JOURNAL OF VIROLOGY, Jan. 2006, p. 51–61 Vol. 80, No. 1
0022-538X/06/$08.00+0 doi:10.1128/JVI.80.1.51–61.2006

Entry of Vaccinia Virus and Cell-Cell Fusion Require a Highly Conserved Cysteine-Rich Membrane Protein Encoded by the A16L Gene

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The vaccinia virus A16L open reading frame encodes a 378-amino-acid protein with a predicted C-terminal transmembrane domain and 20 invariant cysteine residues that is conserved in all sequenced members of the poxvirus family. The A16 protein was expressed late in infection and incorporated into intracellular virus particles with the N-terminal segment of the protein exposed on the surface. The cysteine residues were disulfide bonded via the poxvirus cytoplasmic redox system. Unsuccessful attempts to isolate a mutant virus with the A16L gene deleted suggested that the protein is essential for replication. To study the role of the A16 protein, we made a recombinant vaccinia virus that has the *Escherichia coli lac* operator system regulating transcription of the A16L gene. In the absence of inducer, A16 synthesis was repressed and plaque size and virus yield were greatly reduced. Nevertheless, virus morphogenesis occurred and normal-looking intracellular and extracellular virus particles formed. Purified virions made in the presence and absence of inducer were indistinguishable, though the latter had 60- to 100-fold-lower specific infectivity. A16-deficient virions bound to cells, but their cores did not penetrate into the cytoplasm. Furthermore, A16-deficient virions were unable to induce low-pH-triggered syncytium formation. The phenotype of the inducible A16L mutant was similar to those of mutants in which synthesis of the A21, A28, H2, or L5 membrane protein was repressed, indicating that at least five conserved viral proteins are required for entry of poxviruses into cells as well as for cell-cell fusion.

Vaccinia virus (VACV) is a member of the poxvirus family of large, double-stranded DNA viruses that replicate entirely in the cytoplasm (17). VACV strain Western Reserve (WR), the prototype member of the orthopoxvirus genus, contains approximately 200 genes of which 44 have a putative transmembrane domain (Poxvirus Bioinformatics Resource Center [www.poxvirus.org]). VACV membrane proteins have diverse essential and nonessential roles in virus-host interactions, virion assembly, intracellular movement, and cell-to-cell spread. A novel class of poxvirus membrane proteins, required for cell entry but not for virion assembly, was recently identified (20–22, 27, 28). These four VACV proteins (A21, A28, H2, and L5) are conserved in all sequenced poxviruses, have common features including N-terminal or near N-terminal transmembrane domains and cysteines that form two to four intramolecular disulfide bonds, and are located on the surfaces of infectious intracellular mature virions (IMVs). Each of the four proteins is also required for cell-cell fusion, implying that cell entry involves a related or identical fusion mechanism. Fusion of IMVs with the plasma membrane was previously suggested on the basis of biochemical and microscopic studies (4, 7, 8, 11, 15). Cellular receptor proteins that participate in cell entry, however, remain to be identified.

Following virus morphogenesis, IMVs are completely enveloped by an additional membrane containing an entirely different set of viral proteins; these proteins play roles in intracellular transport, egress, and cell-to-cell spread (reviewed in reference 25). The presence of multiple membranes has complicated models of VACV entry. However, the finding that the same proteins required for IMV entry are also required for cell-to-cell spread by extracellular virions necessitates the removal of the additional membrane prior to entry of the IMV (20–22, 27, 28). Disruption of the extra membrane in the low-pH environment of endosomes has been suggested (14, 29).

As a continuation of our studies of VACV proteins with putative transmembrane domains, we analyzed the product of the A16R (VACV WR 136) open reading frame (ORF). The A16R ORF is conserved in all poxviruses and has 20 cysteine residues predicted to form disulfide bonds and a C-terminal transmembrane domain (19). A previous study had determined that the A16 ORF is expressed late in infection as a soluble, myristylated protein (16). Here we show that the A16 protein is associated with the membrane of intracellular mature virions and that virions lacking A16 were unable to enter cells or induce low-pH-triggered cell-cell fusion. During the course of this analysis, A16 was detected as part of a complex containing the four other entry/fusion proteins (19b). These results, indicating that A16 is an additional member of the group of poxvirus entry/fusion proteins, was unanticipated because its predicted structural features differ from those of A21, A28, H2, and L5.

(This work was done to partially fulfill the Ph.D. thesis requirements of S. Ojeda at the University of Chile, Santiago, Chile.)

MATERIALS AND METHODS

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 (1) and vA16Li were propagated in HeLa S3 cells as described previously (12) and stored at −80°C.

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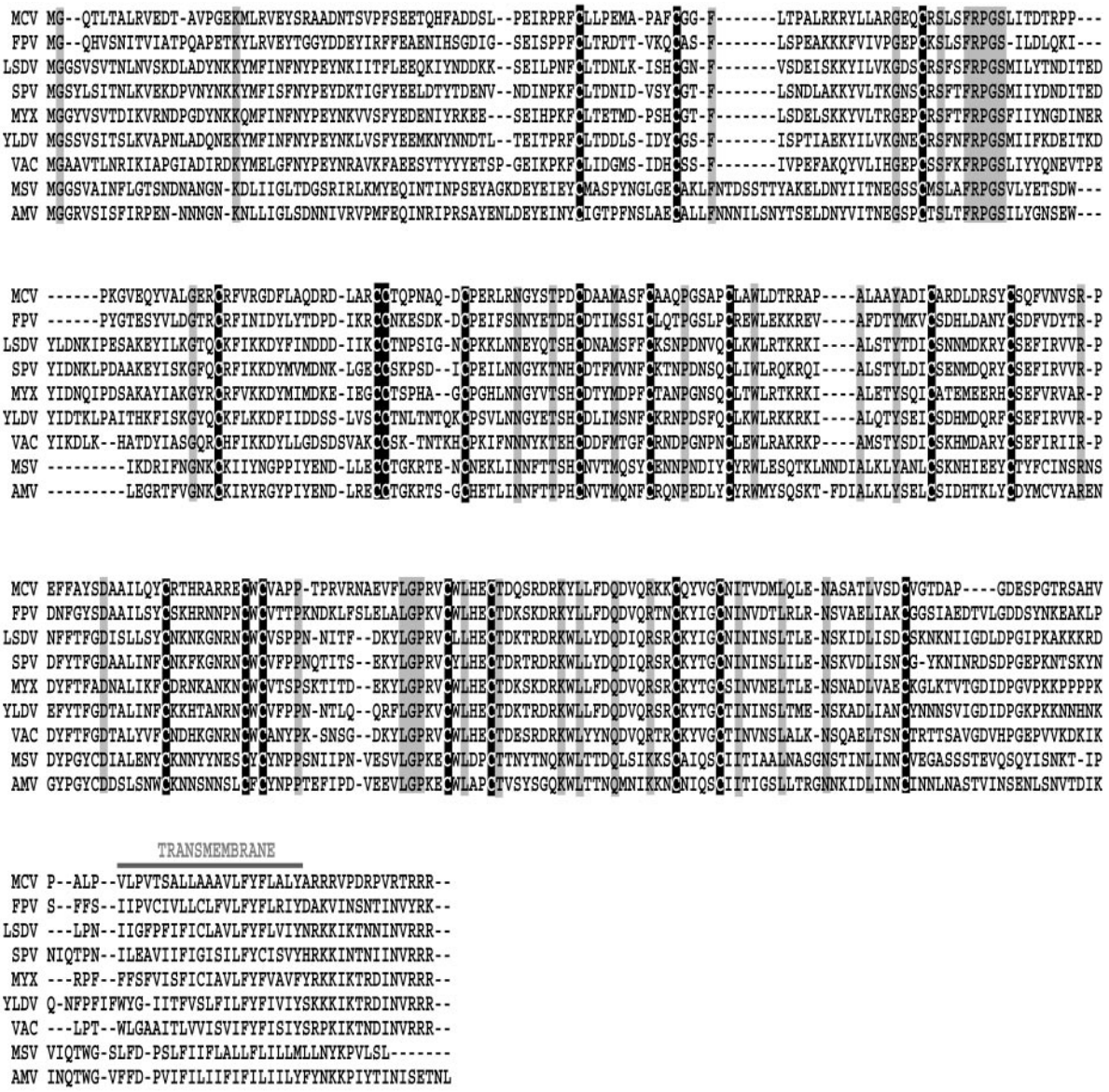

FIG. 1. Multiple-sequence alignment of A16 orthologs in poxviruses. One representative sequence from each genus of *Chordopoxvirinae* and the two complete sequences from each genus of *Entomopoxvirinae* are included in the alignment. MCV, molluscum contagiosum (*Molluscipoxvirus*); FPV, fowlpox virus (*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 by white letters on black background; other conserved residues are shown on a shaded background. Gaps introduced to maximize alignment (dashes) and the predicted transmembrane domain are indicated.

Generation of vA16Li. VT7lacOI, the parental virus of vA16Li, contains the *Escherichia 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 A16L ORF, (ii) a copy of the A16L gene regulated by a T7 promoter and *lac* operator, and (iii) the gene encoding β-glucuronidase under a synthetic early or late VACV promoter was prepared by overlapping PCR. The arrangement of genes is shown below (see Fig. 2A). A second inducible virus, vV5A16Li, which expresses an inducible A16 with a V5 epitope tag at the N terminus, was constructed in the same manner.

Antibodies. Rabbit anti-A16 serum was produced by immunizing a rabbit with a synthetic peptide corresponding to amino acids 364 to 378 (SRPKIKTNDINV RRR) of the predicted A16L ORF with an additional cysteine residue for protein conjugation (Covance Research Products, Inc., Denver, Pa.). Antisera to A27 and A28, made by immunizing rabbits with recombinant proteins, were provided by Gary Cohen and Gretchen Nelson, respectively. Rabbit polyclonal antibodies against the following VACV proteins were used: anti-A21 and anti-L5 (27, 28), anti-A14 (5), anti-A4 (10), and anti-p4b/4b (R. Doms and B. Moss, unpublished). Mouse monoclonal antibody to L1 was prepared from a hybridoma kindly provided by Alan Schmaljohn (31).

Western blot analysis. Cells were lysed in sodium dodecyl sulfate (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% nonfat dry milk in phosphate-buffered saline containing Tween 20 (9 g/liter of NaCl and 0.01% [vol/vol] Tween 20). The membranes were incubated with a 1:1,000 dilution of anti-A16 serum, and proteins were detected using a chemiluminescence kit (West-Pico; Pierce).

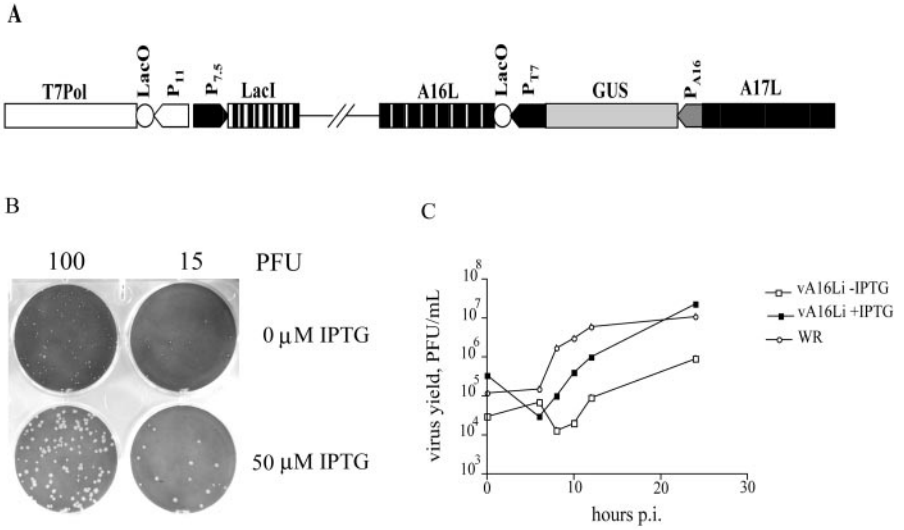

FIG. 2. Construction of an inducible A16L VACV mutant. (A) Genome of vA16Li. Abbreviations: P_{11}, a vaccinia virus late promoter; $P_{7.5}$, a vaccinia virus early-late promoter; *lacO*, *E. coli lac* operator; *lacI*, *E. coli lac* repressor gene; T7Pol, bacteriophage T7 RNA polymerase gene; P_{T7}, bacteriophage T7 promoter; P_{A16}, A16 promoter; GUS, β-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°C 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 vA16Li in the presence (+) or absence (−) of 50 μM IPTG. Cells were harvested at the indicated time (hours postinfection [p.i.]). Virus titers were determined by plaque assay in the presence of 50 μM IPTG.

Disulfide bond analysis. Cells were collected by centrifugation, solubilized in nonreducing SDS gel loading buffer (Invitrogen) containing 20 mM 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMS; Molecular Probes) or *N*-ethylmaleimide (NEM; Sigma). 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 immunoglobulin G (IgG) conjugated to horseradish peroxidase (Invitrogen), and detected by chemiluminescence.

Electron microscopy. Infected-cell monolayers were fixed with 2% glutaraldehyde, embedded in Epon resin, and viewed by transmission electron microscopy.

Immunofluorescence microscopy. HeLa cells were infected with 5 PFU of VACV per cell. After 12 h, the cells were washed and then fixed in 4% paraformaldehyde for 30 min at 4°C. The coverslips were washed in phosphate-buffered saline, and the cells were permeabilized in 0.1% Triton X-100. The cells were incubated with A16 peptide antibody (1:500) followed by an anti-rabbit IgG fluorescein isothiocyanate conjugate. Diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes) staining was used to visualize DNA in nuclei and viral factories. Images were collected on a Leica TCS NT laser-scanning confocal microscope.

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°C, and then 1 mM phenylmethylsulfonyl fluoride was added to stop the reaction. The supernatant and pellet fractions were separated by centrifugation for 30 min at 4°C and immediately boiled. Proteins were analyzed by Western blotting as described above.

Northern blotting. RNA was extracted by using the RNeasy Mini kit (QIAGEN), resolved by electrophoresis on a 1% agarose glyoxal gel, and transferred to a nylon membrane. DNA probes were labeled with [α-^{32}P]dCTP using the DECA prime kit (Ambion) and analyzed with a phosphorimager.

RESULTS

A16R is a highly conserved essential gene. The A16R 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 nonpoxvirus homologs were detected by a position-specific iterative BLAST search. A multiple-sequence alignment of A16R orthologs shows several conserved features, including a penultimate N-terminal glycine, 20 invariant cysteines, and a C-terminal hydrophobic domain (Fig. 1). The conserved glycine is consistent with previous evidence that the A16 protein is myristylated (16).

The conservation of A16R orthologs in all poxviruses, including a VACV strain that was highly attenuated by in vitro passage (3), suggested that the gene is essential for virus replication. Indeed, our attempts to isolate a deletion mutant by insertion of DNA encoding green fluorescent protein into the A16R ORF were unsuccessful. As an alternative, we made a recombinant virus called vA16Li by using the *E. coli lac* operator system to regulate A16R transcription as previously described for other VACV genes (23, 32). As depicted in Fig. 2A, vA16Li contains the *lac* repressor expressed continuously by an early/late VACV promoter and the T7 RNA polymerase gene adjacent to a late VACV promoter, which is regulated by the *lac* operator. The A16R gene was modified so that it is driven by a bacteriophage T7 promoter and regulated by the *lac* operator. In the absence of isopropyl-β-D-thiogalactopyranoside (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 A16R ORFs. In the presence of IPTG, however, the repressor should be inactivated to allow expression of T7 RNA polymerase and transcription of the A16R ORF.

vA16Li 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 (Fig. 2B). In a one-step growth experiment, replication of vA16Li was delayed and reduced by about 1.5 log units in the absence of inducer (Fig. 2C). Since a mutant with the deleted gene could

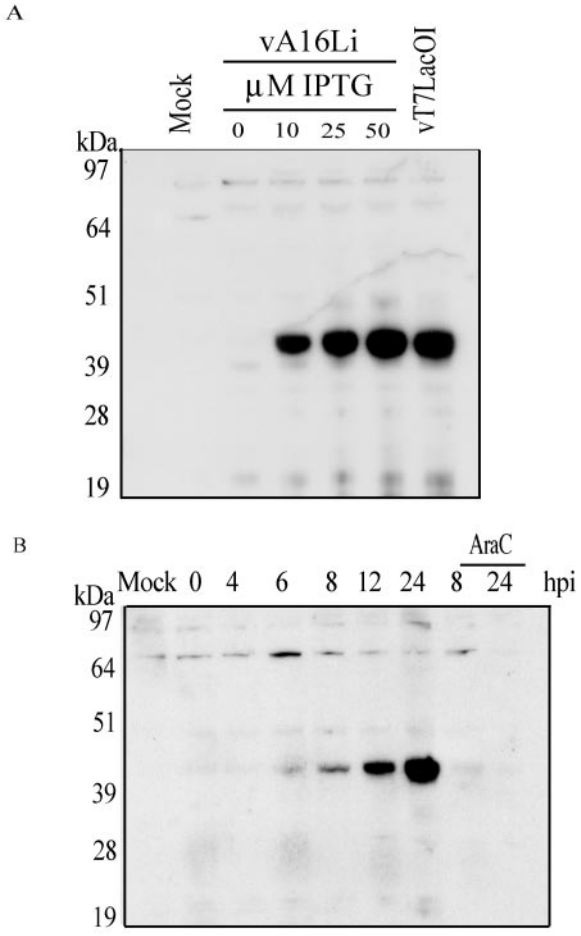

FIG. 3. Synthesis of A16 protein by vA16Li and VACV WR. (A) BS-C-1 cells were mock infected or infected with vA16Li in the presence of the IPTG concentrations shown above the gel. After 24 h, the cells were harvested and the total cell lysates were analyzed by SDS-PAGE and Western blotting using antibody 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 time (hours postinfection [hpi]), cells were harvested and analyzed by Western blotting as described above for panel A. The masses (in kilodaltons) of marker proteins in panels A and B are indicated on the left of the gels.

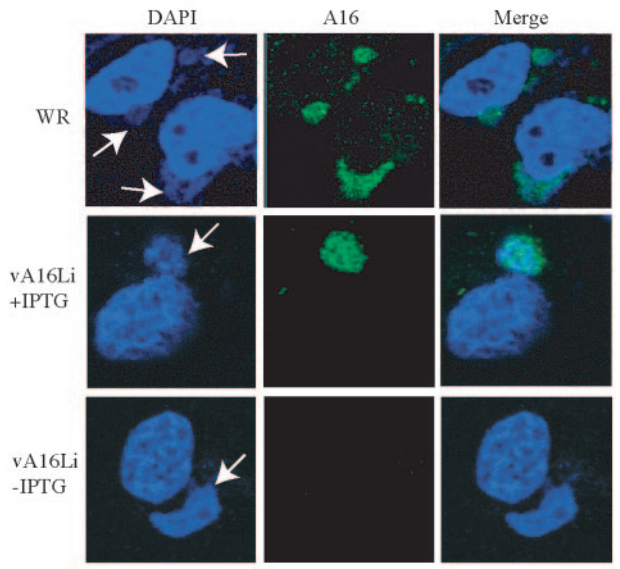

FIG. 4. Intracellular location of A16 determined by immunofluorescence. HeLa cells were infected for 12 h with vA16Li in the presence (+) or absence (−) of the inducer (IPTG) or infected with VACV WR. Cells were fixed, permeabilized, and stained with A16 peptide antibody and fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G, followed by DAPI. Confocal microscopy images are shown. Arrows point to virus factories stained with DAPI.

not be isolated, the low degree of replication could be due to incomplete repression of A16.

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 (Fig. 3A). The 43-kDa protein is the size predicted from the A16R ORF. In the absence of IPTG, a trace amount of the A16 protein was detected. If very little A16 were 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 (Fig. 3A).

Synthesis of A16 during a normal infection. The TAAATG transcription/translation initiator element characteristic of late promoters is present at the start of the A16R ORF, suggesting that the protein is expressed at the postreplicative stage of VACV infection. To investigate the kinetics of A16 synthesis, cells infected with VACV were harvested at different times 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 (Fig. 3B). A faint 23-kDa species, also seen when A16 expression was regulated by the T7 promoter, was detected, raising the possibility of an inefficient downstream translation initiation site, partial posttranslational cleavage, or a weak alternative promoter within the A16L gene. The presence of an appropriately located downstream translation site was not supported by examination of the A16R ORF, nor was a consensus vaccinia virus I7 proteinase cleavage site found (2, 6). A transcript was produced when a plasmid containing a C-terminal fragment of the A16R gene was transfected into cells infected with VACV consistent with a cryptic atypical promoter, but the significance of this is uncertain (S. Ojeda, unpublished). Neither the 43- nor the 23-kDa band was detected when viral DNA replication was inhibited with 1-β-D-arabinofuranosylcytosine (AraC) (Fig. 3B), consistent with the kinetic data indicating that the A16L gene belongs to the late expression class.

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 vA16Li in the presence or absence of IPTG, HeLa cells were fixed, permeabilized, and incubated successively with the anti-A16 peptide antibody and fluorescein isothiocyanate-conjugated secondary antibody followed by DAPI to stain DNA in

nuclei and viral factories next to the nuclei. The A16 protein was detected within viral factories of cells infected with wild-type VACV or vA16Li in the presence of IPTG (Fig. 4). No A16 staining was observed in the viral factories of cells infected with vA16Li in the absence of IPTG, confirming the specificity of the antibody. These results suggested that A16 is associated with viral, rather than cellular, structures.

Association of A16 with purified virions. To investigate the possible association of A16 with virions, IMVs were purified by sedimentation through two sucrose cushions followed by a sucrose gradient. The virions were disrupted by 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 (Fig. 5B). The A16 protein was partially solubilized from intact particles with a combination of 1% NP-40 and 140 mM NaCl in Tris buffer (Fig. 5A). For 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 (Fig. 5A). The solubility of A16, but not A14, was reduced when NaCl was omitted (not shown) or when dithiothreitol (DTT) was present in addition to NP-40 (Fig. 5A). 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 (21, 22), but it is difficult to find an explanation for it.

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 (Fig. 5B). The no-trypsin control shows the dominant 46-kDa band and the minor 23-kDa band. At 1 μg per ml of trypsin, a digestion product of about 10 kDa was present, but the amount of this product decreased at higher trypsin concentrations. At 10 and 100 μg per ml, the dominant products were 34- and 5-kDa proteins. Despite their cleavage, the products remained associated with virions, presumably through the putative membrane anchor sequence or protein-protein interactions. The A16 protein contains numerous potential trypsin cleavage sites, some of which could account for 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 and the A4 protein were completely susceptible to trypsin (Fig. 5B and data not shown). 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 IMV membrane with the long N-terminal segment exposed.

A16 contains disulfide bonds formed by the 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 vV5A16Li, with a V5 epitope tag at the N terminus of an inducible A16L gene, which was constructed as described above for vA16Li. Replication of vV5A16Li in the presence of IPTG was similar to that of vA16Li, indicating that the tag did not have a deleterious effect. 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. 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 (Fig. 6A), 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 mo-

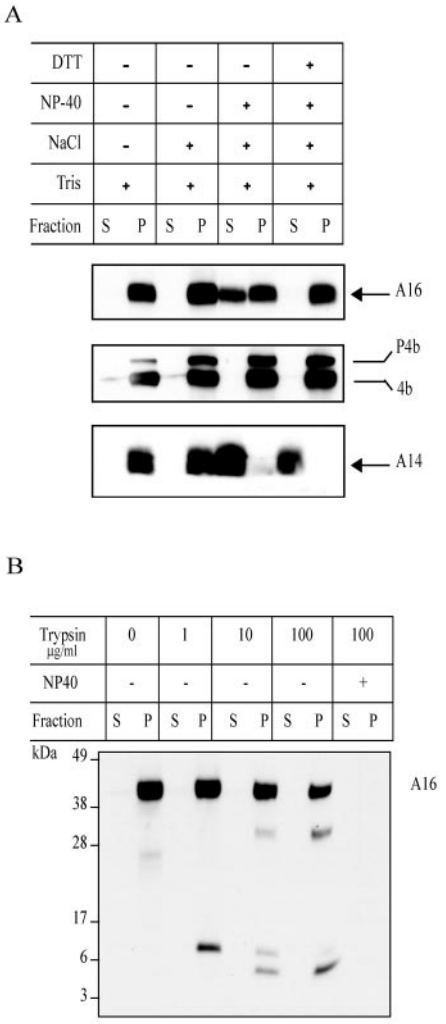

FIG. 5. Detergent extraction and trypsin sensitivity of A16 associated with purified IMVs. (A) Sucrose gradient-purified VACV was incubated with buffer containing one or more of the following components: Tris-HCl (pH 7.4), 1% NP-40, 140 mM NaCl, and 50 mM DTT as indicated by plus or minus signs. After 1 h at 37°C, 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) IMVs were purified as described above for panel A and treated with the indicated concentrations of trypsin without (−) or with (+) NP-40. Equivalent portions of supernatant and pellet fractions were analyzed by SDS-NuPAGE (4 to 12% NuPAGE) and Western blotting with the A16 peptide antibody. The masses (in kilodaltons) of protein markers are indicated on the left. The position of the full-length A16 protein is indicated on the right.

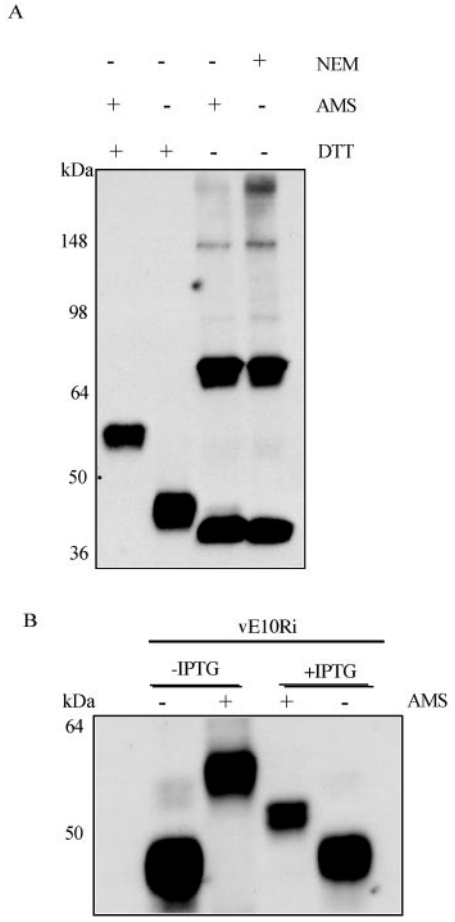

FIG. 6. Formation of disulfide bonds. (A) Disulfide bonds in A16. Cells were infected with vV5A16Li in the presence of IPTG for 18 h. The cells were collected by centrifugation and solubilized in buffer containing DTT, 20 mM AMS, or 10 nM NEM as indicated by plus or minus signs. In addition, a portion of the extract solubilized in the presence of DTT was alklylated with excess AMS. Proteins were resolved by 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 described above for panel A with an anti-V5 mouse monoclonal antibody. The masses (in kilodaltons) of marker proteins are indicated on the left.

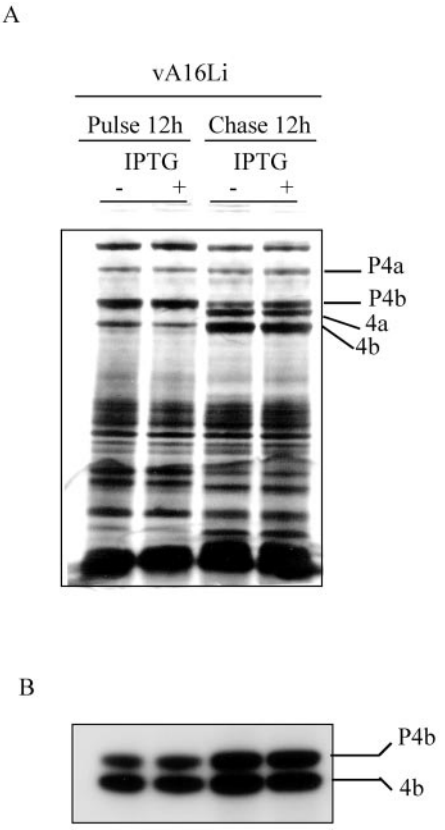

FIG. 7. Synthesis and processing of viral proteins. (A) BS-C-1 cells were infected with 5 PFU per cell of vA16Li in the presence (+) or absence (−) of 50 μM IPTG for 12 h and labeled with a mixture of [^{35}S]methionine and [^{35}S]cysteine for 30 min. Cells were either harvested immediately (pulse) or incubated with excess unlabeled methionine for an additional 12 h The proteins were analyzed by SDS-PAGE and visualized by autoradiography. The positions of migration of precursors (P4a and P4b) and their cleavage products (4a and 4b) are shown on the right. (B) In parallel, the proteins were transferred to a membrane, and Western blotting was performed using anti-P4b/4b polyclonal antibody.

nomeric and dimeric forms were detected in each case (Fig. 6A). 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 nonreduced band migrated more rapidly than DTT-reduced A16, suggesting a more-compact structure consistent with intramolecular disulfide bonds. The slower-migrating band was 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 results of trypsin digestion experiments suggested that the long N terminus of the A16 protein, which contains all 20 cysteines, is on the surface of the virus particle 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 IMV membrane proteins (24). 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 a 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

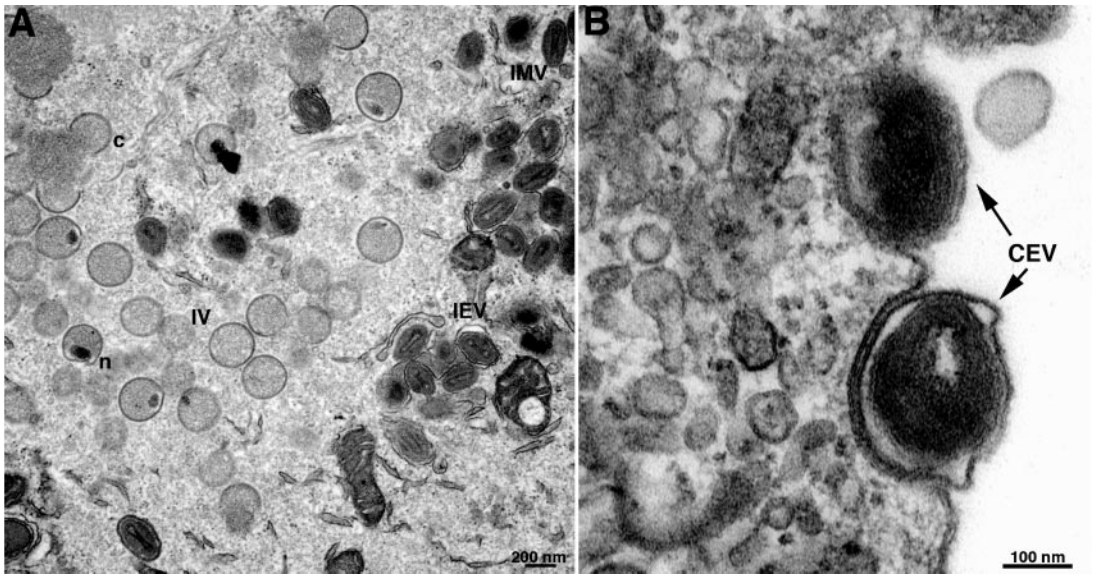

FIG. 8. Electron microscopy of infected cells. BS-C-1 cells were infected with vA16Li in the absence of IPTG. After 21 h, cells were fixed and embedded in Epon. Ultrathin sections were prepared for transmission electron microscopy. (A) A low-power image of a viral factory region displaying crescents (c), immature virions (IV) (some with nucleoids [n]), IMVs, and intracellular enveloped virions (IEV). (B) Surface of a cell with two cell-associated enveloped virions (CEV).

bonds (Fig. 6B). In contrast, AMS increased the mass of the A16 protein made in the presence of IPTG by about 2 kDa, which corresponds to four reactive cysteines and indicates that 8 of the 10 disulfide bonds had formed (Fig. 6B). 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 (21). 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.

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 vA16Li 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 (Fig. 7A). Processing of the P4b protein was also demonstrated by Western blotting (Fig. 7B). 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 (Fig. 8A and B) or presence (not shown) of inducer.

Comparison of −A16 and +A16 IMVs. The location of A16 in the IMV membrane and the apparent absence of any role in assembly suggested to us thatA16 might be needed for virus entry. Up till now, all infections were done with vA16Li 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 (not shown) and by SDS-PAGE and silver staining (Fig. 9A). Western blotting of proteins from virions formed with and without IPTG indicated that the latter contained only a trace of A16 but normal amounts of other IMV membrane proteins, including A28, A21, L5, and A27 (Fig. 9B). Furthermore, the in vitro transcriptional activities of −A16 and +A16 virions were similar to each other and proportional to the number of particles (Fig. 10A). 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.

Role of A16 in virus entry. In the previous section, we demonstrated that −A16 and +A16 virions had similar abilities to synthesize RNA in vitro. 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, cells were infected 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 complemen-

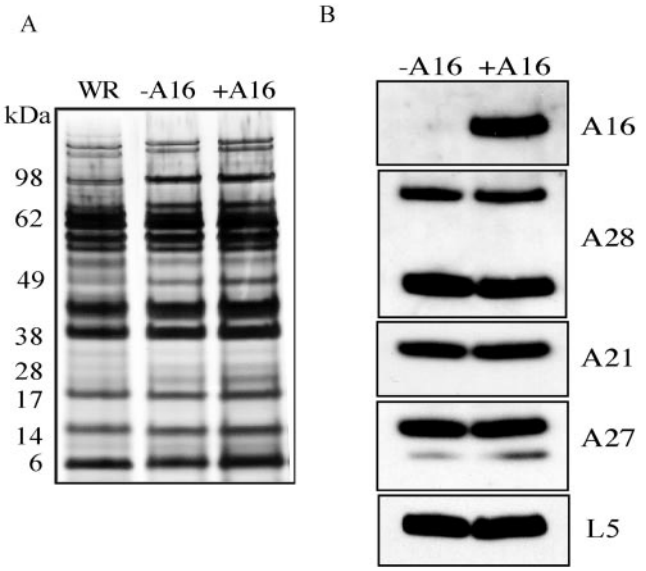

FIG. 9. Protein composition of purified virions. IMVs were purified by sucrose gradient sedimentation from cells infected with VACV WR or with vA16Li in the presence (+A16) or absence (−A16) of IPTG. Equal numbers of particles (determined by optical density at 260 nm) were analyzed by SDS-PAGE and silver staining. The masses (in kilodaltons) of marker proteins are shown on the left. (B) Western blotting of samples prepared as described above for panel A and probed with antibodies to the A16, A28, A21, L5, and A27 proteins.

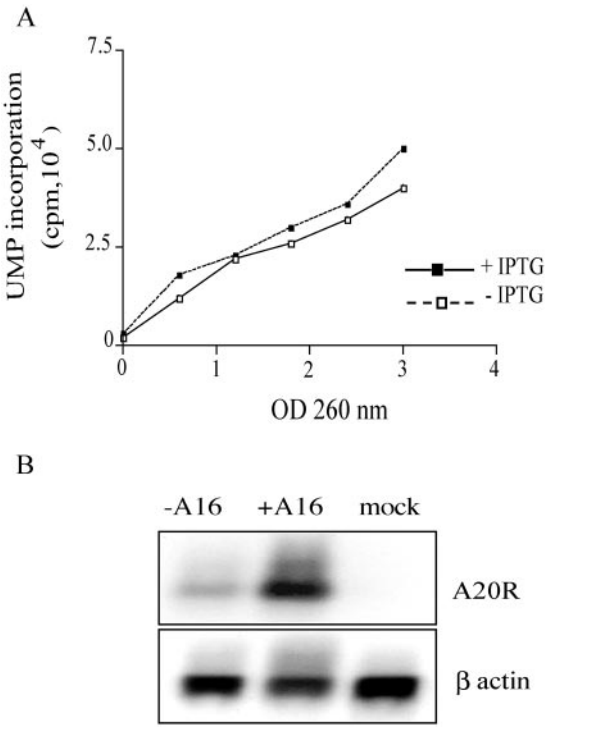

FIG. 10. In vitro and in vivo RNA synthesis. (A) In vitro transcription by permeabilized −A16 and +A16 virions. Lysates were made from cells infected with vA16Li in the presence (+) or absence (−) of IPTG, and virions were purified by sucrose gradient centrifugation. The indicated number of OD_{260} (optical density at 260 nm) units of purified virions were incubated in a reaction mixture containing [α-^{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} units 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 virus gene or β-actin, and analyzed by autoradiography.

tary 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 (Fig. 10B). β-Actin mRNA was analyzed as a control for RNA integrity and loading (Fig. 10B).

To directly examine the ability of −A16 virions to enter cells, we used an assay originally described by Vanderplasschen et al. (29). 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 surfaces of cells and cores in the cytoplasm, respectively. As shown in Fig. 11, anti-L1-stained 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 (Fig. 11). The few cores detected under the latter conditions colocalized with L1 staining, suggesting that they were located on the surface of the cell (Fig. 11, merge). Thus, −A16 virions exhibited a defect in a step of virus entry after binding.

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 IMV particles (called fusion from without) (11, 13). 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 (20, 22, 27, 28).

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 (Fig. 12A), 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 (Fig. 12B). As expected, fusion from within or without did not occur with +A16 virions when the low-pH treatment was omitted (not shown).

DISCUSSION

Our inability to isolate a deletion mutant during attempts to knock out the A16 gene by insertion of DNA encoding green fluorescent protein strongly suggested that A16 expression is essential for virus replication. Because of the ability to identify tiny plaques comprised of only a few green

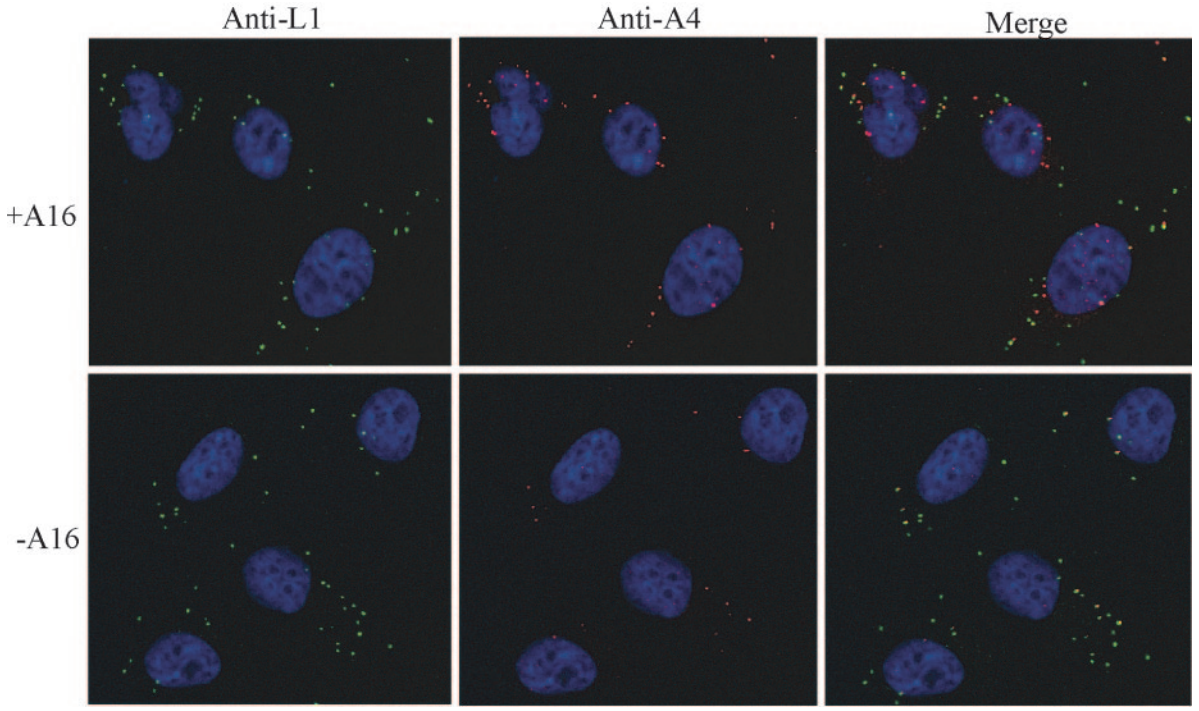

FIG. 11. Cell binding and penetration of purified IMVs. HeLa cells were incubated with 5 PFU per cell of purified +A16 virus or the equivalent OD_{260} (optical density at 260 nm) units of −A16 virus in the presence of 300 μg of cycloheximide per ml for 1 h at 4°C. The cells were washed, incubated for an additional 2 h at 37°C, 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), and merging of the last two.

fluorescent cells, this method has proved to be superior to antibiotic selection for deleting other genes, such as the VACV topoisomerase gene (9) and A27L gene (30) that severely reduce plaque size. To obtain a null mutant, we constructed a recombinant virus with an inducible A16L gene. The inducible mutant made tiny plaques in the absence of inducer, and virus yields were severely reduced. Because of the low replication under nonpermissive conditions, there remains the possibility that A16 is not absolutely required. However, complete repression of gene expression is difficult to achieve with inducible systems and the degree of inhibition of virus replication depends on the amount of the gene product needed. Therefore, either A16 is essential (as we believe) or is needed for efficient replication.

Despite some low-level replication, the phenotype of the A16 mutant was clear. In the absence of inducer, virus morphogenesis appeared normal, including the formation of extracellular virions. Moreover, virions made in the absence of inducer were indistinguishable from +A16 virions by SDS-PAGE, and they contained other membrane proteins, including A28, A21, and L5, which are involved in entry, and A27, which is required for intracellular wrapping of IMVs. The structural integrity of the −A16 virions was indicated by their in vitro transcriptional activity. Nevertheless, the specific infectivity of purified IMVs made in the absence of inducer was only 1 to 2% of that of IMVs made in the presence of inducer. Furthermore, very little viral early mRNA was made in cells infected with −A16 virions, suggesting reduced virus entry. This entry block was confirmed by confocal microscopy, which showed that the −A16 virions could attach to cells but release of cores into the cytoplasm was inhibited. In addition, the virions did not mediate low-pH-triggered cell-cell fusion.

The major conclusion of this study is that A16, like A21, A28, H2, and L5 (20–22, 27, 28), is required for entry of poxviruses into cells and low-pH-triggered cell-cell fusion. None of these five proteins are related in sequence to each other, and each one is independently required for entry and fusion, indicating no structural or functional redundancy. Nevertheless, the proteins do have common features: each protein (i) is conserved in all sequenced poxviruses, suggesting a common fusion mechanism, (ii) is expressed late in infection during the time of virion assembly, (iii) contains a single transmembrane domain, (iv) is present in the IMV membrane as a nonglycosylated species, (v) has one or more intramolecular disulfide bonds formed by the poxvirus cytoplasmic redox system, and (vi) is not required for virion morphogenesis. A16 is about twice as long as the other VACV entry proteins and differs from them in having a C-terminal transmembrane domain instead of an N-terminal one, 20 invariant cysteines instead of 2 to 4, and a myristylated glycine.

The finding that at least five conserved IMV membrane proteins are required for entry and cell-cell fusion is strong evidence that the two processes operate by related mechanisms. We presume that cell-cell fusion occurs in two steps: fusion of the viral membrane with the plasma membrane of one cell and then fusion of that cell with another (18). The

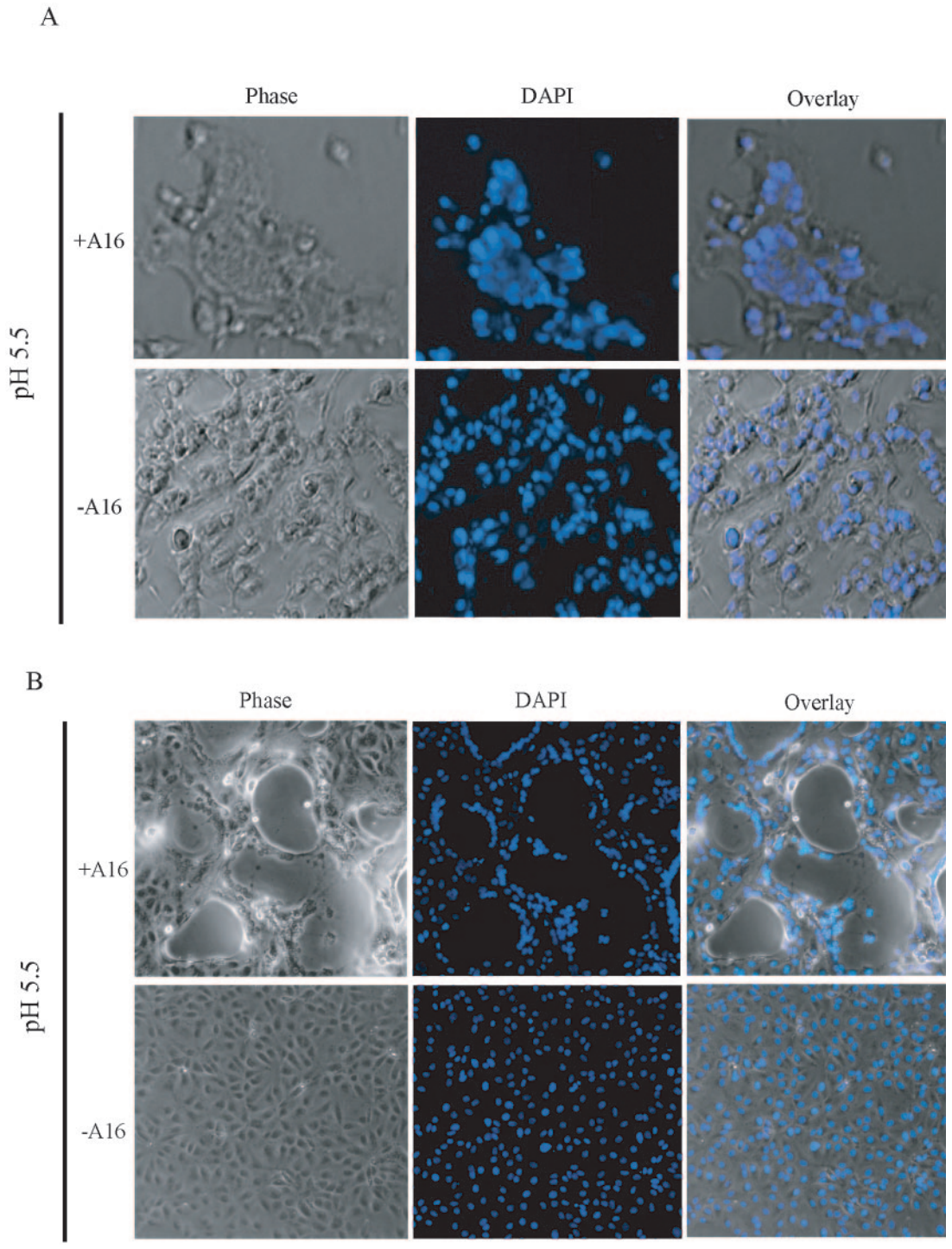

FIG. 12. Fusion from within and without. (A) Fusion from within. BS-C-1 cells were infected with 2 PFU of vA16Li per cell in the absence or presence of IPTG for 18 h at 37°C. The medium was replaced with pH 5.5 or pH 7.4 buffer (not shown) and incubated for 2 min at 37°C. The buffer was aspirated, and the cells were incubated for 3 h at 37°C in fresh culture medium at a neutral pH. 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 optical density at 260 nm units of −A16 virions for 1 h at 4°C. 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°C in neutral pH medium with 300 μg of cycloheximide per ml. Cells were stained and examined as described above for panel A.

reason why so many poxvirus proteins are required for entry and fusion is perplexing. Two of the entry/fusion proteins were reported to associate with each other (20), and there is evidence that the others, including A16, are part of the same complex (19b). Nevertheless, as found here for A16, A21, A28, and L5 and elsewhere for A28 and H2 (20), the entry proteins seem capable of trafficking independently to the viral membrane. It is possible that the proteins in the complex have multiple, nonredundant roles, including membrane fusion per se, activation of fusion, receptor recognition, and a scaffolding function. 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. Only members of the *Herpesviridae* family approach poxviruses with regard to the number of proteins involved in entry (26). 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 nonconserved receptor-binding proteins, such as gD for most alphaherpesviruses.

ACKNOWLEDGMENTS

We thank Andrea Weisberg for electron microscopy, Owen Schwartz for help with confocal microscopy, and Norman Cooper for cell cultures.

This research was supported in part by the Intramural Research Program of the NIAID of the NIH.

REFERENCES

1. **Alexander, W. A., B. Moss, and T. R. Fuerst.** 1992. Regulated expression of foreign genes in vaccinia virus under the control of bacteriophage T7 RNA polymerase and the *Escherichia coli lac* repressor. J. Virol. **66:**2934–2942.
2. **Ansarah-Sobrinho, C., and B. Moss.** 2004. Role of the I7 protein in proteolytic processing of vaccinia virus membrane and core components. J. Virol. **78:**6335–6343.
3. **Antoine, G., F. Scheiflinger, F. Dorner, and F. G. Falkner.** 1998. The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. Virology **244:**365–396.
4. **Armstrong, J. A., D. H. Metz, and M. R. Young.** 1973. The mode of entry of vaccinia virus into L cells. J. Gen. Virol. **21:**533–537.
5. **Betakova, T., E. J. Wolffe, and B. Moss.** 1999. Regulation of vaccinia virus morphogenesis: phosphorylation of the A14L and A17L membrane proteins and C-terminal truncation of the A17L protein are dependent on the F10L protein kinase. J. Virol. **73:**3534–3543.
6. **Byrd, C. M., T. C. Bolken, and D. E. Hruby.** 2002. The vaccinia virus I7L gene product is the core protein proteinase. J. Virol. **76:**8973–8976.
7. **Carter, G. C., M. Law, M. Hollinshead, and G. L. Smith.** 2005. Entry of the vaccinia virus intracellular mature virion and its interactions with glycosaminoglycans. J. Gen. Virol. **86:**1279–1290.
8. **Chang, A., and D. H. Metz.** 1976. Further investigations on the mode of entry of vaccinia virus into cells. J. Gen. Virol. **32:**275–282.
9. **Da Fonseca, F., and B. Moss.** 2003. Poxvirus DNA topoisomerase knockout mutant exhibits decreased infectivity associated with reduced early transcription. Proc. Natl. Acad. Sci. USA **100:**11291–11296.
10. **Demkowicz, W. E., J. S. Maa, and M. Esteban.** 1992. Identification and characterization of vaccinia virus genes encoding proteins that are highly antigenic in animals and are immunodominant in vaccinated humans. J. Virol. **66:**386–398.
11. **Doms, R. W., R. Blumenthal, and B. Moss.** 1990. Fusion of intra- and extracellular forms of vaccinia virus with the cell membrane. J. Virol. **64:**4884–4892.
12. **Earl, P. L., N. Cooper, S. Wyatt, B. Moss, and M. W. Carroll.** 1998. Preparation of cell cultures and vaccinia virus stocks, p. 16.16.1–16.16.3. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 2. John Wiley and Sons, New York, N.Y.
13. **Gong, S. C., C. F. Lai, and M. Esteban.** 1990. Vaccinia virus induces cell fusion at acid pH and this activity is mediated by the N-terminus of the 14-kDa virus envelope protein. Virology **178:**81–91.
14. **Ichihashi, Y.** 1996. Extracellular enveloped vaccinia virus escapes neutralization. Virology **217:**478–485.
15. **Janeczko, R. A., J. F. Rodriguez, and M. Esteban.** 1987. Studies on the mechanism of entry of vaccinia virus into animal cells. Arch. Virol. **92:**135–150.
16. **Martin, K. H., D. W. Grosenbach, C. A. Franke, and D. E. Hruby.** 1997. Identification and analysis of three myristylated vaccinia virus late proteins. J. Virol. **71:**5218–5226.
17. **Moss, B.** 2001. Poxviridae: the viruses and their replication, p. 2849–2883. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia, Pa.
18. **Moss, B.** Poxvirus entry and membrane fusion. Virology, in press.
19. **Senkevich, T. G., E. V. Koonin, J. J. Bugert, G. Darai, and B. Moss.** 1997. The genome of molluscum contagiosum virus: analysis and comparison with other poxviruses. Virology **233:**19–42.

19b. **Senkevich, T. G., S. Ojeda, A. Townsley, G. E. Nelson, and B. Moss.** Poxvirus multiprotein entry-fusion complex. Proc. Natl. Acad. Sci. USA, in press.

20. **Senkevich, T. G., and B. Moss.** 2005. Vaccinia virus H2 protein is an essential component of a complex involved in virus entry and cell-cell fusion. J. Virol. **79:**4744–4754.
21. **Senkevich, T. G., B. M. Ward, and B. Moss.** 2004. Vaccinia virus A28L gene encodes an essential protein component of the virion membrane with intramolecular disulfide bonds formed by the viral cytoplasmic redox pathway. J. Virol. **78:**2348–2356.
22. **Senkevich, T. G., B. M. Ward, and B. Moss.** 2004. Vaccinia virus entry into cells is dependent on a virion surface protein encoded by the A28L gene. J. Virol. **78:**2357–2366.
23. **Senkevich, T. G., A. Weisberg, and B. Moss.** 2000. Vaccinia virus E10R protein is associated with the membranes of intracellular mature virions and has a role in morphogenesis. Virology **278:**244–252.
24. **Senkevich, T. G., C. L. White, E. V. Koonin, and B. Moss.** 2002. Complete pathway for protein disulfide bond formation encoded by poxviruses. Proc. Natl. Acad. Sci. USA **99:**6667–6672.
25. **Smith, G. L., and M. Law.** 2004. The exit of vaccinia virus from infected cells. Virus Res. **106:**189–197.
26. **Spear, P. G., and R. Longnecker.** 2003. Herpesvirus entry: an update. J. Virol. **77:**10179–10185.
27. **Townsley, A., T. G. Senkevich, and B. Moss.** 2005. The product of the vaccinia virus L5R gene is a fourth membrane protein encoded by all poxviruses that is required for cell entry and cell-cell fusion. J. Virol. **79:**10988–10998.
28. **Townsley, A., T. G. Senkevich, and B. Moss.** 2005. Vaccinia virus A21 virion membrane protein is required for cell entry and fusion. J. Virol. **79:**9458–9469.
29. **Vanderplasschen, A., M. Hollinshead, and G. L. Smith.** 1998. Intracellular and extracellular vaccinia virions enter cells by different mechanisms. J. Gen. Virol. **79:**877–887.
30. **Ward, B. M.** 2005. Visualization and characterization of the intracellular movement of vaccinia virus intracellular mature virions. J. Virol. **79:**4755–4763.
31. **Wolffe, E. J., S. Vijaya, and B. Moss.** 1995. A myristylated membrane protein encoded by the vaccinia virus L1R open reading frame is the target of potent neutralizing monoclonal antibodies. Virology **211:**53–63.
32. **Zhang, Y., and B. Moss.** 1991. Inducer-dependent conditional-lethal mutant animal viruses. Proc. Natl. Acad. Sci. USA **88:**1511–1515.