTG followed by a brief incubation with phosphate buffer pH 7.0

or 5.0 for 2 min at 37^{0}C. Buffer was replaced with culture medium, DNA was stained with Hoescht and cells were fixed with 4% paraformaldehyde.

12. Fusion from without.

BS-C-1 cells were inoculated with either 200 PFU of purified +A16/ +G9 virions or the corresponding OD260nm of -A16/-G9 virions at 4^{0}C for 1 hour. Cells were exposed to pH 7.0 or 5.0 for 2 min, buffers were replaced with media containing 300 µg/mL of cycloheximide and incubated for 3 hours at 37^{0}C. Cells were fixed and stained with Alexafluor 594 phalloidin or DAPI to visualize actin filaments and DNA respectively.

13. Surface biotinylation of purified virions.

Purified virions were reacted with 10 mM sulfosuccinimidyl-2(biotinamido) ethyl-1,3-dithiopropionate (sulfo-NHS-SS-biotin; Pierce) in 1X PBS for 1 h at 4^{0}C. The reaction was quenched with 100 mM glycine and the virions were pelleted at 20, 000 x g for 30 min. The virions were incubated in SDS-PAGE loading buffer for 5 min at 85^{0}C and then loaded on to a NeutrAvidin gel column (Pierce). The column was washed and the biotinylated proteins were released by incubating the gel with SDS-PAGE sample buffer with added DTT for 1 h at room temperature.

14. Immunoaffinity purification.

BS-C-1 cells were infected with 5 PFU of vV5A16i or vG9i in the presence or absence of IPTG or with control vT7LacOI. After 24 h infection, cells were lysed and the proteins in the NP-40-soluble fraction were incubated with agarose beads to remove proteins that bind nonspecifically. After this soluble fraction was incubated with anti-V5 MAb or anti-HA immobilized to agarose beads and incubated for 4 h followed by washes. The bound proteins were eluted in the presence of SDS loading buffer and heat

at 85^{0}C for 5 min. Eluted proteins were analyzed by SDS PAGE-silver staining and Western blotting. Samples before IP (pre-IP) or after IP immunoaffinity purification were analyzed by Western blotting using the V5 epitope tag and the A28, A21, L5-polyclonal antibodies describe above.

RESULTS

1. Characterization of the A16L vaccinia open reading frame.

1.1 A16L is a highly conserved essential gene.

The A16L open reading frame (VACV WR 136) is predicted to encode a 43.4 kDa protein. Orthologs of the protein are present in all poxviruses sequenced to date, but no non-poxvirus homolog were detected by a position-specific iterative BLAST search. The conservation of A16L orthologs in all poxviruses (Figure 3), including a VACV strain that was highly attenuated by in vitro passage (Antoine et al., 1998), suggested that the gene is essential for virus replication.

```
MCV  MG--QTLTALRVEDT-AVPGEKMLRVEYSRAADNTSVPFSEETQHFADDSL--PEIRPRFCLLPEMA-PAPCGG-F-------LTPALRKRYLLARGEQCRSLSFRPGSLITDTRPP---
FPV  MG--QHVSNITVIATPQAPETKYLRVEYTGGYDDEYIRFFEAENIHSGDIG--SEISPPFCLTRDTT-VKQCAS-F-------LSPEAKKKFVIVPGEPCKSLSFRPGS-ILDLQKI---
LSDV MGGSVSVTNLNVSKDLADYNKKYMFINFNYPEYNKIITFLEEQKIYNDDKK--SEILPNFCLTDNLK-ISHCGN-F-------VSDEISKKYILVKGDSCRSFSFRPGSMILYTNDITED
SPV  MGSYLSITNLKVEKDPVNYNKKYMFISFNYPEYDKTIGFYEELDTYTDENV--NDINPKFCLTDNID-VSYCGT-F-------LSNDLAKKYVLTKGNSCRSFTFRPGSMIIYDNDITED
MYX  MGGYVSVTDIKVRNDPGDYNKKQMFINFNYPEYNKVVSFYEDENIYRKEE---SEIHPKFCLTETMD-PSHCGT-F-------LSDELSKKYVLTRGEPCRSFTFRPGSFIIYNGDINER
YLDV MGSSVSITSLKVAPNLADQNEKYMFINFNYPEYNKLVSFYEEMGNYNNDTL--TEITPRFCLTDDLS-IDYCGS-F-------ISPTIAEKYILVKGNECRSFNFRPGSMIIFKDEITKD
VAC  MGAAVTLNRIKIAPGIADIRDKYMELGFNYPEYNRAVKFAEESYTYYYETSP-GEIKPKFCLIDGMS-IDHCSS-F-------IVPEFAKQYVLIHGEPCSSFKFRPGSLIYYQNEVTPE
MSV  MGGSVAINFLGTSNDNANGN-KDLIIGLTDGSRIRLKMYEQINTINPSEYAGKDEYEIEYCMASPYNGLGECAKLFNTDSSTTYAKELDNYIITNEGSSCMSLAFRPGSVLYETSDW---
AMV  MGGRVSISFIRPEN-NNNGN-KNLLIGLSDNNIVRVPMFEQINRIPRSAYENLDEYEINYCIGTPFNSLAECALLFNNNILSNYTSELDNYVITNEGSPCTSLTFRPGSILYGNSEW---

MCV  ------PKGVEQYVALGERCRFVRGDFLAQDRD-LARCCTQPNAQ-DCPERLRNGYSTPDCDAAMASFCAAQPGSAPCLAWLDTRRAP----ALAAYADICARDLDRSYCSQFVNVSR-P
FPV  ------PYGTESYVLDGTRCRFINIDYLYTDPD-IKRCCNKESDK-DCPEIFSNNYETDHCDTIMSSICLQTPGSLPCREWLEKKREV----AFDTYMKVCSDELDANYCSDFVDYTR-P
LSDV YLDNKIPESAKEYILKGTQCKFIKKDYFINDDD-IIKCCTNPSIG-NCPKKLNNEYQTSHCDNAMSFFCKSNPDNVQCLKWLRTKRKI----ALSTYTDICSNKMDKRYCSEFIRVVR-P
SPV  YIDNKLPDAAKEYISKGFQCRFIKKDYMVMDNK-LGECCSKPSD--ICPEILNNGYKTNHCDTFMVNFCKTNPDNSQCLIWLRQKRQI----ALSTYLDICSENMDQRYCSEFIRVVR-P
MYX  YIDNQIPDSAKAYIAKGYRCRFVKKDYMIMDKE-IEGCCTSPHA--GCPGHLNNGYVTSHCDTYMDPFCTANPGNSQCLTWLRTKRKI----ALETYSQICATEMEERHCSEFVRVAR-P
YLDV YIDTKLPAITEKFISKGYQCKFLKKDFIIDDSS-LVSCCTNLTNTQKCPSVLNNGYETSHCDLIMSNFCKRNPDSFQCLKWLRKKRKI----ALQTYSEICSDEMDQRFCSEFIRVVR-P
VAC  YIKDLK--HATDYIASGQRCHFIKKDYLLGDSDSVAKCCSK-TNTKHCPKIFNNNYKTEHCDDFMTGFCRNDPGNPNCLEWLRAKRKP----AMSTYSDICSKEMDARYCSEFIRIIR-P
MSV  ---------IKDRIFNGNKCKIIYNGPPIYEND-LLECCTGKRTE-NCNEKLINNFTTSHCNVTMQSYCENNFNDIYCYRWLESQTKLNNDIALKLYANLCSKNHIKEYCTYFCINSRNS
AMV  ---------LEGRTFVGNKCKIRYRGYPIYEND-LRECCTGKRTS-GCHETLINNFTTPHCNVTMQNFCRQNPEDLYCYRWMYSQSKT-FDIALKLYSELCSIDETKLYCDYMCVYAREN

MCV  EFFAYSDAAILQYCRTHRARREGWCVAPP-TPRVRNAEVFLGPRVCWLHECTDQSRDRKYLLFDQDVQRKKCQYVGCNITVDMLQLE-NASATLVSDCVGTDAP----GDESPGTRSAHV
FPV  DNFGYSDAAILSYCSKERNNPNCWCVTTPKNDKLFSLELALGPKVCWLHECTDKSKDRKYLLFDQDVQRTNCKYIGCNINVDTLRLR-NSVAELIAKCGGSIAEDTVLGDDSYNKEAKLP
LSDV NFFTFGDISLLSYCNKNKGNRNCWCVSPPN-NITF--DKYLGPRVCLLHECTDKTEDRKWLLYDQDIQRSRCKYIGCNININSLTLE-NSKIDLISDCSKNKNIIGDLDPGIPKAKKKRD
SPV  DFYTFGDAALINFCNKFKGNRNCWCVPPPNQTITS--EKYLGPRVCYLHECTDRTRDRKWLLYDQDIQRSRCKYTGCNININSLILE-NSKVDLISNCG-YKNINRDSDPGEPKNTSKYN
MYX  DYFTFADNALIKFCDRNKANKNCWCVTSPSKTITD--EKYLGPRVCWLHECTDKSKDRKWLLFDQDVQRSRCKYTGCSINVNELTLE-NSNADLVAECKGLKTVTGDIDPGVPKKPPPPK
YLDV EFYTFGDTALINFCKKHTANRNCWCVPPPN-NTLQ--QRFLGPKVCWLHECTDKTRDRKWLLFDQDVQRSRCKYTGCTININSLTME-NSKADLIANCYNNNSVIGDIDPGKPKKNNENK
VAC  DYFTFGDTALYVFCNDHKGNRNCWCANYPK-SNSG--DKYLGPRVCWLHECTDESRDRKWLYYNQDVQRTRCKYVGCTINVNSLALK-NSQAELTSNCTRTTSAVGDVHPGEPVVKDKIK
MSV  DYPGYCDIALENYCKNNYYNESCYCYNPPSNIIPN-VESVLGPKECWLDPCTTNYTNQKWLTTDQLSIKKSCAIQSCIITIAALNASGNSTINLINNCVEGASSSTEVQSQYISNKT-IP
AMV  GYPGYCDDSLSNWCKNNSNNSLCFCYNPPTEFIPD-VEEVLGPKECWLAPCTVSYSGQKWLTTNQMNIKKNCNIQSCIITIGSLLTRGENKIDLINNCINNLNASTVINSENLSNVTDIK

              TRANSMEMBRANE
MCV  P--ALP--VLPVTSALLAAAVLFYFLALYARRRVPDRPVRTRRR--
FPV  S--FFS--IIPVCIVLLCLFVLFYFLRIYDAKVINSNTINVYRK--
LSDV ---LPN--IIGFPFIFICLAVLFYFLVIYNRKKIKTNNINVRRR--
SPV  NIQTPN--ILEAVIIFIGISILFYCISVYHRKKINTNIINVRRR--
MYX  ---RPF--FFSFVISFICIAVLFYFVAVFYRKKIKTRDINVRRR--
YLDV Q-NFPFIFWYG-IITFVSLFILFYFIVIYSKKKIKTRDINVRRR--
VAC  ---LPT--WLGAAITLVVISVIFYFISIYSRPKIKTNDINVRRR--
MSV  VIQTWG-SLFD-PSLFIIFLALLFLILLMLLNYKPVLSL-------
AMV  INQTWG-VFFD-PVIFILIIFIFILIILYFYNKKPIYTINISETNL
```

Figure 3. Multiple alignment of A16 orthologs in poxviruses. One representative sequence from each genus of *Chordopoxvirinae* and the two complete *Entomopoxvirinae* are included in the alignment. MCV, molluscum contagiosum (*Molluscipoxvirus*); FPV, fowlpox virus (*Avipoxvirus*); LSDV, lumpy skin disease virus (*Capripoxvirus*); SPV, swinepox virus (*Suipoxvirus*); MYX myxoma virus (*Leporipoxvirus*); YLDV, Yaba-like disease virus (*Yatapoxvirus*; VAC, vaccinia virus; MSV Melanoplus sanguinipes entomopoxvirus (*Entomopoxvirus B*); AMV, Amsacta moorei entomopoxvirus (*Entomopoxvirus B*). Conserved cysteines are indicated in white with black background; other conserved residues are shown with a shaded background. The predicted transmembrane domain is indicated.

1.2 A16 is required for efficient virus replication.

Experiments were designed to determine the requirement of A16 protein for viral replication. First, we replaced the original A16L gene by homologous recombination with a GFP reporter gene under the control of a VACV late promoter. Indeed, our attempts to isolate a deletion mutant were unsuccessful. As an alternative, we made a recombinant virus called vA16i by using the *E. coli* lac operator system to regulate A16L transcription as previously described for other VACV genes (Zhang and Moss, 1991; Senkevich, TG 2000). As depicted in Figure 4A, vA16i contains the lac repressor expressed continuously by a VACV early/late promoter and the T7 RNA polymerase gene adjacent to a VACV late promoter, which is regulated by the lac operator. The A16L gene was modified so that it is driven by a bacteriophage T7 promoter and regulated by the lac operator. In the absence of IPTG, the lac repressor is expected to inhibit expression by binding to lac operators adjacent to the promoters of both the T7 RNA polymerase and the A16L ORFs. In the presence of IPTG, however, the repressor should be inactivated to allow expression of T7 RNA polymerase and transcription of the A16L ORF.

vA16i was clonally purified in the presence of 50 μM IPTG. The virus made tiny plaques in the absence of inducer and nearly normal size plaques in its presence (Figure 4B). In a one-step growth experiment, replication of vA16i was delayed and reduced by about 1.5 logs in the absence of inducer (Figure 4C). The presence of small amount residual virus bound to cell surface could explain the differences observed immediately after adsorption and washing. Since a mutant with the gene could not be isolated, the

low degree of replication could be due to incomplete repression of A16.

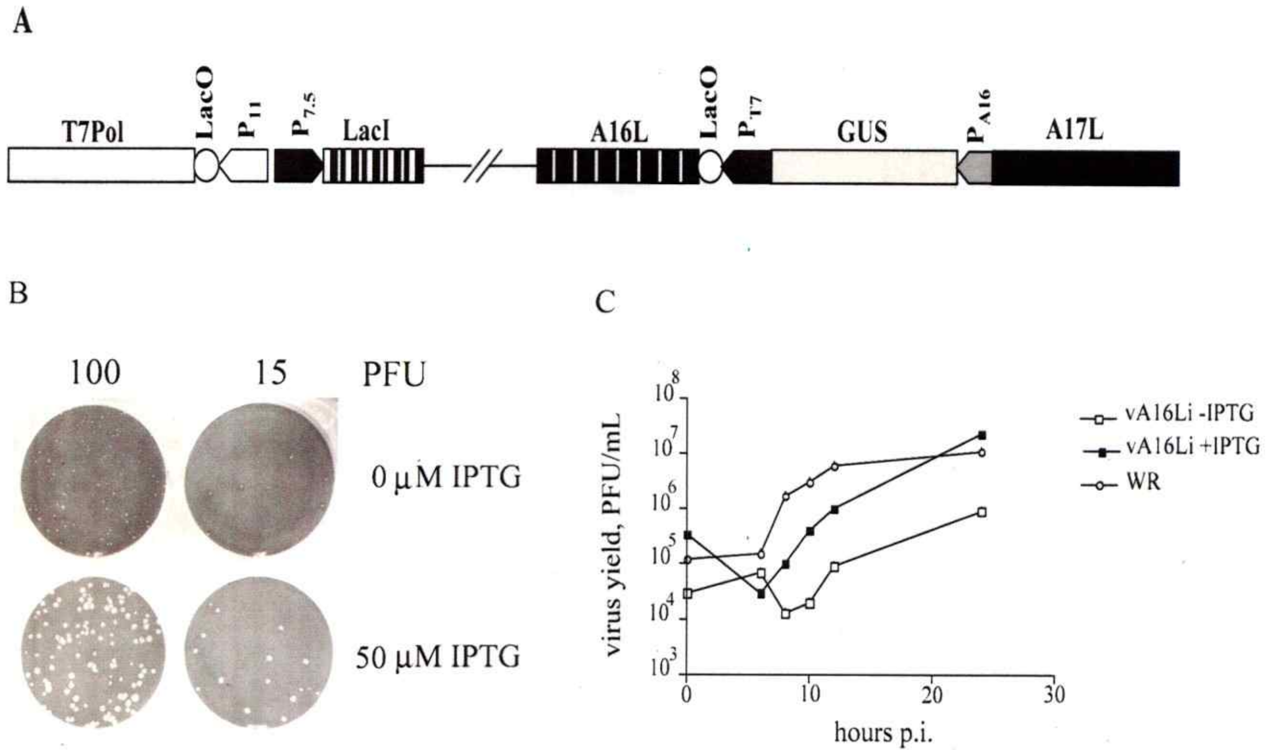


Figure 4. Construction of an inducible A16i VACV mutant. (A) The genome of vA16Li is shown. Abbreviations: P11, a vaccinia virus late promoter, P7.5, a vaccinia virus early-late promoter; *lacO*, *E.coli lac* operator; *lacI*, *E.coli lac* repressor gene; T7 pol, bacteriophage T7 RNA polymerase gene; PT7, bacteriophage T7promoter; GUS, b glucuronidase. (B) IPTG-dependence of plaque formation. Cell monolayers were infected with approximately 100 and 15 PFU of vA16Li and overlaid with medium containing 0 or 50 μM IPTG. After 37^0C for 48 h, the plates were stained with crystal violet. (C) Time course of virus production. BS-C-1 cells were infected with VACV WR or vA16i in the presence or absence of 50 μM IPTG. Viral yield at 0 time indicates after one hour virus adsorption. Cells were harvested at the indicated hours post infection (p.i.). Virus titers were determined by plaque assay in the presence of 50 μM IPTG.

1.3 Synthesis of A16 during a normal infection.

Antibody to a peptide representing the 15 amino acids at the C-terminus of A16 was generated in order to evaluate the effect of IPTG on expression of A16 and for further characterization of the protein. As shown by western blotting, a major polypeptide of ~43-kDa and a minor one of ~23-kDa increased with IPTG concentration (Figure 5A). The 43-kDa-protein is the size predicted from the A16L ORF. In the absence of IPTG, a trace amount of the A16 protein was detected. If very little A16 is needed, this could explain the formation of tiny plaques and the low degree of replication. At 50 μM IPTG, the amount of A16 was similar to that made under the control of the natural A16L promoter as shown for vT7lacOI (Figure 5A).

The characteristic TAAATG transcription/translation initiator element characteristic of late promoters is present at the start of the A16L ORF, suggesting that the protein is expressed at the post-replicative stage of VACV infection. To investigate A16 expression, cells infected with VACV were harvested at intervals and the proteins were analyzed by western blotting using the C-terminal peptide antibody. The major 43-kDa-polypeptide was detected at 6 h and accumulated over a 24-h period (Figure 5B). A faint 23-kDa species was also seen but neither the 43- nor the 23-kDa bands were detected when viral DNA replication was inhibited with AraC (Figure 5B), consistent with the kinetic data indicating that the A16L gene belongs to the late expression class.

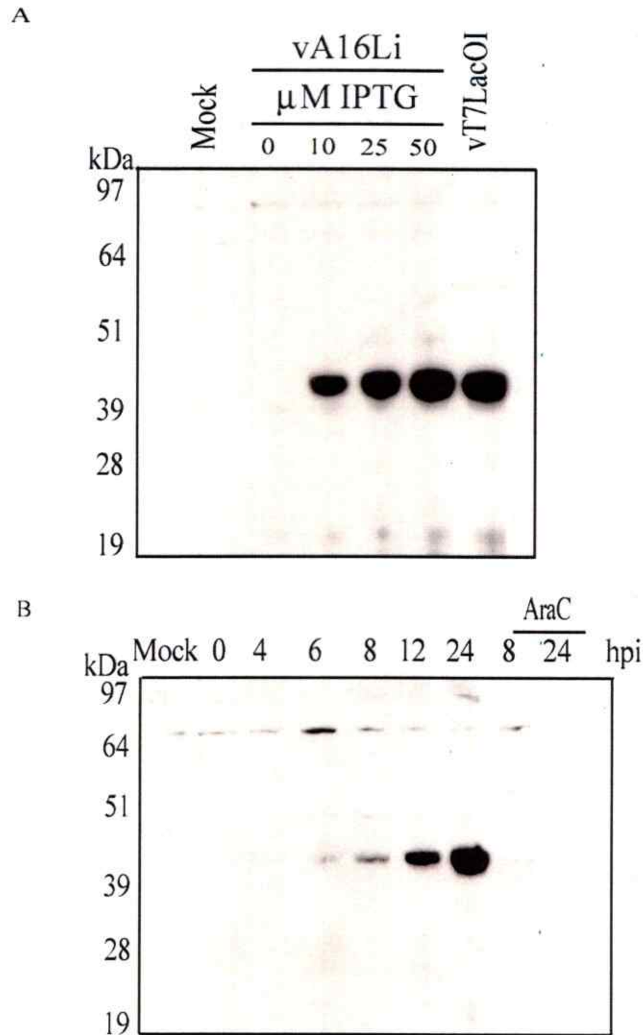


Figure 5. Synthesis of A16 protein by vA16i and VACV WR. (A) BS-C-1 cells were infected with vA16i in the presence of indicated concentrations of IPTG. After 24 h, the cells were harvested and the total cell lysates were analyzed by SDS-PAGE and western blotting using antibody to the predicted C-terminal 15 amino acids of the A16L ORF. Proteins were detected by chemiluminescence. (B) BS-C-1 cells were mock infected for 8 h or infected with 5 PFU per cell of VACV in the absence or presence of AraC. At the indicated hours post infection (hpi), cells were harvested and analyzed by western blotting as in panel A. The masses of marker proteins in Panels A and B are indicated on the left.

1.4 Analysis of alternative initiator methionine in A16L ORF.

The detection of 23-kDa species, also seen when A16 expression was regulated by the T7 promoter, raised the possibility of partial post-translational cleavage, an inefficient downstream translation initiation site, or a weak alternative promoter within the A16L gene. The first alternative was not supported by the absence of a consensus vaccinia virus I7 proteinase cleavage site (Ansarah-Sobrinho, C., 2004; Byrd, C. M., 2002). On the other hand, the A16L ORF and two possible initiator methionine codons separated by 72 nucleotides. Both fragments contained the last C-terminal 15 amino acids epitope recognized by the anti-A16 peptide polyclonal. However, their promoter sequences did not resemble the typical sequence of viral late promoter. A PCR product including these upstream regions, the respective polypeptide encoding sequence (aminoacids 171-378 and 196-378) and a V5-epitope tag at the C-terminus were cloned into a plasmid. The differences in mobility between the endogenous non-taged polypeptide and the C-terminal V5-tag fragment allowed us to distinguish between them. As shown in Figure 6 , a transcript was produced when either a plasmid containing the 171-378 or 196-378 C-terminal fragment of the A16L gene was transfected into cells infected with VACV consistent with the presence of a cryptic atypical promoter. However, the biological significance of this is uncertain.

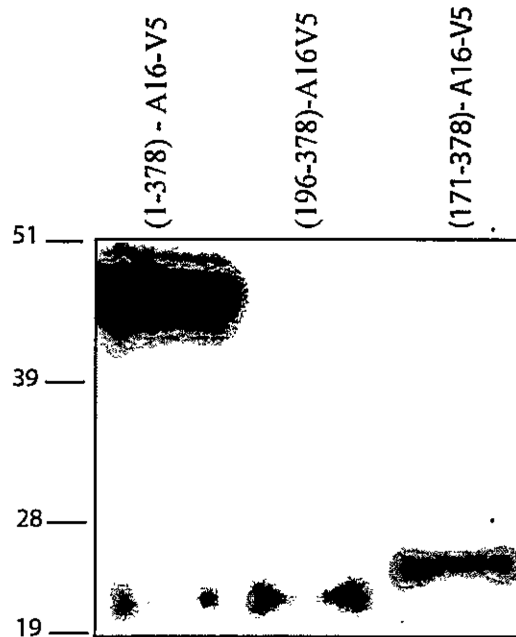


Figure 6. Analysis of expression of A16 from alternative translation initiator.
BS-C-1 cells were infected with 5 PFU of VACV WR strain and simultaneously transfected with a plasmid containing a V5 C-terminal tag of A16 full length (lane 1), truncated A16 (196-378) (lane 2) and truncated A16 (171-378) (lane 3). Cells were lysated in SDS loading buffer and samples were loaded in acrylamide gel followed by western blotting using an anti-V5 Mab.

1.5 Intracellular localization of A16 protein.

The presence of a C-terminal hydrophobic region, suggested that A16 might be associated with viral or cellular membranes. Immunofluorescence microscopy was used to determine the intracellular distribution of A16. At 12 h after infection with wild type VACV or vA16i in the presence or absence of IPTG, HeLa cells were fixed, permeabilized, and incubated successively with the anti-A16 peptide antibody and fluorescein conjugated secondary antibody followed by DAPI to stain DNA in nuclei and juxtanuclear viral factories. The A16 protein was detected within viral factories of cells infected with wild type VACV or vA16i in the presence of IPTG (Figure 7). Viral factories are the first morphological signs of the infection. They are large cytoplasmic structures enriched in DNA in the infected host cell, easily visualized by DNA dyes (Cairns, 1960). No A16 staining was observed in the viral factories of cells infected with vA16i in the absence of IPTG, confirming the specificity of the antibody. These results suggested that A16 is associated with viral rather than cellular structures.

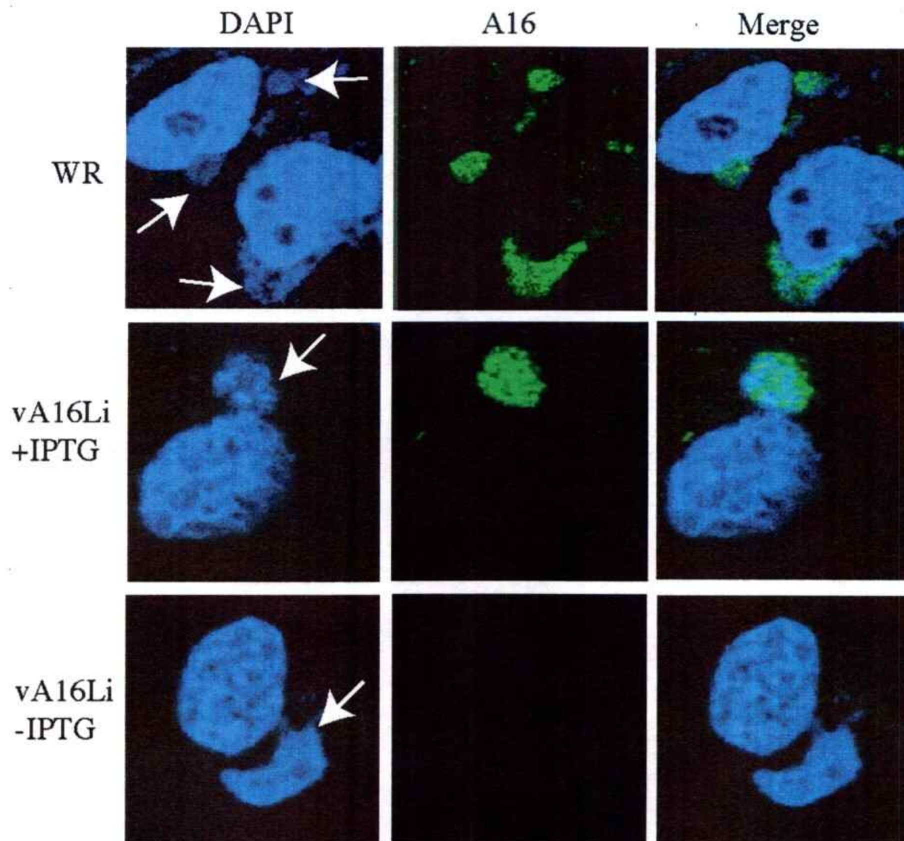


Figure 7. Intracellullar location of A16 determined by immunofluorescence. HeLa cells were infected for 12 h with vA16i in the presence or absence of inducer or with VACV WR. Cells were fixed, permeabilized and stained with A16 peptide antibody and fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G followed by DAPI. Confocal microscopy images are shown. Arrows point to virus factories stained with DAPI.

1.6 Association of A16 with purified virions.

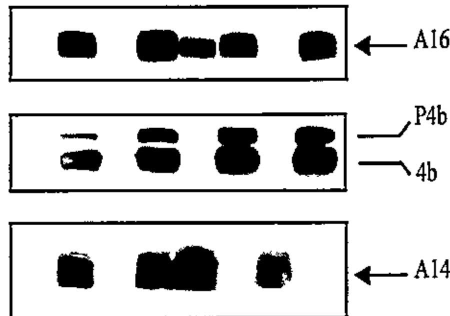
To investigate the possible association of A16 with virions, MVs were purified by sedimentation through 2 sucrose cushions followed by a sucrose gradient. The virions were disrupted with SDS and the presence of A16 was determined by western blotting with antibody to the C-terminal peptide. Both the 46- and 23-kDa polypeptides were detected, although the latter was present in only a trace amount (as will be shown in Figure 5B). The A16 protein was partially solubilized from intact particles with a combination of 1% NP-40 and 140 mM NaCl in Tris buffer (Figure 8A). As positive and negative controls, we showed that the A14 membrane protein was almost completely solubilized under the latter conditions, whereas the A3L (4b and precursor P4b) core protein remained insoluble (Figure 8A). The solubility of A16 but not A14 was reduced when NaCl was omitted or when DTT was present in addition to NP-40 (Figure 8A). Although difficult to explain, we had previously noted a negative effect of DTT on the extraction of A28, a protein that contains intramolecular disulfide bonds and is required for cell entry (Senkevich et al 2004; Senkevich et al., 2004).

To determine the topology of the A16 protein, purified virions were partially digested with trypsin. The supernatant and pellet fractions were analyzed by SDS-PAGE and western blotting with antibody to the C-terminus of A16 (Figure 8B). The 0 trypsin control shows the dominant 46-kDa band and the minor 23-kDa band. At 1 μg per ml of trypsin, there was a digestion product of about 10-kDa, which decreased at higher trypsin concentrations. At 10 and 100 μg per ml, the dominant products were 34- and 5-kDa. Despite their cleavage, the products remained associated with virions, presumably

through the putative membrane anchor sequence or protein-protein interactions. The A16 protein is predicted to contain numerous lysine and arginine residues, some of which could be cleaved to generate the observed partial digestion products that retain the transmembrane domain and antibody epitope. Under these digestion conditions, the core protein A4 was trypsin-resistant, indicating the integrity of the virus particles (not shown). When NP-40 was added to disrupt the membrane, the A16 protein was completely susceptible to trypsin (Figure 8B). Taken together, the partial release of A16 with detergent and NaCl and the trypsin sensitivity of A16 suggested that the protein is anchored in the MV membrane with the long N-terminal segment exposed.

A

DTT	-		-		-		+	
NP-40	-		-		+		+	
NaCl	-		+		+		+	
Tris	+		+		+		+	
Fraction	S	P	S	P	S	P	S	P



B

Trypsin µg/ml	0		1		10		100		100	
NP40	-		-		-		-		+	
Fraction	S	P	S	P	S	P	S	P	S	P

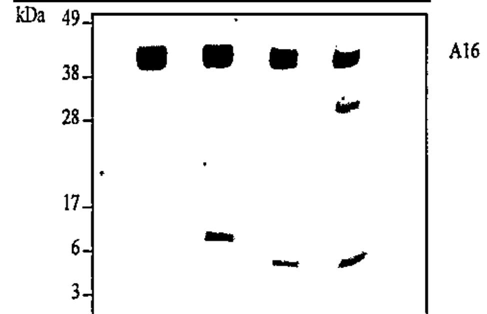


Figure 8. Detergent extraction and trypsin sensitivity of A16 associated with purified MV. (A) Sucrose gradient-purified VACV was incubated with buffer containing one or more of the following components: Tris-HCl (pH 7.4), 1% NP-40, 140 mM NaCl, and 50 mM DTT as indicated by plus or minus signs. After 1 h at 37^0C, samples were centrifuged and supernatant (S) and pellet (P) fractions were analyzed by western blotting using antisera to A16, A3 (P4b/4b) and A14 peptides. (B) IMV were purified as in panel A and treated with indicated concentrations of trypsin without (-) or with (+) NP-40. Equivalent portions of supernatant and pellet fractions were analyzed by 4-12% NuPAGE-SDS and western blotting with the A16 peptide antibody. Masses of protein markers in kDa are indicated on the left. The position of the full-length A16 protein is indicated on the right.

1.7 A16 contains disulfide bonds formed by viral redox pathway.

The presence of 20 conserved cysteine residues in A16 raised the likelihood of intra- or intermolecular disulfide bonds. To investigate this, we compared the mobilities of A16 in cells lysed in the presence of reducing or alkylating agents. In this experiment we used a recombinant VACV called vV5A16i , with a V5 epitope tag at the N-terminus of an inducible A16L gene, which was constructed as described for vA16i. Replication of vV5A16i in the presence of IPTG was similar to that of vA16Li indicating that the tag did not have a deleterious effect.

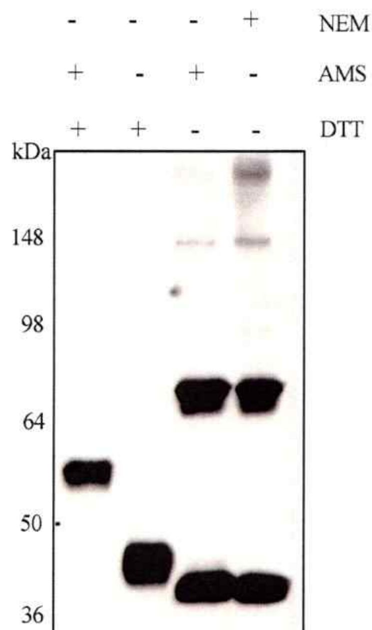
The study of the redox state of a cysteine residue is complicated by the reversibility of the process. The recognition of the original redox state requires an irreversible reaction that block free thiols by alkylation rapidly upon cell lysis.

Two alkylating agents were used in this study: NEM and AMS. Because of its large size, AMS adds an additional 0.536 kDa for each cysteine that is alkylated, whereas the smaller NEM causes a negligible increase in mass. This methodology offers important advantages compare to mass spectrometry analysis: (i) it allows to work with small amount of protein mixture; (ii) modified molecules can be rapidly detected by Western Blot (Camerini, S, 2005). There was an approximately 10-kDa difference in mobility between reduced A16 that was treated with DTT alone and alkylated A16 that was treated with DTT and AMS (Figure 9A), which is consistent with reduction and subsequent alkylation of all 20 cysteines. When A16 was treated with AMS or NEM without prior reduction, bands corresponding to monomeric and dimeric forms were detected in each case (Figure 9A). The mobilities of the bands were the same regardless

of whether AMS or NEM was used, indicating the absence of reactive cysteines. If free cysteines had existed then the AMS-alkylated protein would have migrated more slowly than the NEM-alkylated protein, which was not the case. The faster unreduced band migrated more rapidly than DTT reduced A16, suggesting a more compact structure consistent with intramolecular disulfide bonds. The slower migrating band is the size expected for an A16 dimer. The SDS-resistance of the dimer could be due to intermolecular disulfide bonds that either formed naturally or by a rapid disulfide interchange that occurred even in the presence of alkylating agent.

The trypsin digestion experiments suggested that the long N-terminus of the A16 protein, which contains all 20 cysteines, is on the surface of virus particles and therefore would face the cytoplasm in infected cells. Poxviruses encode components of a unique redox pathway that operates in the cytoplasm and forms intramolecular disulfide bonds in some MV membrane proteins (Senkevich et al., 2002). The three viral proteins that form this redox pathway are A2.5, E10 and G4. An inducible E10 virus (vE10Ri) was used to determine whether the viral redox pathway is required for formation of the disulfide bonds of A16. Cells were infected with vE10Ri in the presence or absence of IPTG to regulate E10 expression and transfected with an expression plasmid containing the full-length A16L sequence, with a V5 tag appended to its N-terminus, regulated by its natural promoter (which is unaffected by IPTG). Without reducing agent, AMS increased the mass of the A16 protein made in the absence of IPTG by about 10-kDa indicating that all 20 cysteines were reactive and hence there were no disulfide bonds (Figure 9B). In contrast, AMS increased the mass of the A16 protein made in the

presence of IPTG by about 2-kDa corresponding to four reactive cysteines and indicating that 8 of the 10 disulfide bonds had formed (Figure 9B). Incomplete disulfide bond formation in the presence of IPTG is probably related to the somewhat artificial induction and transfection system, as noted previously for the A28 protein under similar conditions (Senkevich et al., 2004). The formation of disulfide bonds in A16 via the viral redox pathway is consistent with the deduced membrane topology in which the long N-terminus of the membrane-anchored protein is exposed to the cytoplasm.



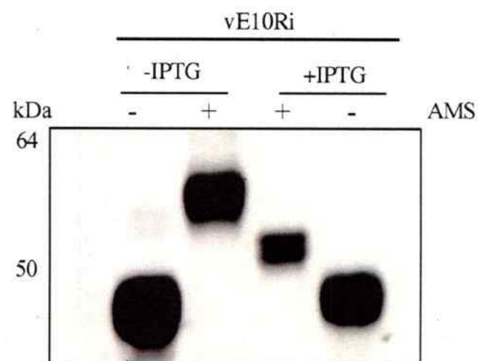


Figure 9. Formation of disulfide bonds. (A) Disulfide bonds in A16. Cells were infected with vV5A16i in the presence of IPTG for 18 h. The cells were collected by centrifugation and solubilized in buffer containing DTT, 20 mM AMS or NEM as indicated by plus or minus signs. In addition, a portion of the extract solubilized in the presence of DTT was alklylated with excess AMS. Proteins were resolved by non-reduced SDS-PAGE, transferred to a nitrocellulose membrane; incubated with anti-V5 mouse antibody conjugated to horseradish peroxidase and detected by chemiluminescence. (B) E10 expression is required for formation of disulfide bonds. Cells were infected with vE10Ri in the absence or presence of 100 μM IPTG and transfected with a plasmid encoding the A16L ORF regulated by its own promoter and containing a C-terminal V5-tag. Proteins from total cell extracts were alkylated with AMS (+AMS) or treated with the reducing agent Tris-(2-carboxyethyl) phosphine (-AMS) and resolved by SDS-PAGE. Western blotting was performed as in panel A with an anti-V5 mouse monoclonal antibody.

1.8 Virus morphogenesis is unaffected by repression of A16.

Some viral membrane proteins are required for particle assembly and morphogenesis; consequently processing of core proteins fails to occur in their absence. To investigate such a role for the A16 protein, a pulse-chase experiment was carried out. Cells were infected with vA16i in the presence or absence of IPTG, pulse-labeled at 12 h (when host protein synthesis is turned off) with [^{35}S]methionine, and then chased with excess methionine for an additional 12 h. Processing of core proteins, determined by SDS-PAGE and autoradiography, was unaffected by IPTG suggesting that A16 is not required for early steps in assembly (Figure 10A). Processing of the P4b protein was also demonstrated by western blotting (Figure 10B). The relative amounts of precursor and product were similar in the presence and absence of A16 expression.

Electron microscopy was used to further investigate a role of A16 in the later stages of virus morphogenesis, which would not affect protein processing. However, no defect was noted as normal looking immature and mature intracellular and extracellular virus particles were observed in the absence (Figure 11A,B) of inducer.

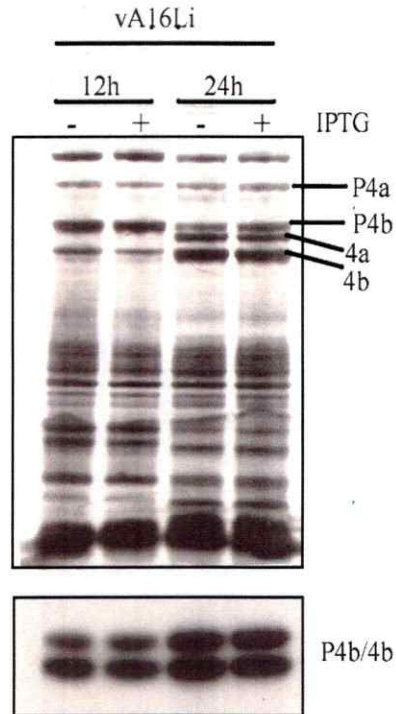


Figure 10. Synthesis and processing of viral proteins. BS-C-1 cells were infected with 5 PFU per cell of vA16i in the presence or absence of 50 μM IPTG for 12 h and labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine for 30 min. Cells were either harvested immediately (pulse) or incubated with excess unlabeled methionine for an additional 12 h The proteins were analyzed by SDS-PAGE and visualized by autoradiography (A). The positions of migration of precursors (P4a and P4b) and their cleavage products (4a and 4b) are shown on the right. In parallel, the proteins were transferred to a membrane and western blotting was performed using anti-P4b/4b polyclonal antibody (B).

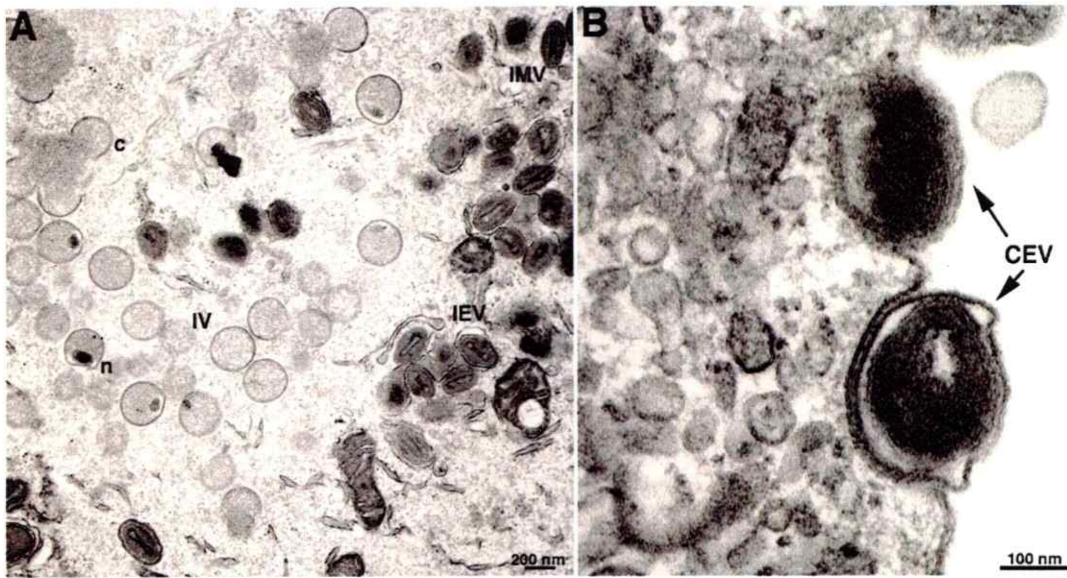


Figure 11. Electron microscopy of infected cells. BS-C-1 cells were infected with vA16i in the absence of IPTG. After 21 h, cells were fixed and embedded in Epon. Ultrathin sections were prepared for transmission electron microscopy. Panel A shows a low power image of a viral factory region displaying crescents (c), immature virions (IV) some with nucleoids (n), MV, and intracellular enveloped virions (EV). Panel B shows the surface of a cell with two cell-associated enveloped virions (CEV).

1.9 Comparison of –A16 and +A16 MVs.

The location of A16 in the MV membrane and the apparent absence of any role in assembly, suggested to us that A16 might be needed for virus entry. Up till now, all infections were done with vA16i stocks that were propagated in the presence of IPTG and therefore contained A16. In order to investigate the hypothesis that A16 is involved in entry, we purified virions from cells infected in the absence of IPTG (-A16 virions) and compared them to virions made in the presence of IPTG (+A16 virions). The yields of virus particles prepared in the presence and absence of IPTG were similar as determined by optical density. Moreover, the two types of particles were indistinguishable by electron microscopy after negative staining (Figure 12) as well as by SDS-PAGE and silver staining (Figure 13A). Western blotting of virions formed with and without IPTG indicated that the latter contained only a trace of A16 but normal amounts of other MV membrane proteins including A28, A21, L5 and A27 (Figure 13B). Nevertheless, plaque assays revealed that the specific infectivity of –A16 virions were 60- to 100-fold less than that of +A16 virions. The variation probably reflects differences in the amounts of residual inoculum virus. The low specific infectivity of –A16 virions was sufficient to account for the decreased yield of infectious virus.

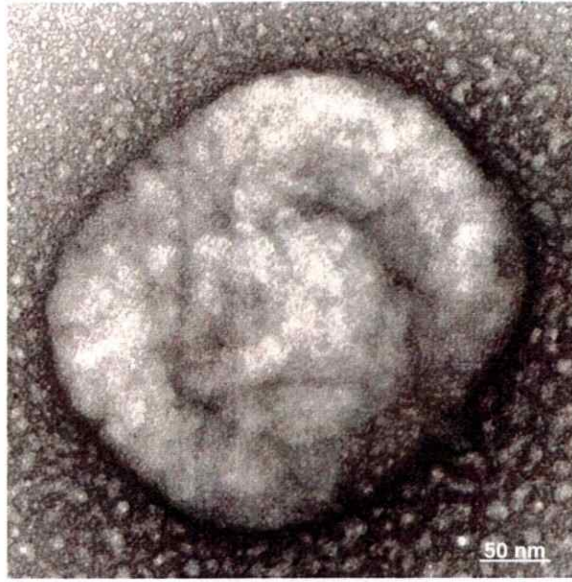


Figure 12. Negative stain image of purified MV virions.

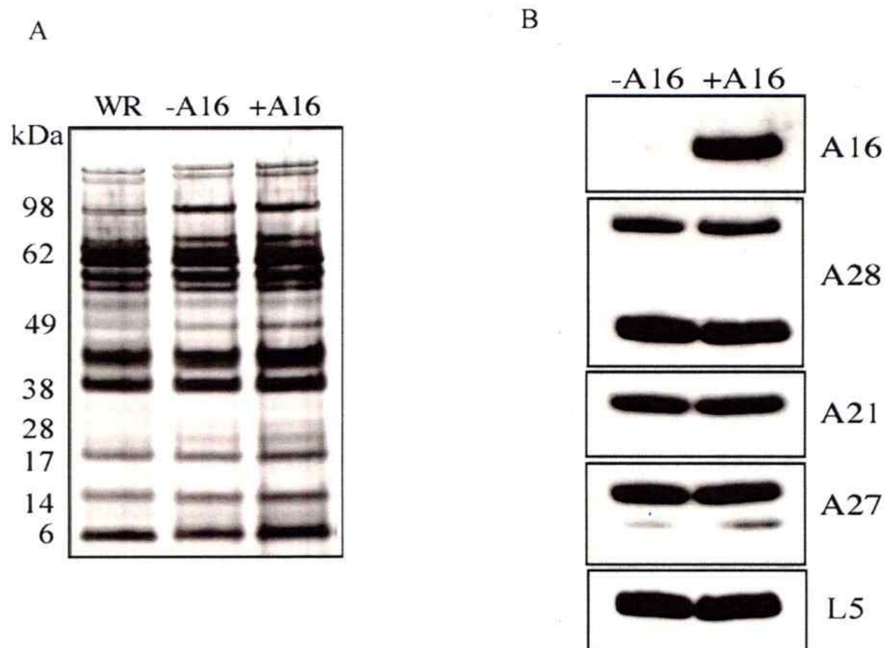
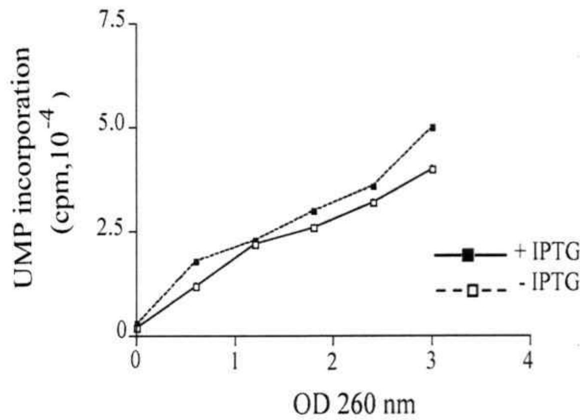


Figure 13. Protein composition of purified virions. MVs were purifed by sucrose-gradient sedimentation from cells infected with VACV WR or with vA16i in the presence (+A16) or absence (-A16) of IPTG. Equal numbers of particles (determined by OD_{260}) were analyzed by SDS-PAGE and silver staining. The masses in kDa of marker proteins are shown on the left. (B) Western blotting of samples prepared as in Panel A and probed with antibodies to the A16, A28, A21, L5 and A27 proteins.

1.10 Role of A16 in virus entry.

In the previous section, we showed that -A16 and +16 virions had similar overall polypetide composition and morphology but their specific infectivity was different. Since transcription of early genes is initiated soon after the delivery of cores into the cytoplasm, failure of -A16 virus to synthesize RNA would suggest a block in entry. To evaluate viral early RNA synthesis we used two different approaches. First, we used equal amounts of –A16 and +A16 virions, later they were NP-40 permeabilized and incubated with [alpha-P^{32}] UTP. As shown in Figure 13A both had similar abilities of synthetize RNA in vitro. Second, we infected cells were with equal amounts of purified -A16 or +A16 virions in the presence of AraC to prevent DNA replication and late stage RNA synthesis. Total RNA was extracted and northern blotting was performed using a radioactive probe complementary to the viral A20R early mRNA. We observed an intense band of 0.5 kB corresponding to the full-length transcript from cells infected with +A16 virions but a much weaker one from cells infected with -A16 virions (Figure 14 B). ß actin mRNA was analyzed as a control for RNA integrity and loading (Figure 14 B).

A



B

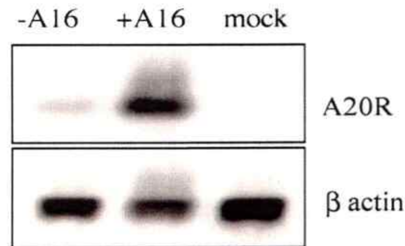


Figure 14. In vitro and in vivo RNA synthesis. (A) In vitro transcription by permeabilized -A16 and +A16 virions. Lysates were made from cells infected with vA16i in the presence or absence of IPTG and virions were purified by sucrose gradient centrifugation. The indicated number of OD_{260} units of purified virions were incubated in a reaction mixture containing [a-^{32}P]UTP. Incorporation of radioactivity into RNA was determined. (B) Northern blot analysis. BS-C-1 cells were treated with 40 μg per ml of AraC for 1 h. Total RNA was extracted from cells at 3 h after mock infection or infection with 5 PFU of purified +A16 virions or the equivalent OD_{260} of –A16 virions. The RNA was resolved by agarose gel electrophoresis, transferred to a membrane, probed with radioactively labeled DNA complementary to the A20R early vaccinia gene or b actin and analyzed by autoradiography.

In the previous section we showed that –A16 and +A16 virions can synthetize RNA but the amount of early mRNA made after they enter cells was different which suggested a defect in viral entry. To directly examine the ability of -A16 virions to enter cells, we used an assay originally described by Vanderplasschen et al. (Vanderplasschen, et al., 1998). In our adaptation, purified virions were adsorbed to cells for 1 h at 4°C and then the temperature was raised to 37°C for 2 h to allow penetration. The protein synthesis inhibitor cycloheximide was present in order to prevent cytopathic effects and core disassembly. The cells were stained with antibodies to the L1 membrane protein and the A4 core protein to detect virions on the surface of cells and cores in the cytoplasm, respectively. As shown in Fig, anti-L1-staining virions were associated with cells infected with -A16 or +A16 virions, indicating that A16 is not required for binding. Numerous stained cores were seen in the cytoplasm of cells infected with +A16 virions but were infrequent in cells infected with -A16 virions (Figure 15). The few cores detected under the latter conditions co-localized with L1 staining suggesting that they are located on the surface of the cell (not shown). Thus, -A16 virions exhibited a defect in a post-binding step of virus entry.

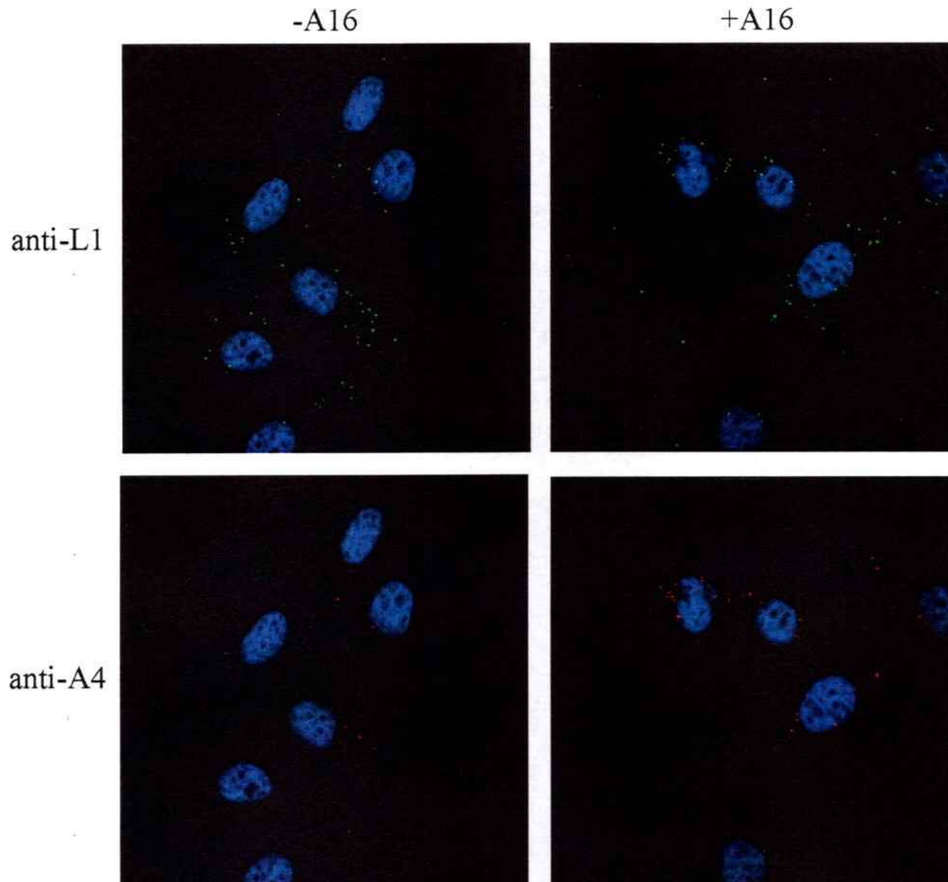


Figure 15. Cell binding and penetration of purified MV. Hela cells were incubated with 5 PFU per cell of purified +A16 virus or the equivalent OD_{260} of –A16 virus in the presence of 300 μg of cycloheximide per ml for 1 h at 4^0C. The cells were washed, incubated for an additional 2 h at 37^0C and then fixed and stained with anti-L1 mouse monoclonal antibody and anti-A4 rabbit polyclonal antibody, followed by fluorescein isothiocyanate-conjugated goat anti-rabbit and Rhodamine Red-X- conjugated anti-mouse IgG. The cells were then stained with DAPI to show nuclear DNA. Confocal microscopy images show DNA (blue), anti-L1 (green), anti-A4 (red).

1.11 Low pH-induced fusion from within and without.

Cell-cell fusion can be triggered by briefly exposing cells to low pH at late times after VACV infection, when progeny virions are on the cell surface (called fusion from within), or soon after infecting cells with large numbers of MVs particles (called fusion from without) (Doms, R. W., 1990, Gong, S. C., 1990). Previous studies, with conditional lethal mutants impaired in expression of the A21, A28, H2 and L5 proteins, showed a correlation between the inability of virions to enter cells and the inability to induce cell-cell fusion (Townsley, A., 2005; Senkevich, T. G., 2004, Senkevich, T. G., 2005; Townsley, A., 2005;).

To determine whether A16 expression is required for fusion from within, we infected cells with vA16Li in the presence or absence of IPTG and briefly exposed the cells to pH 5.5 at 18 h after infection. Large syncytia were observed only in the presence of IPTG (Figure 16A), indicating that the expression of A16 is essential for fusion. Similarly, low pH-triggered fusion from without occurred when cells were infected with purified +A16 virions but not with -A16 virions (Figure 16B). As expected, fusion from within or without did not occur with +A16 virions when the low pH treatment was omitted (not shown).

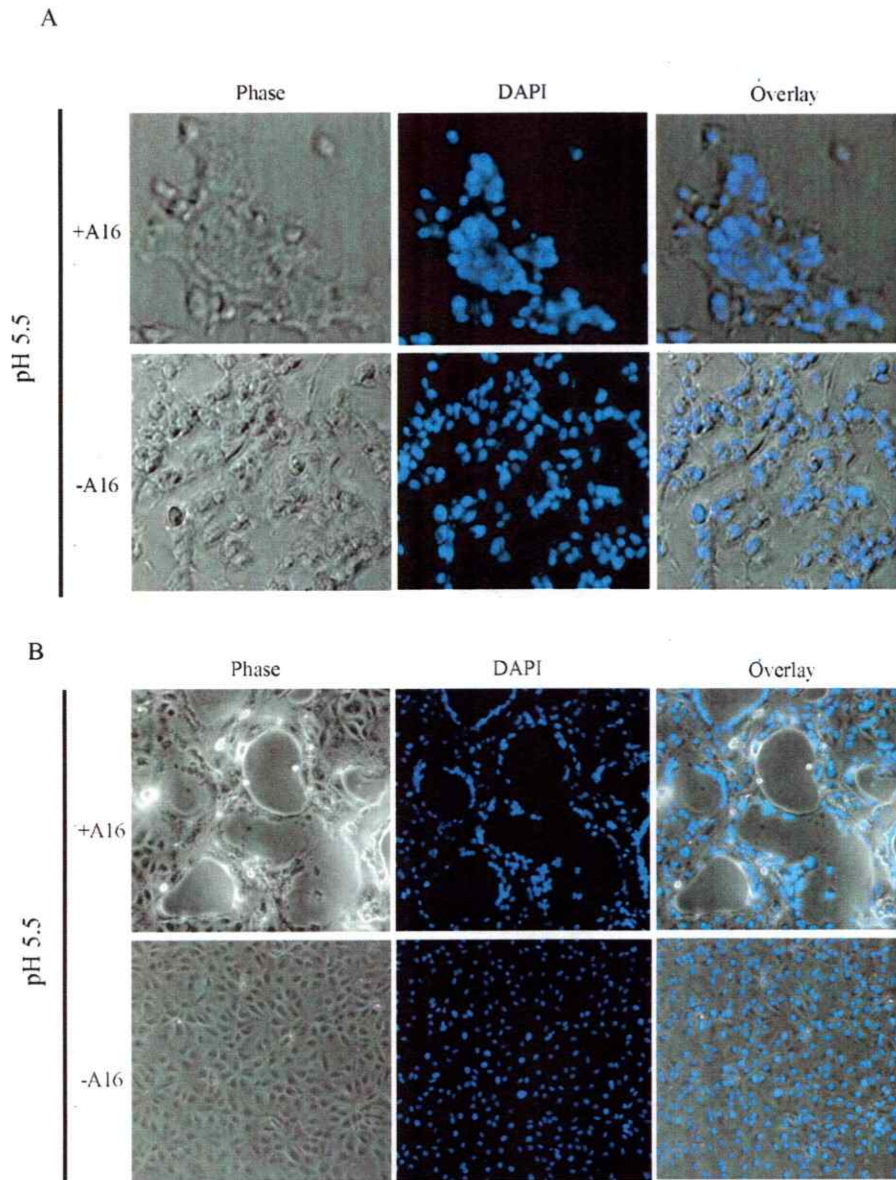


Figure 16. Fusion from within and without. (A) Fusion from within. BS-C-1 cells were infected with 2 PFU of vA16Li per cell in the absence or presence of IPTG for 18 h at 37^0C. The medium was replaced with pH 5.5 or 7.4 buffer (not shown) and incubated for 2 min 37^0C. The buffer was aspirated and the cells were incubated for 3 h at 37^0C in fresh neutral pH culture medium. Cells were stained with Hoechst dye, fixed with paraformaldehyde and examined by phase contrast and fluorescence microscopy. (B) Fusion from without. BS-C-1 monolayers were incubated with 200 PFU of purified +A16 virions per cell or the equivalent OD_{260} of -A16 virions for 1 h at 4^0C. The cells were washed and incubated for 2 min with pH 5.5 buffer or pH 7.4 buffer (not shown) and then for 3 h at 37^0C in neutral pH medium with 300 μg of cycloheximide per ml. Cells were stained and examined as in panel A.

2. Characterization of G9R vaccinia open reading frame.

G9 is highly conserved throughout the poxvirus family. The G9R ORF (VACWR087) is predicted to encode a 39.8-kDa protein with the following structural features that are conserved in all poxviruses: fourteen cysteine residues, a site for N-terminal myristoylation and a C-terminal transmembrane domain (Figure 16). A16 and J5 are structurally related to G9, although the sequence similarity is low (Senkevich et al., 1997) and both G9 and A16 are myristoylated (Martin et al., 1997). No non-poxvirus homologs of G9 were detected by a position-specific iterative BLAST search.

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LSDV --------------------MGSSFTVPEKIKISNRPKKETMEMRLNVDNMYEQIKTLNQINDELYIGIINEEK-----KKQLIKQFPEFLFIESGPGN    74
YLDV --------------------MGSTFTIPENVKRNPPNKNPTSEMKLNVDNMYKLISYLTQISDTLYVGSVSEEK-----KNQLKTIFPEFLFLESGPGN    74
 SPV --------------------MGSSFAVPEDAKRQPLPRKPTYEMNLDVNKMYELIASISRINDEIYIGKVDDYT-----LNQLRYRFPEFLFIESGPGK    94
 MYX --------------------MGASFVVPENVKRAP-PKHNTSHMALNVDEMYELISILNKG-EELYLGKLTKEK-----KAVLEQRFPEFKFVESGPGS    72
 VAC --------------------MGG-GVSVELPKRDPPPGVPTDEMLLNVDKMHDVIAP-AKLLEYVHIGPLAKDK-----EDKVKKRYPEFRLVNTGPGG    72
 MCV --------------------MGNSVQLPAQ---EPPGPMLTAEMELTLMDMLDVIAQARHGELVHTGALITEEPRFTEIRQLLRSMLPEFDLVETGRG-    75
 FPV --------------------MGGGLVLPTR---DPPKEQDTSETATNIPKLLKSIPGVKLGQQIRIGYKPGPET---------AKAFPEFDIKEVSNG-    66
 MSV --------------------MGQSIEIHPK----YWGVNVSQEMQISYADILNTLYTAEIDDRVAIGQYATDPN---------SYTNVPEFNPIIKPGG    66
 AMV --------------------MGGSVDIEAR----YTGSSNFQETYLSFSNLINTIYILTRDERIPIGIFSNNPD---------DYRNYRGYTAIFKPGG    66

LSDV LHKVIRSKYNNDKKYCCKSMNLFSYWQD-NNGNITDFYKPNSILSSCDPDIQNSG---FCDDTLLSWCDTATQ---SNSKLCYDWINSALNRNN------   161
YLDV LTKVIRSKYNNDADLCCKSFNLTSYWK--NNDKISLSYKPNFILNSCDPDLKNSG---YCDNTLFNWCQKDKY----DKNICNDWMKSLLNRGN------   159
 SPV LYKAVRSKYTNDERYCCKNLKLQSYWKD-DNGVISILYRPNTILNSCDPDLLTST---LCDNLLLSLCNDAYKS-VSESNLCDHWISTLLERYN------   183
 MYX LFKAVRVSYKNDINYCCKDLQIHGYWID-KNGDISLFYRPNTVLNSCKPNMQSSG---VCDTFMFKRCEDRLD----TNACHDWISSMLNRED------   157
 VAC LSALLRQSYNGTAPNCCRTFNRTHYWK--KDGKISDKYEEGAVLESCWPDVHDTG---KCDVDLFDWCQGDTFD----RNICHQWIGSAFNRSNRTVE-G   162
 MCV LFGMQRRVFSGELGFCCTHR-EHHYWEDTRTREHAAAYAPGRALRTCAPWTLTKAS--PCDQTLFANCVREDSD----TELCKQWLGAAFAREELVPPGA   168
 FPV LYELSRKSYLGDTKTCCINPSLSYYWED-SKNKIFDEYATGRSLKTCDPLTKTISGSTLCDNILTSLCLDEKSG--VDRTMCNEWMGYALNRPD------   157
 MSV YTELHRKSYLGPDEICCLN-DIRYGWVD-ANGNVTSNANLNKTLLSCDPTTIKVGSSNICDVPLYNTCIVDKN--QEYKGKCGVWLNGLFKEWQIDFS--   160
 AMV YKELLKVNDLGPDDLCCIY-DWRYANVD-ENNILSQNASVNKNLFTCDPRTIQVGTNNICDNSMYRACILDFNNHRYLEAKCGVWLDGLFKRFATASN--   162

LSDV -SSSEKLINGLITTCSNNANTPICNIFLHCLRVKNTETFDNVIDYILYSQSDDFKRTYMKCSYPSNKIIKESLKFFEARECWDPNCSESNLNFLLTKNYK   260
YLDV -GNSEEFIYKLISLCSSNANTPICENFLHTLRVQNTEKTDNIIDYILYQQSNNFKSKFMKCSFPTKDKLQESLKYLELRECWDPECANANVNFLLTENYN   258
 SPV -ISSN-LIDTIINICSKNANLDICQSFIRTLRIRNTEKFDNIIDYILYSQSDIFKQKYMKCSYPTQNKIDESLKYVEKRECWDPECATGNINLLLTNNYR   281
 MYX -DIGARFVNNFVEKCSRDASTPLCETFLHNLRAKNTQPYDNLIDYLLMAQKDDFKSKYMRCSFPTKEKKEESLKYSETRECWDPECANANVNFLLTENYR   256
 VAC QQSLINLYNKMQTLCSKDASVPICESFLEHLRAHNTEDSKEMIDYILRQQSADFKQKYMRCSYPTRDKLEESLKYAEPRECWDPECSNANVNFLLTRNYN   262
 MCV SSSLDKLVGLFARRCAQDVRAEGCEDWLHAMRVANEPRFDRVIDAVLAQQDPEFKRTHMACSYPSHRSLELARRVAEPRECWDPQCQRANPNFLLSENYH   268
 FPV LSIPKSINDRYTKLCSKGANNIVCKDWLEHLRIIGGKENDEVIDNVLMQQTPEFKEKYMKCSFPSENTVFLADRVIEPRECWDQECITSNVHFLLSKNYH   257
 MSV --IIASINKIMSEHCSQNYKNDNCDIWLAAIRNSTNSEYYNIADNVLYAQTD---KSELSCAFPPEYIVRQSNKINTPKECWYRPCAFSPNYLLLSSNID   255
 AMV --IINNTNNILLQSCSNNINNDLCIKWLIAIRNSGNPTFFSLADNVLNAQTD---KTNLKCAFSPSYITDTQNRLNVPKECWYRECAFSPNYLLLTDNIT   257

                                                                   TRANSMEMBRANE
LSDV NLGLCKIDRCNISINNLFFDEGSSVRMSCNDES---IKSKFPINKEKVILHNVDNSFQLKIHLLTILSLFVIWVLIVAI----   336
YLDV NLGLCTINRCNVSINHLDMDSTSNISMSCNGNN---LMNPFPINREKVILHNINSSLNLKIHLITILSILIIWILIVAI----   334
 SPV NLGLCNINRCNIGINYLNLDNKSKLRMSCGKDT---VIN-KPINREKVIKHKMDHSFKLRIHELTILSILVMWILIVAI----   356
 MYX NLGLCTINRCNVDINHLNITPRSKLRMTCGTEA---LYSPLPVNKEKVIKHNVDNSFQLHVYEITILFVLVIWILIVAI----   332
 VAC NLGLCNIVRCNTSVKNLQMDKTSSLRLSCGLSNS-DRFSTVPVNRAKVVQHNIKHSFDLKLHLISLLSLLVIWILIVAI----   340
 MCV ALAGCHIYRCNVSIGTLLMDKRSKLRISCHDPS-----TRVQLNKLGVVQDNVRRAPRVRTELLAALALFLVWVLIVAL----   342
 FPV NLTLCHIYRCNISINNLLIDGKSSVKISCHDENISNKDKPKARNKAKFIDDILGSSFNINFGFFFVIFIMIALILIVLL----   336
 MSV NKNNCLLSECNININNLNIISNTELSIICKN-QVIYRSSKEQSN----IIQEEAEFNRFLIPNYTVYFILILIFLIMIIFSKN   333
 AMV LKNNCSLSECNINIGNLDIVSASEVTITCNNNKSNTVSSRQKLD----ILLRESEDYRFLLTNNILILILLFIFLIFLIIREN   336
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Figure 17. Multiple-sequence alignment of G9 orthologs. One representative sequence from each genus of *Chordopoxvirinae* and two from *Entomopoxvirinae* are included in the alignment. MCV, molluscum contagiosum (*Molluscipoxvirus*); YLDV, Yaba-like disease virus (*Yatapoxvirus*); LSDV, lumpy skin disease virus (*Capripoxvirus*); SPV, swinepox virus (*Suipoxvirus*); MYX, myxoma virus (*Leporipoxvirus*); FPV, fowlpox virus (*Avipoxvirus*); MSV, Melanoplus sanguinipes entomopoxvirus; AMV, Amsacta moorei entomopoxvirus; VAC, vaccinia virus (*Orthopoxvirus*). Invariant conserved cysteines are shown in a black background; other conserved amino acid residues are shown in gray background.

2.1 G9 is required for efficient VACV replication.

To determine the role of G9, we used a modification of previously described methods (Ward, G. A., 1995) to construct a recombinant virus in which expression of G9 is regulated by an inducer. The mutant virus called vG9i, contains (i) an N-terminal HA epitope-tagged G9R ORF regulated by the bacteriophage T7 RNA polymerase promoter and the E. coli lac operator, (ii) the E. coli lac repressor gene expressed constitutively by an early/late VACV promoter and (iii) the bacteriophage T7 RNA polymerase gene controlled by a VACV late promoter and lac operator. The stringency of the system depends on the presence of lac operator sequences located upstream of the T7 RNA pol and G9R ORFs (Ward et al., 1995). In addition, the orientation of the G9R ORF was reversed (to make it G9L) to prevent RNA polymerase read-through from neighboring genes (Figure 18A). When BS-C-1 cells were infected with vG9i in the presence of 50 μM IPTG, plaques similar in size to those of the parental virus were formed, whereas much smaller plaques formed in the absence of inducer (Figure 18B). In a one-step growth experiment, the yield of infectious vG9i was approximately 1.5 logs higher at the optimal IPTG concentration of 50 μM than in the absence of IPTG (Figure 18C). A similar effect on virus yield was previously observed when the synthesis of A16 was repressed in the same manner. The induction of infectious virus formation by IPTG correlated with the effect of the inducer on G9 synthesis, as shown by western blotting (Figure 18D).

The formation of small plaques and low but significant replication in the absence of IPTG led us to consider whether G9 is truly essential. To investigate this question, we attempted to delete the gene using a novel application of the VAC-BAC system in which the complete VACV genome is cloned in a bacterial artificial chromosome and can be rescued by FPV in mammalian cells (Domi and Moss, 2002; Domi and Moss, 2005). The first step, construction of VAC-BACΔG9 in which the G9R ORF was replaced by the ampicillin resistance gene, was carried out in E. coli. CV-1 cells were then infected with helper FPV and transfected with VAC-BACΔG9. After 7 days, the cells were harvested and a plaque assay in BS-C-1 cells was performed to detect any VAC-BACΔG9 virus. However, no plaques were seen at the lowest dilution tested even when viewed with a fluorescent microscope to detect individual cells expressing GFP, which was encoded in the VAC-BAC. To confirm that VAC-BACΔG9 had no additional mutations that would impair rescue, DNA containing the intact G9R gene and flanking region was transfected with VAC-BACΔG9 into cells infected with FPV. Under these conditions recombination occurred between the VAC-BACΔG9 and the G9 DNA resulting in rescue of infectious virus (Figure 18E). Our inability to isolate a deletion mutant indicated that G9 was essential or that the level of replication was too low for isolation by this method.

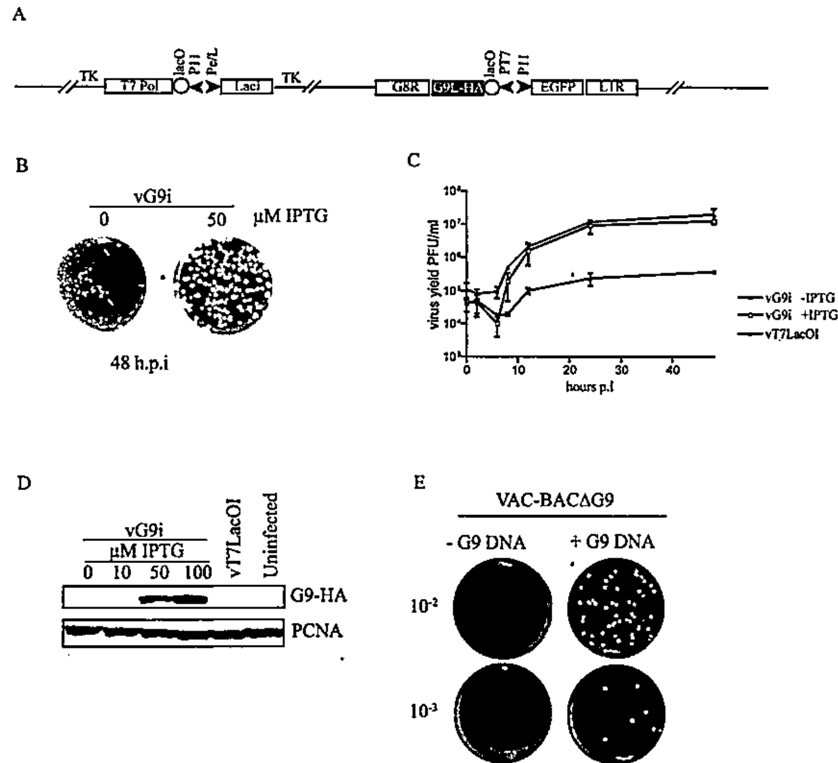


Figure 18. Correlation of G9 expression with virus replication. (A) Construction and characterization of a G9-inducible virus. Diagram of relevant segments of the recombinant vG9i genome. T7 pol, bacteriophage T7 RNA polymerase; PT7, bacteriophage T7 promoter; *lacO*, *E.coli* lac operator; lacI, *E.coli lac* repressor; P11, vaccinia late promoter; Pe/l, P 7.5 early-late promoter; TK, thymdine kinase locus. (B) Plaque formation in the presence and absence of inducer. BS-C-1 monolayers were infected with the vG9i in the presence of 0 or 50 µM IPTG. At 48 h post infection (h.p.i) the cells were stained with crystal violet. (C) Single step virus yield in the presence and absence of inducer. BS-C-1 cells were infected with 5 PFU of vG9i in the presence or absence of IPTG or with the parent virus vT7LacoOI and harvested at the indicated times post infection (p.i.). The crude lysates were treated with 125µg/ml of Trypsin for 30 minutes at 37^0C. Virus yields were determined by plaque assay at 50 µM IPTG. (D) Western blot. BS-C-1 cells were infected with 5 PFU of vG9i in the presence of the indicated amounts of IPTG. After 24 h, whole cell extracts were analyzed by western blotting using an anti-HA Mab and rabbit anti-PCNA. (E) Attempt to delete the G9R ORF. CV-1 cells were infected with FPV and transfected with VACBACΔG9 plasmid (VBΔG9). After 7 days, the cells were lysed and the presence or absence of infectious VBΔG9 virus was determined by plaque assay. In parallel, another plate of CV-1 cells was infected with FPV and transfected with VBΔG9 plus DNA containing the G9 wild type sequence and flanking region (VBΔG9+G9) and assayed in the same manner.

2.2 Association of G9 with the MV.

The predicted hydrophobic domain in the very C-terminal of G9 suggests that it is also MV-associated. However, it was necessary to confirm the MV association of G9 experimentally. Because highly specific antibody to G9 was unavailable, we took advantage of the HA epitope-tagged G9 in vG9i. Virions were purified from cells that were infected in the presence of IPTG and analyzed by western blotting using an anti-HA MAb. We also analyzed whole cell extracts in parallel. Relative to the amount of total protein applied to the polyacrylamide gel, G9 was enriched more than 8-fold in MVs compared to the whole cell extract (Figure 19A). By comparison, A16 was enriched more than 16-fold (Figure 19A). The relatively lower percentage of G9 in MVs compared to A16 may be due to the higher than normal and slightly delayed expression that occurs with the inducible T7 promoter system.

Membrane proteins are generally extractable form virions with NP-40 detergent. Accordingly, we treated the vG9i MVs with NP-40 detergent in the presence or absence of DTT at 37°C. However, no G9 was extracted under either condition (Figure 19B). Previously characterized MV membrane and core proteins, A14 and A3 (P4b) were analyzed as controls. A14 was partially extracted with NP-40 alone and completely extracted with NP-40 plus DTT, whereas 4b was totally insoluble (Figure 19B), as expected for membrane and core proteins, respectively. We had previously found that A16 resisted extraction with NP-40 but could be partially released when 150 mM NaCl was added. However, G9 was not extracted with NP-40 in the presence (Figure 19B). The myristoylated glycine and the C-terminal transmembrane domain may contribute to

the low solubility of G9 and A16; however, L1 is easily extracted with NP-40 and it has a myristoyl residue and a C-terminal transmembrane domain.

We used a second method to investigate the membrane-association of G9 that did not depend on its solubility in a non-ionic detergent. MVs were purified from cells infected with vG9i in the presence of IPTG and biotinylated with sulfo-NHS-SS-biotin, a membrane-nonpermeating reagent. Subsequently, the excess biotin was quenched and the virions were collected by centrifugation and lysed by heating in SDS. Biotinylated and non-biotinylated proteins were separated using NeutrAvidin beads and the bound and unbound fractions were analyzed by western blotting. In mock-treated MVs, G9 was associated with the unbound NeutrAvidin fraction whereas in biotinylated MVs G9 was almost entirely in the bound fraction (Figure 19C). As controls, western blotting was also carried out with antibody to A28, another component of the entry-fusion complex, and with A4, a core protein. The proportion of biotinylated A28 was similar to that of G9 whereas little A4 bound to the NeutrAvidin beads indicating that the MVs were largely intact (Figure 19C). We concluded that G9 is exposed on the surface of the MV membrane but that it is insoluble in non-ionic detergent.

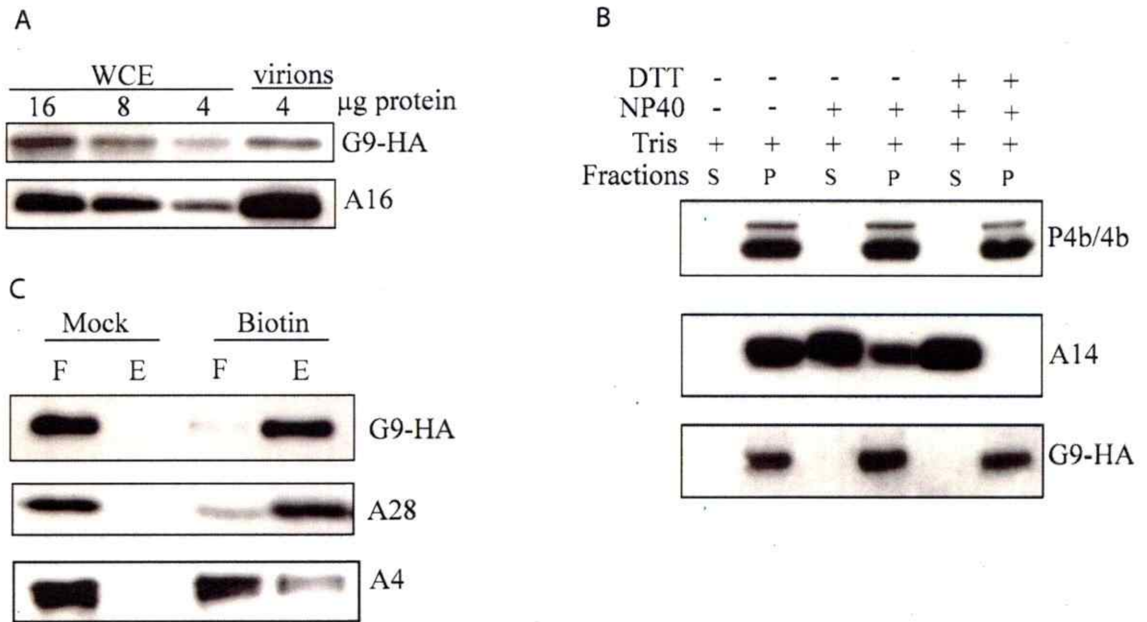


Figure 19. Association of G9 protein with purified virions. (A) Whole cell extracts (WCE) or sucrose gradient purified virions from cells infected with vG9i in the presence of IPTG were analyzed by SDS-PAGE and western blotting using anti-HA MAb. The amounts of total protein (4 to 16 μg) applied to the gel are indicated (B) Extraction of G9 from purified virions. Virions prepared as in panel A were treated with 1% NP-40 with or without 50 mM DTT for 1 h at 37^{0}C and separated into soluble (S) or insoluble (P) fractions. Proteins in both fractions were resolved by SDS-PAGE, followed by western blotting with anti-A3 (P4b/4b) and anti-A14 polyclonal antibody and anti-HA MAb for G9. (C) Biotinylation of surface proteins. Purified virions prepared as in panel A were treated or mock treated with sulfo-NHS-SS-biotin. Excess biotin was quenched and the virions were recovered by sedimentation, disrupted with SDS and DTT and allowed to bind to a NeutrAvidin column, which was then washed. The unbound flow through (F) and the bound and eluted (E) materials were resolved by SDS-PAGE and analyzed by western blotting with anti HA MAb, and anti-A4 and anti-A28 polyclonal antibodies.

2.3 VACV morphogenesis was unaffected by repression of G9.

The processing of core protein precursors to their mature products is coordinated with MV morphogenesis and interference with the latter prevents cleavage (Moss et al., 1973). We found that the ratio of the core protein precursor of 4b to the product 4b was similar in the presence and absence of IPTG (Figure 20A) consistent with normal morphogenesis.

Thin sections of BS-C-1 cells infected with vG9i in the presence or absence of IPTG were examined by transmission electron microscopy to directly assess the role of G9 in morphogenesis. Either with (not shown) or without IPTG, all stages of virion assembly were seen including crescents and immature virions (Figure 19B), MVs (Figure 20 C) and EVs (Figure 20D).

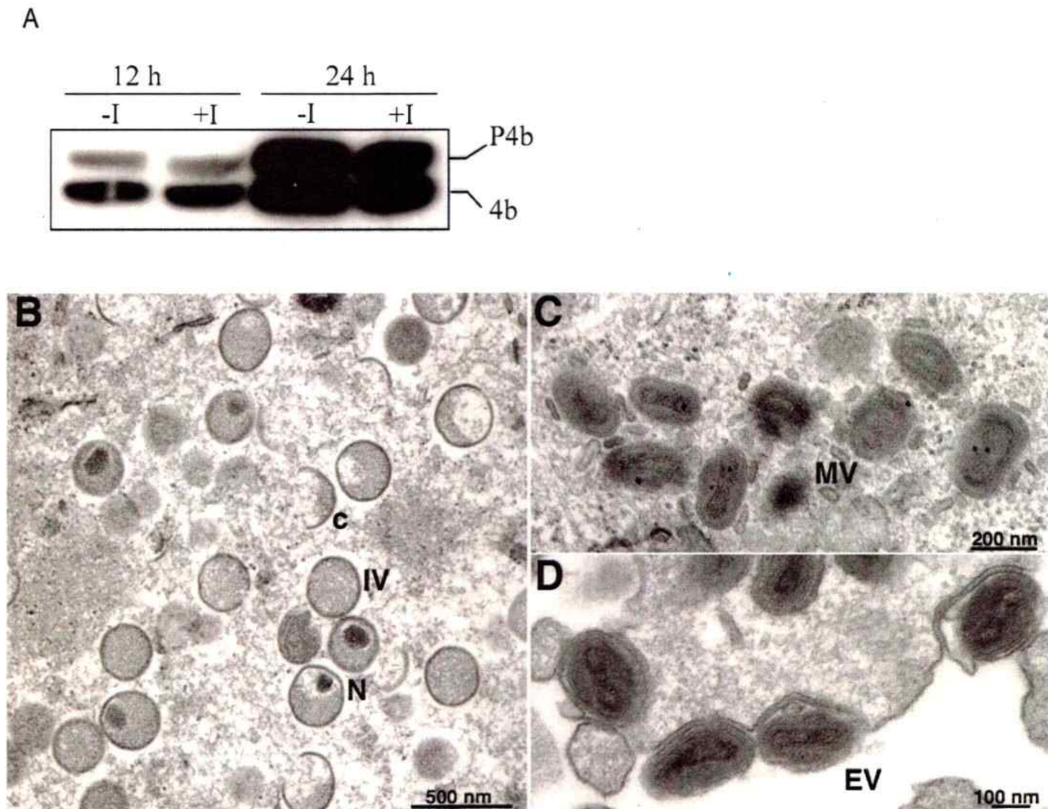


Figure 20. Core protein processing and viral morphogenesis. (A) Cleavage of P4b. BS-C-1 cells were infected with 5 PFU per cell of vG9i in the absence or presence of 50 μM IPTG (I). After 12 and 24 h, whole cell extracts were prepared and analyzed by SDS-PAGE and western blotting with antibody to P4b/4b. (B, C, D) Electron microscopy. BS-C-1 cells were infected with 10 PFU per cell of vG9i for 21 h in the absence of IPTG. Ultrathin sections were prepared and examined by transmission electron microscopy Abbreviations: C, crescent; IV, immature virion; N. immature virions with nucleoid; MV, mature virion; EV, extracellular virion. Magnification indicated by bar.

2.4 Characterization of vG9i MVs made in the absence of IPTG.

Because vG9i must be propagated in the presence of IPTG, the virus used for all infections thus far contained G9; consequently only the effects of repressing further G9 synthesis was studied. It was important, however, to compare the properties of vG9i virus made in the absence of IPTG (called -G9 virus although as will be seen it is not totally lacking G9) with that made in the presence of IPTG (called +G9 virus). In each case, MVs were purified by sedimentation twice through a sucrose cushion and banded once on a sucrose gradient. The specific infectivity, defined as the ratio of PFU to virus particles estimated from the $OD_{260\ nm}$, was more than 20-fold higher for +G9 virions than for -G9 virions. This residual infectivity was explained by the presence of a low amount of G9 in the virions. Although G9 could barely be detected when equal amounts of –G9 and +G9 virions were used for western blotting, the protein was clearly detected when 6 times more –G9 than +G9 virus was analyzed (Figure 21A). Despite the deficiency of G9, the two virus preparations could not be distinguished from each other or from "wild type" VACV WR by silver staining polypeptides following SDS-PAGE (Figure 21B). Furthermore, western blotting with available antibodies to other membrane proteins demonstrated the presence of similar relative amounts of A28, A21, L5, and A16 as well as an additional membrane protein H3 in all three MV preparations (Figure 20C). Note that at this loading, the low amount of G9 was not detected in –G9 virions; G9 was not detected in wild type VACV WR because of the absence of the HA epitope tag (Figure 21C).

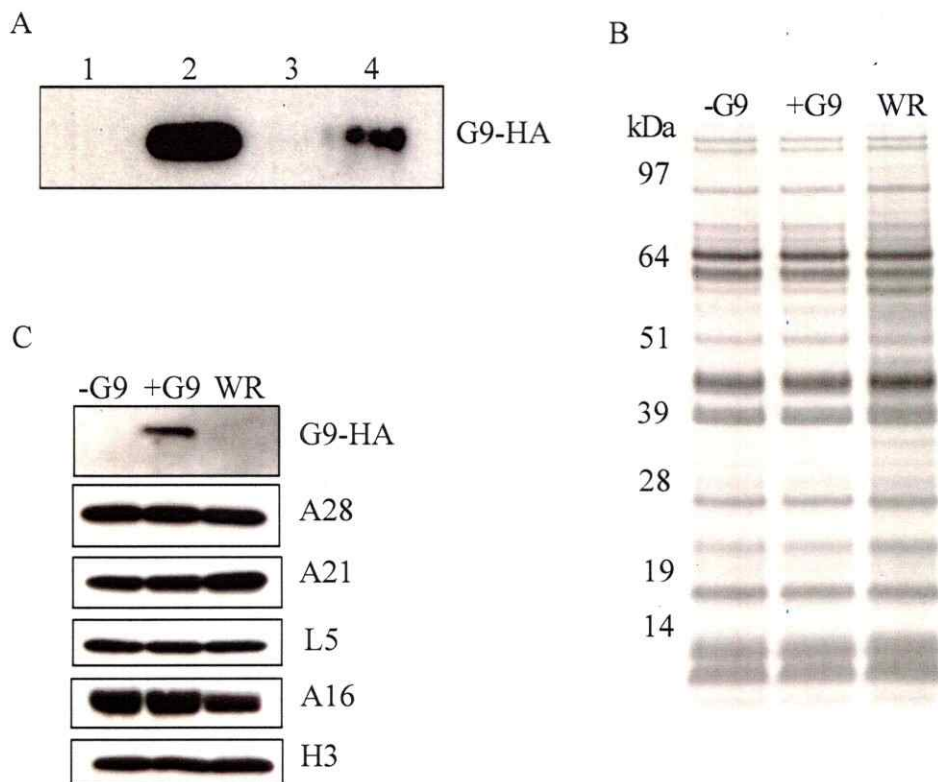


Figure 21. Polypeptide composition of purified virions. (A) SDS-PAGE and western blotting with anti-HA MAb of G9 from purified virions made in the presence (+G9) or absence (-G9) of IPTG. Lanes 1, 2 and 3 were loaded with equal amount of WR (1), +G9 (2) and –G9 (3) virions. Lane 4 correspond six times more of +G9 virions (B) Equal numbers of purified +G9, -G9 and VACV WR virus particles were analyzed by SDS-PAGE and silver staining. The mobilities of standard proteins of indicated mass are shown on the left. (C) SDS-PAGE and western blotting with polyclonal antibodies to A28, A21, L5, A16, and H3 from purified –G9 and +G9 virions. G9 was probed with anti-HA MAb as in panel A.

2.5 MVs deficient in G9 are defective in cell entry.

The normal appearance and polypeptide composition of MVs deficient in G9 and the physical association of G9 with other entry-fusion proteins strongly suggest that G9 has a role in entry. We used two different methods to compare the entry of +G9 and –G9 MVs. The first depended on the presence of the viral RNA polymerase and other transcription factors in the virus core, which allows viral mRNA synthesis to occur soon after penetration into the cytoplasm. Cells were infected with purified vG9i MVs made in the presence or absence of IPTG. After 3 h of incubation in the presence of AraC to prevent viral DNA replication and late gene expression, total RNA was extracted. The mRNAs were resolved by electrophoresis in a denaturing agarose gel, transferred to a membrane, and hybridized to a ^{32}P-labeled DNA probe that was complementary to the transcript of the early A20R gene. An intense band corresponding in size to A20 mRNA was detected in cells infected with +G9 virus, whereas that mRNA was barely detected in cells infected with –G9 virus (Figure 22). Glyceraldehyde-3-phosphate dehydrogenase mRNA, used as a loading control, indicated that equivalent amounts of cytoplasmic mRNA were present in each sample (Figure 22).

As with vA16i, we used another procedure, based on antibody detection of uncoated cores in the cytoplasm. This method is more specific for the entry step but less sensitive than measurement of early transcription. HeLa cells were incubated with purified –G9 and +G9 virions at 4^0C to allow binding. After washing to remove unbound and loosely bound virions, the cells were incubated for 2 h at 37^0C to allow penetration. This step was done in the presence of cycloheximide, a protein synthesis inhibitor, to prevent the dissolution of cores and cytopathic effects. A MAb to the L1 MV membrane protein was

used to detect virions on the cell surface and antibody to the A4 core protein to detect intracellular cores. Similar numbers of +G9 and –G9 MVs were bound to cells after incubation at 4°C indicating no defect in attachment (Figure 23). In contrast, after incubation at 37°C there were numerous cores of +G9 virus in the cytoplasm but very few of -G9 virus (Figure 23).

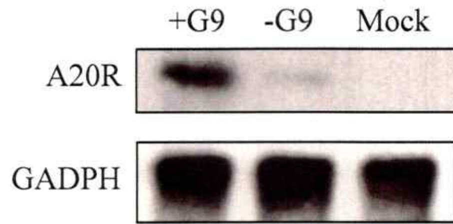


Figure 22. Analysis of early transcription in cells infected with purified –G9 and +G9 virions. BS-C-1 cells were mock infected or infected with 5 PFU per cell of +G9 virions or the equivalent $OD_{260\ nm}$ of –G9 virions in the presence of AraC. Total RNA was extracted at 3 h after infection. Northern blot analysis was carried out using [α^{32}-P]dCTP-labeled DNA probes complementary to the VACV A20R and GADPH cellular transcripts. The radioactivity was quantified using a phosphoimager.

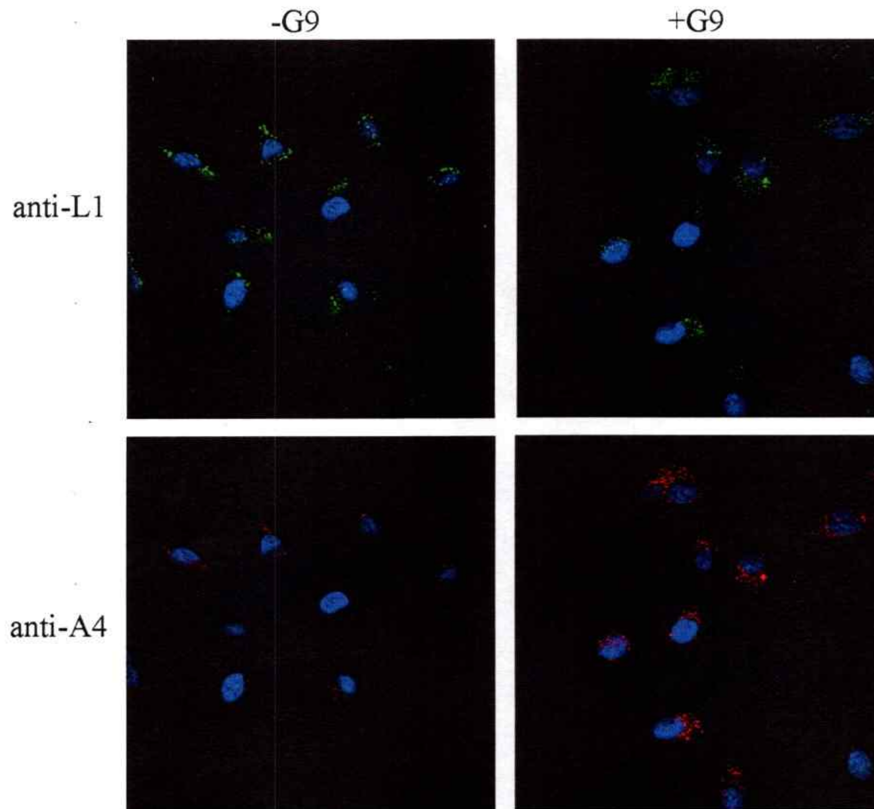


Figure 23. Virus binding and entry. Detection of extracellular virions and intracellular cores. HeLa cells were infected with 5 PFU per cell of +G9 virions or the equivalent $OD_{260\ nm}$ of –G9 virions in the presence of 300 μg of cycloheximide per ml for 1 h at 4^0C. At this time some cells were washed, fixed and permeabilized with 0.1% Triton X-100 and then incubated with anti-L1 mouse MAb and anti-A4 rabbit polyclonal antibody followed by fluorescein isothiocyanate-conjugated goat anti-mouse (green) and rhodamine red-X-conjugated goat anti-rabbit antibody (red), respectively. DNA was visualized by DAPI staining and optical sections were obtained by confocal microscopy. After 1 h at 4^0C, some cells were incubated for an additional 2 h at 37^0C, and then treated and visualized as above.

2.6 G9 is required to induce low pH-triggered cell-cell fusion.

VACV-infected cells form syncytia after brief exposure to low pH (Doms et al., 1990). To determine whether G9 expression is required for fusion from within, BS-C-1 cells were infected with vG9i in the presence or absence of IPTG for 21 h, briefly exposed to low pH, and then incubated for an additional 3 h in normal medium. As shown in Figure 23B, cells infected in the presence of IPTG formed large multinucleated syncytia whereas cells infected in the absence of IPTG did not.

Formation of syncytia can also be triggered by briefly lowering the pH immediately after adding a high multiplicity of MVs to cells (Gong et al., 1990). This phenomenon, called fusion from without, also requires the entry-fusion proteins studied thus far. To determine whether G9 is required for fusion from without, 200 PFU of +G9 virions or the equivalent number of –G9 virions was added per cell. The cells were then briefly treated with pH 5.0 or 7.0 buffer and incubated in regular medium for 3 h in the presence of cycloheximide to prevent cytopathic effects. Extensive cell-cell fusion was observed only in monolayers infected with +G9 virions and exposed to low pH (Figure 24A). Thus, G9 is required for VACV-induced fusion from within and without.

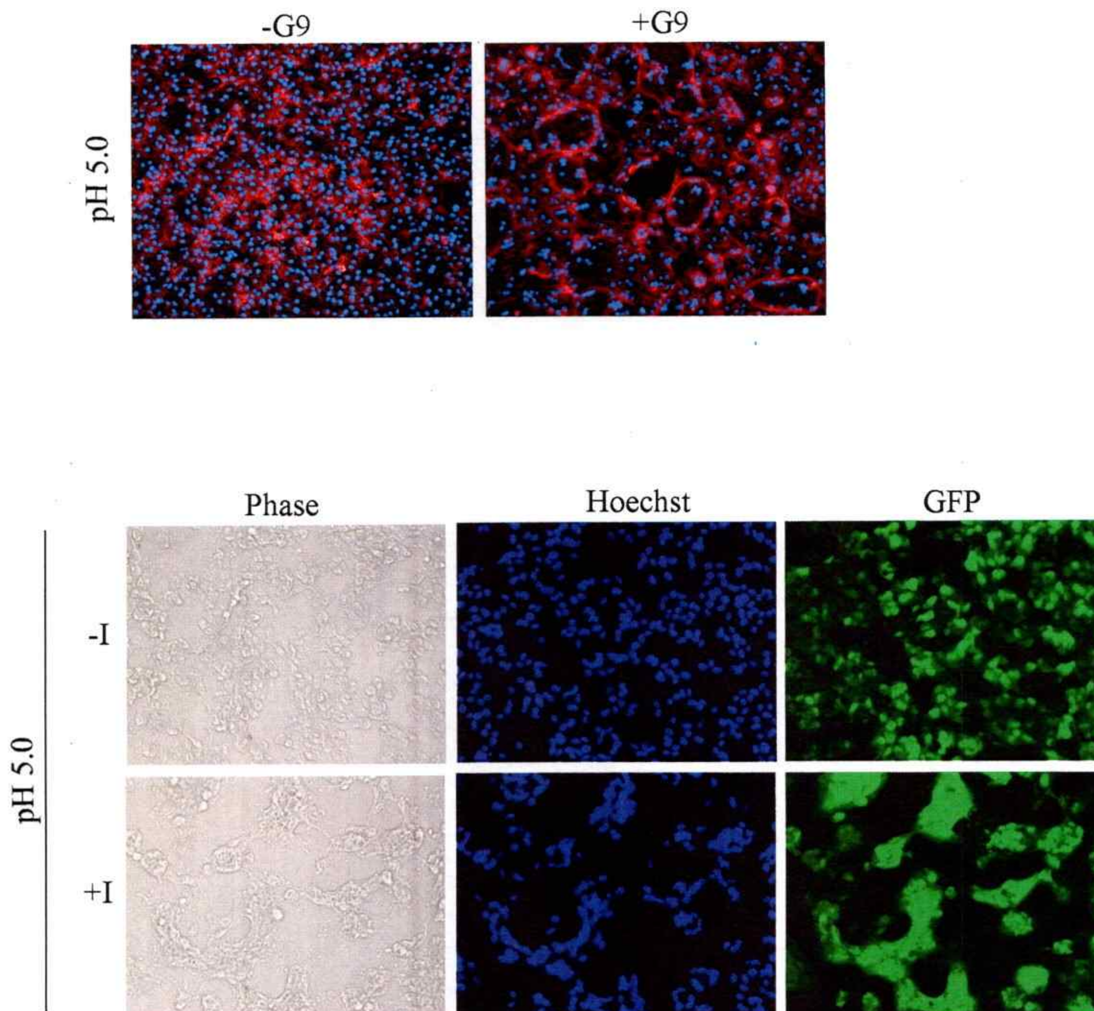


Figure 24. (A) Fusion from within. BS-C-1 cells were infected with 2 PFU per cell of vG9i in the absence (-I) or presence (+I) of 50 μM IPTG for 18 h at 37^{0}C followed by a brief exposure to pH 5.0 or 7.4 buffer. The buffer was replaced with regular medium and the incubation continued for 3 h. The cells were stained with Hoechst dye and visualized by phase and epifluorescence microscopy for DNA and GFP expression. (B) Fusion from without. BS-C-1 monolayers were incubated with 200 PFU per cell of +G9 virions or the equivalent $OD_{260\ nm}$ of –G9 virions at 4^{0}C for 1 h. Unabsorbed and loosely bound virions were removed by washing and the cells were briefly treated with pH 5.0 or 7.4 buffer at 37°C and then incubated in regular medium containing 300 μg of cycloheximide per ml for 3 h. The cells were fixed and stained with DAPI and Alexa Fluor 594-phalloidin to visualize DNA and the actin cytoskeleton, respectively.

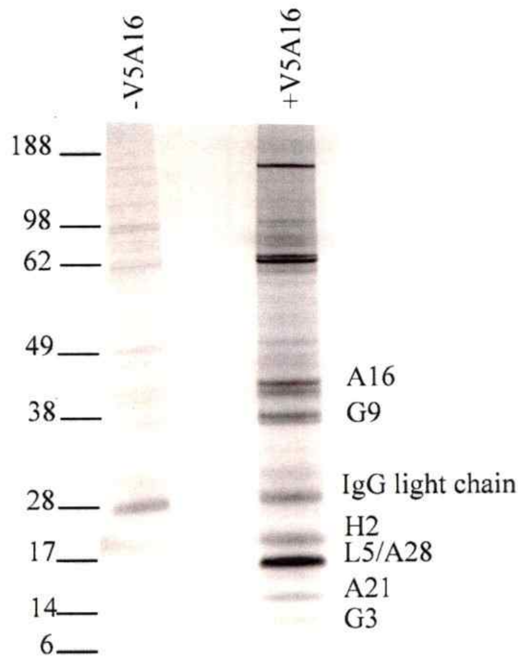
3. Protein-protein interaction studies between A16, G9 and other vaccinia proteins.

Recent reports from the literature provide evidences for the existence of a putative vaccinia entry/fusion complex (Senkevich et al., 2005). Using immunoaffinity purification followed by mass spectrometry, eight vaccinia membrane proteins, namely A16, A21, A28, H2, G9, G3, J5 and L5, were found in such complex. They are conserved in all poxvirus suggesting that they have nonredudant functions. These proteins can be structurally classified in two groups. First group contains A21, A28, G3, H2 and L5 with a N-terminal transmembrane domain, 0-2 intramolecular disulfide bonds and C-terminal segment exposed on the MV surface. Second group contains A16, G9 and J5 with a C-terminal transmembrane domain, 4-10 intramolecular disulfide bonds and an N-terminal exposed outside the particle. Although four of the proteins were shown to form intramolecular disulfide bonds through the vaccinia cytoplasmic redox-pathway (Townsley et al., 2005; Senkevich et al., 2004), reduced forms of the entry-proteins were able to form the complex, suggesting that they will be necessary for function and/o stability of the complex. Interestingly, conditional-lethal null mutants for A28, H2, L5 and A21 had similar phenotype to A16 and G9 mutant virus (Ojeda et al., 2006; Ojeda et al., 2006; Senkevich et al., 2005; Senkevich et al., 2004; Townsley et al., 2005; Townsley et al., 2005). In conditions were their expression was repressed, the morphogenesis was normally, noninfectious MVs attached to cells but their cores cannot penetrate into the cytoplasm. As A16 and G9, they were also required for virus-induced low pH fusion from within and without.

The initial characterization of such complex was carried out using vA28i recombinant virus, which inducibly expresses A28 protein. However, a single large complex or

multiple subcomplexes may be form with A28. To investigate this possibility, we performed immunoprecipitations using the V5-epitope tag on A16 protein. Cells were infected with the vV5A16i recombinant virus that expresses the A16 protein with an N-terminal V5 epitope tag, in the presence or absence of inducer or with vT7LacOI. Analysis of solubilized cell extracts were analyzed by silver staining after SDS-PAGE and by Western blot. As shown in Figure 24A, the pattern of silver stained bands obtained after affinity purification of the complex with V5 antibody from vV5A16i extracts were similar to that obtained with A28 coimmunoprecipitation. The Western blot analysis showed that A21, A28 and L5 were captured with V5-tagged A16, confirming that they were in complex with A16 (Figure 25B). In parallel we did the same experiments using the vG9i recombinant virus, which inducible expresses the G9 protein with an N-terminal HA epitope tag. However, we were unable to immunoprecipitated G9 using the HA-agarose conjugated beads, suggesting that the HA epitope tag of G9 may be unaccesible to the antibody. Since we know that A16 immunoaffinity purification can capture the other components of the entry complex (Figure 25B), we used A16 polyclonal antibody to demonstrate that G9 specifically bind to such complex too. To address this question, BS-C-1 cells were infected with 5 PFU of vG9i in the presence or absence of IPTG. Membrane proteins from whole infected cell were solubilized in 1% NP-40 and clarified extracts were incubated with rabbit polyclonal A16 antibody followed by protein A-agarose beads. Analysis of solubilized extracts by Western blot indicated that G9-HA protein coimmunoprecipitated with A16, confirming that they were in a complex (Figure 26). Thus, proteins in the entry complex

were always detected independently of which protein was used for immuoaffinity purification studies.



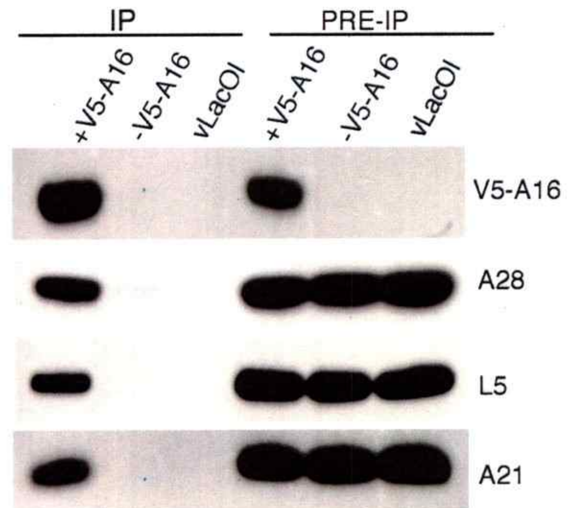


Figure 25. Immunoaffinity purification of proteins associated with V5-tagged A16. BS-C-1 cells were infected with 5 PFU of vV5A16i in the presence or absence of IPTG or with control vT7LacOI. Cells were lysed and the proteins in the NP-40-soluble fraction were incubated with ant-V5 MAb immobilized to agarose beads and for 4 hours followed by washes. Eluted proteins were analyzed by SDS-PAGE followed by silver staining (A) and Western blotting (B). Samples before IP (pre-IP) or after IP immunoaffinity purification were analyzed by Western blotting using the V5 epitope tag and the A28, A21, L5- polyclonal antibodies describe above (B).

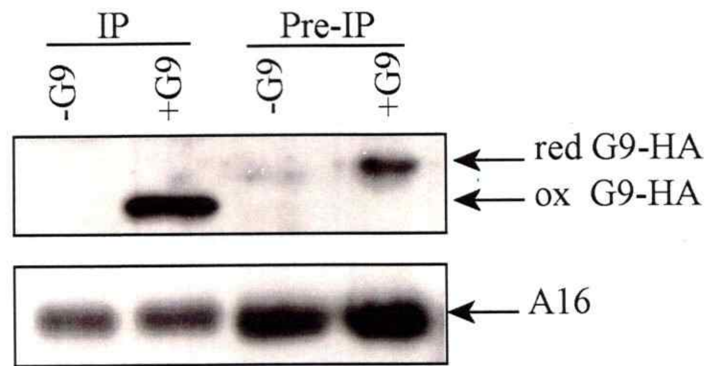


Figure 26. Analysis of the interaction between A16 and G9 proteins. BS-C1 cells were infected with 5PFU of vG9i in the presence or absence of IPTG. Cells were harvested and spinned and cell pellet was resuspended in 10 mMTris-HCl at pH 9.0. Nuclei were separated and soluble fraction was loaded in a 36% sucrose cushion. The enriched membrane was pellet, washed in 1 mM Tris pH 9.0 and solublized in 1% NP-40 detergent and 1X PBS. The proteins in the NP-40 soluble fraction were incubated with agarose beads to remove proteins nonspecifically bound to beads and then with the anti-A16 polyclonal antibody. Beads were washed and proteins were eluted in the presence of SDS-loading buffer and heat. The G9-HA protein co-purifing with A16 was analyzed by Western blotting using anti-HA Mab-HRP conjugated.

DISCUSSION

I have characterized two components of VACV MV membrane: A16 and G9 proteins. Our finding that A16 and G9 are MV proteins is in agreement with a recent mass spectroscopic analysis of the entire MV proteome (Chung, CS, 2006). Both proteins are cystein-rich with a myristic modification on the glycine residue immediately following the initial methionine. A16L ORF encoded for a major polypeptide of ~ 43 kDa and a minor one of ~ 23 kDa. The smaller species contained the C terminal region of A16L ORF and it was also detected when transfected in infected cells, suggesting the presence of a cryptic atypical promoter. In the case of G9 ORF only one major product of ~39 kDa polypeptide corresponding to the G9 full length was detected. Other important feature is their cysteine-rich domain in the C terminal of both proteins. In the A16 protein, 10 of its 20 cysteines formed intramolecular disulfide bonds through the viral cytoplasmic redox pathway. However, the unavailability of specific antibodies against G9 and its low solubility in the assay conditions, did not allow us to study the formation of intramolecular bonds.

A16 and G9 were found in association with the MV-membrane through their very C-terminal transmembrane domain. The trypsin sensitivity of A16 protein on the MV and the labeling of G9 in purified MVs with a membrane non-permeating biotinylation reagent demonstrated that A16 and G9 are exposed on the surface of the particle. Neither A16 nor G9 could be extracted with NP-40 detergent in the presence or absence of DTT unlike other viral membrane proteins. However, we found that the A16 but not G9 protein could be partially solubilized in the presence of detergent and NaCl, suggesting

that some protein-protein interaction may be retaining them in the insoluble fraction. Both proteins were more efficiently solubilized from the membrane fraction of infected cells, which would presumably contain IV membranes, than from purified virions. Similarly, the complex of entry-fusion proteins was more efficiently isolated from infected cell membranes than virions (T. Senkevich, personal communication).

When experiments to study the requirement of both proteins for viral replication were carried out, we were unable to isolate a A16 null mutant by homologous recombination in mammalian cells, suggesting that A16 was essential for viral replication. Similarly, a bacterial artificial chromosome containing the complete VACV genome (Domi, A, 2002), from which the G9L ORF was deleted, could not be rescued by fowlpox from transfected cells. However, a DNA fragment containing the G9R ORF provided in trans allowed rescue of infectious VACV, again suggesting that either G9 expression is essential or deletion of the gene severely impairs infectivity preventing us from isolating deletion mutants.

In order to study the function of the G9 and A16 proteins during the viral cycle, we used a reverse genetic approach to obtain a conditional lethal mutant. Thus, an inducible copy of either gene was used to replace its endogenous copy allowing the regulation of their expression by the addition of an inducer. The inducible mutants vG9i and vA16i made tiny plaques in the absence of inducer and virus yields in a single cycle of replication were severely reduced. The residual growth under non-permissive conditions could be due to incomplete suppression of G9 and A16 expression in the absence of inducer. Nevertheless, we cannot exclude the possibility that neither A16 nor G9 are absolutely required. It is known that a complete repression of gene expression is difficult to achieve

with inducible systems and the degree of inhibition of virus replication depends on positions effects of the target gene within the genome and the amount of the protein needed to fulfill its role in replication. This is consistent with our inability to isolate deletion mutants of each gene, suggesting that both genes are essential (as we believe) or are needed for efficient replication.

Despite low-level replication the phenotype of vA16i and vG9i was clear. In the absence of inducer virus morphogenesis appeared normal, including the presence of EVs on the cell surface, indicating that virus can exit the cell normally. The proteolytic cleavage of core proteins, another marker of normal morphogenesis, proceeded normal in the absence of inducer. Moreover, the overall polypeptide composition of virions made in the absence of inducer (-A16 and –G9) was similar to virions made in the presence of inducer as determined by SDS PAGE. They content similar amounts of other membrane proteins as A21, A28, L5, H3 and A27. The specific infectivity of purified MV deficient of A16 or G9 was 2% and 5% of that of MV containing wild type levels of A16 or G9 protein respectively. We also demonstrated that permeabilized virions made in the absence of A16 synthetized normal amounts of RNA in vitro, suggesting that differences in infectivity could not attributed to a defect in early transcription.

Alternatively, a defect in entry could explain the phenotype of vG9i and A16i viruses under non-permissive conditions. To test this hypothesis, MV entry was observed directly by confocal microscopy using antisera against MV core and membrane proteins. This approach demonstrated that the -A16 and –G9 virions could attach to cells but were unable to release cores into the cytoplasm. Consistently with this finding, the expression

of early viral mRNA, after infection with equal amounts of purified MVs, was greatly reduced despite their inherent normal ability to transcribe RNA in vitro.

Previous reports have shown that MVs membrane proteins A21, A28, H2 and L5 were required for entry and cell-cell fusion, indicating a relation between these two events. We presume that the MVs fuse with the plasma membrane of one cell which them fuse with and another cell using the fusion machinery left behind by the MVs (Moss, 2005). A similar phenotype was found in inducible A16 and G9 virus mutants, suggesting that they are part of the same viral fusion machinery.

A recent characterization of a poxvirus entry-fusion complex, comprising eight conserved vaccinia proteins, identified A16 and G9 as putative members of this complex (Senkevich, T, 2004). Immunoafinity purification studies showed that A28, A21, L5 and G9 proteins copurified with A16, confirming that they formed complex. However, their specific role in the complex appears to be different. Studies using conditional lethal mutants of each of these proteins, indicated that (i) A28 was needed for formation of the complex; (ii) A21 associated with L5 in the absence of A28; (iii) in the absence of G9 or A16 the formation of the protein complex was drastically perturbed (Senkevich, T 2005). Nevertheless, we observed equivalent amounts of the other entry-proteins in –A16 and –G9 virions compared to +A16 and +G9 virions, indicating that neither A16 nor G9 were needed for their expression or for trafficking them to the viral membrane. It is possible that the proteins in the complex have multiple, non-redundant roles including membrane fusion per se, activation of fusion, receptor recognition, and a scaffolding function. The requirement of all these MV membrane proteins for entry and virus-induced cell-cell fusion together with no evidence that the EV membrane is directly

involved in membrane fusion, suggests that MV membrane is the fusogenic membrane. Thus, we believe that the one membrane (MV) and the two membrane (EV) particle will share a common final step that involves the MV membrane containing the entry/fusion complex. However, the molecular mechanism of membrane fusion catalysed by vaccinia viral membrane-fusion proteins is not yet understood. The existence of a putative fusion peptide in one of the fusion proteins or a fusion-protein cooperativity during membrane insertion and fusion, has not been determined. The requirement of several proteins for viral entry has been described only in members of the Herpesviridae (Spear and Lockneker, 2003). Orthologs of three glycoproteins, designated gB, gH, and gL, are essential for entry of all herpesviruses. In addition to the three basic fusion proteins, some herpesviruses require additional non-conserved receptor-binding proteins such as gD for most alpha herpesviruses. Because the number of proteins involved in poxvirus entry seems to be higher than for other viruses, determination of the mechanism is expected to be challenging.

Although not well characterized, two possible mechanisms of entry have been proposed: binding of the MVs to an unknown receptor or receptors on the plasma membrane allowing neutral pH fusion (Armstrong, J. A, 1973; Carter, G. C, 2005; Chang, A., 1976) or via an internalization mechanism (Dales, S., 1964; Payne, L. G., 1978) that is mimicked by low pH-induced syncytia formation (Doms, R. W., 1990; Gong, S. C., 1990). Additional evidence showing that vaccinia entry was sensitive to endosomal acidification inhibitors indicates that MV particles can enter cells through a low-pH endocytic route (Townsley, A, 2006). However, the contribution of both pathways to vaccinia entry seems to be quite different. A large number of plasma membrane fusion

events were observed when virions bound to uninfected cells were briefly exposed to low pH but very few plasma membrane fusion events if they were treated with neutral pH (Townsley, A, 2006). Low pH appears to have multiple roles. Low pH disrupts the EV wrapper (Vanderplasschen, 1998) and synchronizes the fusion of MVs particles with the plasma membrane (Ichihashi, 1996; Townsley, 2006). On the other hand, the VACV-syncytia formation triggered at low pH is dependent on the vaccinia entry proteins A28, H2, A21, L5, A16 and G9. Thus, exposure to a low pH could provide the transition from a metastable pre-fusion state to an activated complex. The existence of a dual pathway for virus penetration may explain why vaccinia virus infects a wide variety of cells.

CONCLUSIONS

. A16 and G9 are conserved in all poxviruses. As structural features they have a C-terminal transmembrane domain, a high number of conserved cysteine residues and they are myristylated on their N-terminus.

. A16L and G9R vaccinia ORF encodes for a 43 and 39 kilodalton polypeptides respectively.

. A16 forms 10 intramolecular disulfide bonds using the viral cytoplasmic redox pathway.

. A16 synthesis start at 6 h post infection and its expression is inhibited in the presence of a DNA replication inhibitor, indicating it belong to the late class of genes.

. A16 and G9 are incorporated in the MV membrane but they are not solubilized with NP-40 detergent.

. Trypsin digestion experiments and virion surface labeling with an impermeant reagent indicates that A16 and G9 are anchored through their C-terminal hydrophobic domain to the viral membrane with the N-terminus exposed on the MV surface.

. Unsucessful attempts to isolate a deletion A16 or G9 mutants suggests that both proteins are essential for viral replication and their functions are nonredundant.

. vA16i and vG9i recombinant virus made smaller plaques in the absence of IPTG.

. In the absence of A16 or G9, the synthesis of viral proteins and the processing of core proteins occur normally.

. Viral morphogenesis is unaffected by repression of either A16 or G9 proteins.

. Virions assembled in the absence of A16 or G9 have polypeptide composition similar to those assembled in the presence of A16 or G9 but the former are less infectious.

. The A16 or G9-deficient MV virions bind to the surface of cells but their cores do not penetrate into the cytoplasm, indicating that A16 and G9 are involved in entry.

. A16 and G9 proteins are required for vaccinia virus-induced fusion from within and without.

. Immuno affinity purification analysis indicates that either A16 or G9 are components of the putative vaccinia entry/fusion complex.

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