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Varicella-Zoster Virus Glycoprotein I Is Essential for Growth of Virus in Vero Cells

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Varicella-zoster virus (VZV) encodes at least six glycoproteins. Glycoprotein I (gI), the product of open reading frame 67, is a 58- to 62-kDa glycoprotein found in VZV-infected cells. We constructed two VZV gI deletion mutants. Immunoprecipitation of VZV gE from infected cells indicated that cells infected with VZV deleted for gI expressed a gE that was larger (100 kDa) than that expressed in cells infected with the parental virus (98 kDa). Cell-associated or cell-free VZV deleted for gI grew to lower titers in melanoma cells than did parental VZV. While VZV deleted for gI replicated in other human cells, the mutant virus replicated to very low titers in primary guinea pig and monkey cells and did not replicate in Vero cells. When compared with the parental virus, rescued viruses, in which the gI deletion was restored with a wild-type allele, showed a similarly sized gE and comparable growth patterns in melanoma and Vero cells. VZV deleted for gI entered Vero cells; however, viral DNA synthesis was impaired in these cells. The VZV gI mutant was slightly impaired for adsorption to human cells. Thus, VZV gI is required for replication of the virus in Vero cells, for efficient replication of the virus in nonhuman cells, and for normal processing of gE.

Varicella-zoster virus (VZV) open reading frames (ORFs) 31, 14, 68, 37, 67, and 60 encode glycoprotein B (gB), gC, gE, gH, gI, and gL, respectively. VZV gI, formally known as gpIV (7), is predicted to contain a 17-amino-acid signal sequence followed by a 278-amino-acid hydrophilic extracellular domain, a 17-amino-acid membrane anchor, and a 59-amino-acid intracellular domain. VZV gI contains O- and N-linked sugars as well as mannose glycans (14, 21). The glycoprotein is phosphorylated at serine 343, and the adjacent proline residues are essential for the kinase recognition sequence (32). Two proteins of 62 and 50 kDa are immunoprecipitated by rabbit antiserum or monoclonal antibody to gI; the latter is thought to represent an incompletely processed form (8, 21, 33). VZV gI is noncovalently associated with gE (33) and is much less abundant in VZV-infected cells than gE.

VZV gI is a target for cytotoxic T cells (15, 27) and induces neutralizing antibodies that are complement dependent (13). Inoculation of guinea pigs with vaccinia virus expressing VZV gI protects the animals from infection with VZV (19).

VZV gI is homologous to herpes simplex virus (HSV) gI. VZV gI exhibits 24% amino acid identity and 43% amino acid similarity to HSV type 1 (HSV-1) gI (18). Comparison of the unique short regions of HSV-1, HSV-2, and pseudorabies virus (PRV) indicates that their gG, gD, and gI genes are related, have cysteine residues in similar locations, and probably have evolved by duplication and subsequent divergence; the gE gene is more distantly related (20). Unlike HSV and PRV, VZV does not have a gG or gD homolog. gD is essential for replication of HSV-1 (16). Since VZV gI is the VZV glycoprotein most similar to HSV gD, VZV gI may be essential for virus replication. HSV and PRV gIs are important for cell-to-cell viral spread (10, 34); since VZV is transmitted only by cell-to-cell contact in tissue culture, we did studies to determine whether VZV gI is essential for viral replication in vitro. Construction of VZV mutants deleted for gI indicated that gI was not required for replication of VZV in human cells in vitro. VZV gI was determined to be important for efficient replication in nonhuman cells and essential for replication of the virus in Vero cells.

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MATERIALS AND METHODS

Cells and cell lines. Human melanoma cells (MeWo) were obtained from Charles Grose, human schwannoma cells (ST88-14) were a gift from Cynthia Morton (24), African green monkey kidney cells (clone 2129) were a gift from Lou Potash, whole human fibroblast cells and guinea pig embryo fibroblasts were obtained from BioWhittaker (Walkersville, Md.), and Vero and BS-C-1 cells were obtained from the American Type Culture Collection.

A MeWo cell line containing the ORF67 gene was constructed by inserting a *Bam*HI-*Afl*III fragment (VZV nucleotides 114,042 to 115,622 [7]) containing ORF67 and its promoter (17) from VZV cosmid MstIIA (4) into the *Bst*1107I site of plasmid pSV2neo (29). Plasmid pSV2neo-ORF67 was linearized with *Aat*II, MeWo cells were transfected with the plasmid by the calcium phosphate procedure, and clones were selected in the presence of G418 (geneticin; Life Technologies, Inc., Grand Island, N.Y.).

Cosmids and plasmids. VZV ORF67 is encoded by VZV nucleotides 114,496 to 115,558 (7). A cosmid with a deletion within ORF67 was constructed by cutting cosmid VZV MstII A with *Avr*II and *Sgr*AI (Boehringer Mannheim), which cuts the cosmid at VZV nucleotides 112,853 and 117,355. The 4.5-kb fragment containing VZV ORF67 was inserted into plasmid pGEM2 (Promega) into which *Sgr*AI and *Avr*II sites had been inserted. The resulting plasmid was cut with *Eco*RV (which cuts at VZV nucleotides 114,518 and 114,857) and *Kpn*I (which cuts at VZV nucleotide 115,127). The large fragment was blunted with T4 DNA polymerase, and an oligonucleotide containing stop codons in all three ORFs, TAGCTAGGCGCGCCTAGCTA, was inserted between nucleotides 114,518 and 115,127. The resulting plasmid was sequenced to verify that the oligonucleotide had been inserted at the expected site and then cut with *Sgr*AI and *Avr*II, and the large fragment was inserted into cosmid MstII A in place of the wild-type sequence. The resulting cosmid, VZV MstII A-67d, has a 609-nucleotide deletion that results in the loss of amino acids 9 to 211, with a stop codon inserted after amino acid 8 (Fig. 1). Two independent clones of MstII A-67d were obtained.

To produce a cosmid with a large deletion in ORF67, the RecA-assisted restriction endonuclease cleavage procedure was used (12). VZV cosmid MstII A (0.8 μg) was incubated with the *Escherichia coli* RecA protein (10 μg) and two 60-mers (90 ng each), AACCCGTTAAGGCAAATAGGGTACAAACGCG CAATGTTTTGAAATACTAATATAAATAAC and ACCCGTTTGTTAA ATAGAACTAATTATCCCGGATTTTATATTAAATAAACTATATGCGTT, centered around the *Hpa*II sites at VZV nucleotides 114,435 and 115,572 that flank ORF67. After incubation for 10 min at 37°C, the remaining unblocked *Hpa*II sites were methylated by using *Hpa*II methylase for 20 min at 37°C. The methylase was heat inactivated, and the RecA complex was dissociated. The

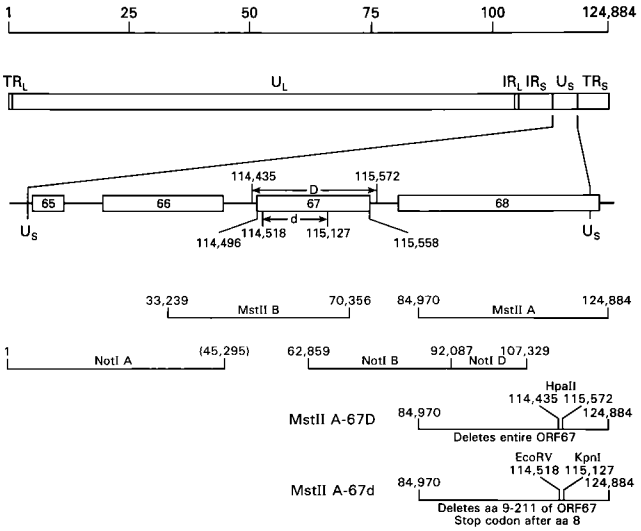

FIG. 1. Construction of VZV gI mutants. The VZV genome consists of 124,884 bp of DNA (line 1) containing long (L) and short (S) terminal and internal repeats (TR and IR, respectively) and unique long and short DNA domains (U_L and U_S, respectively) (line 2). The U_S region with the sites of the ORF67 large (D) and small (d) deletions is shown (line 3). The MstII and NotI cosmids used to construct parental VZV are shown (lines 4 and 5, respectively). The nucleotide positions are numbered based on the prototype strain Dumas (7). Cosmid MstII A-67D has a deletion beginning before the first codon and ending after the last codon of ORF67 (line 6). Cosmid MstII A-67d has a deletion of codons 9 to 211 of ORF67 and a stop codon inserted after codon 8 (line 7). aa, amino acid.

cosmid was then digested with *Hpa*II (which cuts at nucleotides 114,435 and 115,572), and the large fragment was gel purified and ligated to itself. The resulting cosmid, VZV MstII A-67D, has a 1,137-nucleotide deletion that results in the loss of the entire ORF67 coding sequence, beginning 57 nucleotides before the start codon and ending 11 nucleotides after the stop codon (Fig. 1). Two independent clones of MstII A-67D were obtained, and the deletion was verified by sequencing the junction region of the cosmid.

To restore the deletions in ORF67, a plasmid containing ORF67 with additional flanking sequences was constructed. Cosmid VZV MstII A was cut with *Avr*II and *Eco*RI, and the 4.2-kb fragment containing ORF67 was inserted into the *Avr*II and *Eco*RI sites of plasmid LITMUS 28. The resulting plasmid, pORF67, contains VZV nucleotides 112,854 to 117,034.

Transfections. MeWo cells, or MeWo cell clones stably transfected with plasmid pSV2neo-ORF67 that had not been screened for expression of VZV gI were used for transfections. MeWo cells were transfected with pCMV62, VZV cosmids NotI A, NotI B, and MstII B, and either MstII A, MstII A-67d, or MstII A-67D by the calcium phosphate procedure (4). Six days after transfection, the cells were seeded into 75-cm^2 flasks and monitored for cytopathic effects (CPE).

To rescue the deletion in viruses containing ORF67 deletions, pORF67 was linearized with *Avr*II and *Eco*RI and the VZV DNA containing ORF67 was isolated. MeWo cells were transfected with 0.5 μg of virion DNA from ORF67 deletion mutants, 1.5 μg of ORF61 DNA, and 50 ng of pCMV62 by the calcium phosphate method. The transfected cells were passaged into flasks 6 days after transfection and subsequently passaged in MeWo and Vero cells.

Southern blots, immunoprecipitations, and immunofluorescence assays. Viral DNA was purified from nucleocapsids, cut with *Eco*RI or *Bam*HI, fractionated on 0.8% agarose gels, transferred to nylon membranes, and probed with [^{32}P]dCTP-labeled VZV cosmids corresponding to the whole viral genome or with a radiolabeled probe from the *Bam*HI K fragment (VZV nucleotides 114,043 to 118,004) containing VZV gI.

For immunoprecipitations, MeWo cells were infected with VZV-infected cells, and after 2 days, the cells were radiolabeled with [^{35}S]methionine for 2 h and lysed. The supernatant was incubated with a monoclonal antibody to VZV gE (Chemicon, Temecula, Calif.) followed by protein A-Sepharose or with a monoclonal antibody to VZV gI (Viro Research, Inc., Rockford, Ill.) followed by goat anti-mouse immunoglobulin G coupled to Sepharose (Organon Teknika Corp., Durham, N.C.). Immune complexes were fractionated on sodium dodecyl sulfate–6 to 15% gradient polyacrylamide gels.

Immunofluorescence detection of VZV ORF62 protein was performed by fixing cells 48 h after infection with 50% methanol-50% acetone (vol/vol), incubating them with a murine monoclonal antibody to the ORF62 protein (Chemicon, Temecula, Calif.), and staining them with fluorescein isothiocyanate-conjugated anti-mouse antibody. Slides were washed with phosphate-buffered saline, and fluorescence microscopy was performed.

Growth properties of recombinant VZV. Cell-free VZV was prepared as described previously (28). Growth curves were obtained by infecting cells with either cell-free virus or with melanoma cells containing about 200 PFU of VZV. The cells were harvested 1, 2, 3, 4, and 5 days after infection, and serial dilutions were prepared and used to inoculate uninfected melanoma cells. Plaques were stained and counted 7 days after infection.

Plaque size was measured with the aid of an inverted microscope. The mean size of 20 plaques was determined for each VZV mutant, and the Tukey multiple comparison test was used to determine the statistical significance of the differences.

Kinetics of viral DNA synthesis. Melanoma or Vero cells were infected with melanoma cells containing about 200 PFU of VZV, and cells were harvested at various times after infection. The cells were pelleted and then resuspended in 400 μl of Tris-EDTA buffer containing 0.5% SDS and 20 μg of RNase/ml for 1 h at 37°C. Proteinase K was added to 100 μg/ml, and after a 3-h incubation at 50°C, DNA was extracted with phenol-chloroform and precipitated with ethanol. Serial dilutions of control VZV cosmid DNAs, representing the entire genome, were used to quantify the amount of VZV DNA in the cells. Aliquots of DNA were denatured as described elsewhere (23), applied to a slot blot apparatus, and hybridized with [^{32}P]dCTP-labeled VZV cosmids corresponding to the whole viral genome. The signal was quantitated with a PhosphorImager (Molecular Dynamics), and the amount of DNA for each data point was determined by comparison with the signal from the control DNA.

VZV adsorption to cells. Cell-free VZV (50 to 100 PFU) was incubated with melanoma cells in six-well plates at 37°C. At 5, 10, 20, 30, 60, or 180 min after infection, the inoculum was removed, the monolayers were washed twice with medium, and fresh medium was added. Plaques were counted 7 days after infection. The percent adsorption was measured as the number of plaques at each time point divided by the number of plaques obtained after adsorption for 180 min.

VZV penetration into cells. Cell-free VZV (50 to 100 PFU) was incubated with melanoma cells at 4°C. After 2 h, the inoculum was removed, warm medium was added, and the cells were incubated at 37°C for 0, 20, 40, 60, or 120 min. At each time point, the medium was removed, and the cells were incubated with 0.1 M glycine-HCl (pH 3.0) for 2 min and then washed twice with medium before fresh medium was added. As a control, cells were incubated for 2 h at 4°C with the same inoculum, and after a 120-min incubation in fresh medium, the cells were washed with medium twice (without incubation in low-pH buffer) and fresh medium was added. Plaques were counted 7 days later, and the percent penetration was measured as the number plaques at each time point divided by the number of plaques obtained with the control.

Polyethylene glycol treatment was used to enhance penetration of VZV into Vero cells. Vero cells were infected with either cell-free VZV deleted for gI or parental virus at 37°C for 2 h, briefly exposed to polyethylene glycol, and washed extensively as previously described (26). The cells were incubated for 10 days, and plaques were counted microscopically.

RESULTS

Generation of VZV deleted for gI. Transfection of MeWo cells with four cosmids spanning the entire VZV genome results in the production of infectious virus (4). Initial attempts to produce VZV deleted for gI by using cosmids with deletions in gI failed to produce infectious virus. Therefore, cell lines containing ORF67 were constructed and then transfected with the three parental cosmids and either the gI deletion mutant cosmids or a parental cosmid containing gI. Nine to 10 days after transfection, CPE were observed in one cell line (67-11-2) transfected with the four parental cosmids. Thirteen days after transfection, CPE were observed in 67-11-2 cells transfected with the three parental cosmids and MstII A-67D. Two independent clones of this virus, ROka67D1 and ROka67D2, were obtained from two independently derived cosmids. Eighteen days after transfection, CPE were observed in cells transfected with the three parental cosmids and MstII A-67d. Two clones of this virus, ROka67d1 and ROka67d2, were obtained from independently derived cosmids.

VZV gI is dispensable for viral replication in vitro. VZV ROka67D or ROka67d was propagated in 67-11-2 cells, cell-free VZV was obtained by sonicating cells, and MeWo cells that do not contain gI were infected. CPE were noted 10 days after infection, and the virus was subsequently propagated in MeWo cells.

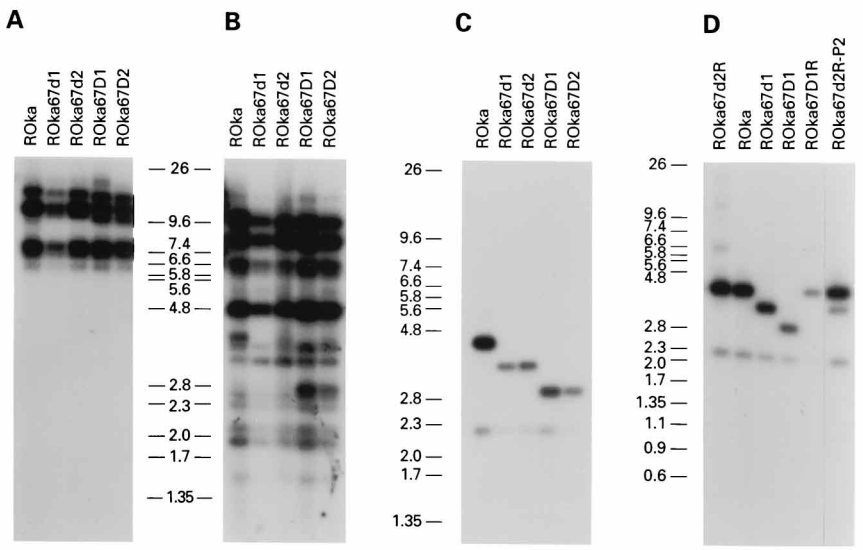

FIG. 2. Southern blots of virion DNAs from VZV gI mutants. Virion DNA was digested with *Eco*RI (A) or *Bam*HI (B) and probed with all four radiolabeled VZV cosmids. Virion DNA was also digested with *Bam*HI and probed with a radiolabeled VZV *Bam*HI K fragment (C and D). Each of the rescued viruses (ROka67D1R and ROka67d2R) in panel D was passaged four times in Vero cells, except for ROka67d2R-P2, which was passaged only twice in these cells. The numbers indicate the sizes of DNAs in kilobase pairs.

Additional experiments indicated that infectious virus could be produced by direct transfection of MeWo cells with the three parental cosmids and either MstII A-67D or MstII A-67d. VZV ROka67D1, which was used in subsequent experiments, was derived from transfection of MeWo cells, while VZV ROka67D2, ROka67d1, and ROka67d2 were derived from transfection of 67-11-2 cells, and cell-free virus was obtained and subsequently propagated in MeWo cells. The mean diameters of plaques produced by ROka67d and ROka67D in MeWo cells (0.38 and 0.26 mm, respectively) were similar or slightly larger than the plaque sizes seen in 67-11-2 cells (0.24 and 0.28 mm, respectively). These experiments indicate that the 67-11-2 cell line does not complement the growth of the gI deletion mutant.

To verify that VZV deleted for gI had the expected genome structure, viral DNA was prepared from parental recombinant VZV and the gI deletion mutants and then cut with *Eco*RI or *Bam*HI. Southern blot analysis of *Eco*RI digests probed with DNA from the entire VZV genome showed identical bands (Fig. 2A). *Bam*HI digests probed with the whole VZV genome showed similar bands, except a new band of 2.8 kb was noted for ROka67D DNA (Fig. 2B) (see below). Digestion of ROka DNA with *Bam*HI followed by probing with the *Bam*HI K fragment (which contains the gI gene) yielded the 4.0-kb *Bam*HI K fragment. In contrast, digestion of ROka67d DNA with *Bam*HI resulted in a 3.4-kb band while digestion of ROka67D DNA yielded a 2.8-kb band, due to the deletions in the gI gene (Fig. 2C). An additional, faint band of 2.2 kb was present in each lane due to hybridization of a small portion of the probe with a different-sized *Bam*HI fragment which resulted from inversion of the unique short region of the VZV genome.

To verify that cells infected with ROka67D or ROka67d did not express VZV gI, cells were radiolabeled and immunoprecipitated with monoclonal antibody to gI. Cells infected with parental VZV (ROka) expressed 58- and 47-kDa gI proteins corresponding to mature and immature (high-mannose) forms of gI (33), respectively (Fig. 3A). A band of 98 kDa corresponding to gE was also present as a complex with gI. Neither gE nor gI was immunoprecipitated from cells infected with either ROka67d or ROka67D by the monoclonal antibody to gI.

Cells infected with each of the viruses expressed VZV gE, indicating that the absence of gI expression was not due to lack of VZV gene expression. Cells infected with ROka67d or ROka67D expressed a VZV gE that was larger (100 kDa) than that expressed by cells infected with ROka (98 kDa), presumably due to an alteration in glycosylation as previously observed when gE was expressed in the absence of gI (33). VZV gI (58 kDa) was also immunoprecipitated from ROka-infected cells by the monoclonal antibody to gE.

Construction of rescued viruses in which the gI deletion mutation has been restored. Since the transfection process might result in a virus which has a mutation at an additional, unanticipated site, we created viruses in which the deletions in gI were restored with a wild-type allele. MeWo cells were cotransfected with virion DNA from ROka67D1 and with a 100-fold molar excess of plasmid DNA containing the gI gene with 1.6 kb of flanking sequence on the 5′ end and 1.5 kb of flanking sequence on the 3′ end. CPE typical of VZV were observed, and the resulting virus, ROka67D1R, was propagated in MeWo cells. Cell-free virus was prepared and used to infect Vero cells. Vero cells were used to amplify the rescued virus from the background of gI-deleted virus, since VZV deleted for gI does not replicate in Vero cells (see below). The rescued virus was passaged four times in Vero cells. Another rescued virus, ROka67d2R, derived from ROka67d2 virion DNA, was constructed by the same procedure.

To verify that the gI gene was restored in the rescued viruses, viral DNA was prepared, cut with *Bam*HI, and probed with the *Bam*HI K fragment containing the gI gene. After four passages in Vero cells, viral DNA from ROka67D1R and ROka67d2R each contained a restored wild-type gI allele (4.0 kb) and the mutant gI alleles (3.4 or 2.8 kb) were not detectable (Fig. 2D). In contrast, after only two passages in Vero cells, both a wild-type and a mutant gI allele were present in DNA from ROka67d2R (Fig. 2D, last lane).

To verify that cells infected with the rescued virus expressed

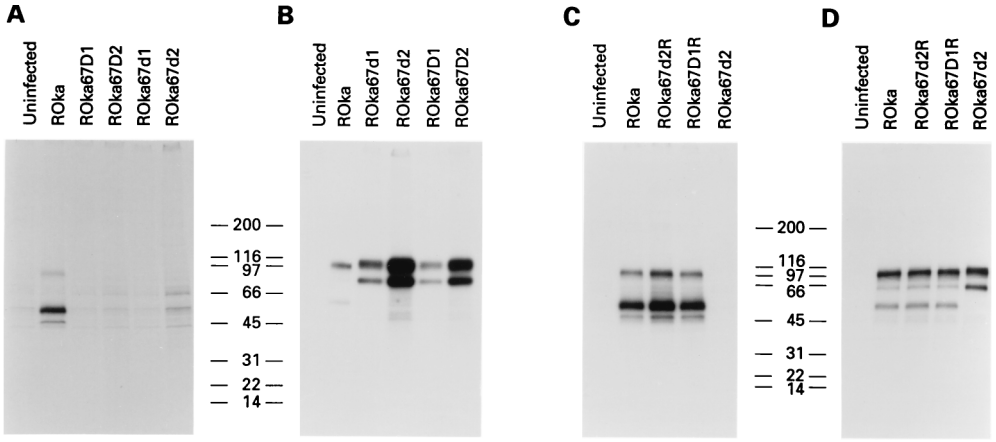

FIG. 3. Immunoprecipitation of radiolabeled protein extracts from cells infected with parental VZV or VZV gI deletion mutants by monoclonal antibodies to VZV gI (A and C) or gE (B and D). (A) Cells infected with VZV ROka express proteins of 98, 58, and 47 kDa that are immunoprecipitated with antibody to gI. (B) Cells infected with VZV ROka express 98- and 58-kDa proteins while cells infected with ORF67 deletion mutants express 100- and 80-kDa proteins that are immunoprecipitated with antibody to gE. Cells infected with ROka, ROka67D1R, or ROka67d2R express proteins of similar size that are immunoprecipitated with antibody to gI (C) or gE (D). The numbers indicate the molecular weights of proteins in kilodaltons.

VZV gI, cells were labeled with [^{35}S]methionine and immunoprecipitated with monoclonal antibody to VZV gI or gE. Cells infected with ROka, ROka67D1R, or ROka67d2R expressed proteins of 58 and 47 kDa, corresponding to mature and immature forms of gI, as well as a band of 98 kDa corresponding to gE (Fig. 3C).

Cells infected with ROka, ROka67D1R, or ROka67d2R expressed a gE protein of 98 kDa that was smaller than the 100-kDa gE protein in cells infected with ROka67d2, presumably due to the difference in glycosylation (see above). A 58-kDa protein, corresponding to gI, was also immunoprecipitated from cells infected with ROka, ROka67D1R, or ROka67d2R, but not ROka67d2, by the monoclonal antibody to gE.

Deletion of VZV gI impairs virus growth initiated by cell-to cell infection in vitro. MeWo cells were infected with cells containing VZV ROka, ROka67d, or ROka67D for 7 days, and the resultant plaque morphologies were compared. Cells infected with VZV ROka67d or ROka67D produced much smaller plaques than those infected with VZV ROka. The mean diameter ($\pm$ the standard deviation) of plaques after infection of MeWo cells with cells containing VZV ROka was 1.08 $\pm$ 0.18 mm, while the mean size of plaques produced by VZV ROka67d was 0.26 $\pm$ 0.14 mm ($P < 0.01$). The mean size of plaques produced by VZV ROka67D was 0.38 $\pm$ 0.14 mm, which was also significantly different ($P < 0.01$) from the mean size of plaques produced by VZV ROka.

To further determine whether deletion of gI impairs the ability of VZV to grow in vitro after infection with cell-associated virus, we measured the production of VZV ROka, ROka67d, and ROka67D during a 5-day growth analysis. MeWo cells were inoculated with virus-infected cells, and at various time points the cells were harvested and the virus titer was determined. By the first day after infection, a marked difference was apparent in the virus titers of cells infected with parental or gI-deleted virus; this difference persisted throughout the 5-day growth curve (Fig. 4A). Thus, both the plaque morphologies and growth curves indicated that the absence of gI impairs the growth of VZV.

gI has been shown to be particularly important for neuron-to-neuron spread of HSV (11) and for transneuronal spread of PRV from the retina to the visual centers (31). HSV and PRV establish latent infections in neurons. Since satellite cells may be the site of latency for VZV (6), and since Schwann cells are the peripheral nervous system equivalent of satellite cells (22), we studied virus replication in a schwannoma cell line. VZV deleted for gI grew to lower titers than parental VZV in schwannoma cells (Fig. 4B), although the difference was not as striking as that seen in MeWo cells. VZV deleted for gI was also impaired for growth in human fibroblasts (Fig. 4C).

Deletion of VZV gI impairs virus growth initiated by infection with extracellular virus in vitro. HSV gI facilitates cell-to-cell spread of the virus in cultured cells (1, 10) but is not thought to be required for infection of cells by extracellular virus. The experiments with the VZV mutants described above

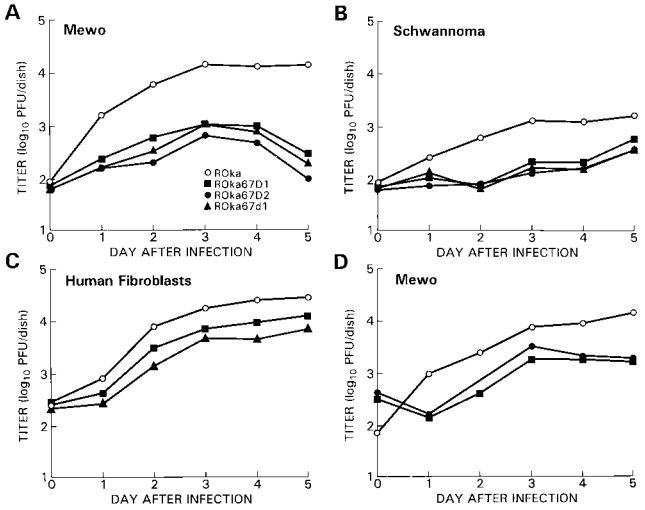

FIG. 4. Growth curves of cell-associated or cell-free VZV gI deletion mutants in human cells. Melanoma (A), schwannoma (B), and fibroblast (C) cells were inoculated with VZV-infected cells, and melanoma cells (D) were infected with cell-free virus. Aliquots, obtained by treating the cells with trypsin, were obtained on days 1, 2, 3, 4, and 5 after infection, and the titers of virus were determined by plating on melanoma cells. Day 0 indicates the virus titers in the inocula (titered on melanoma cells). The titer ($\log_{10}$ of the mean number of PFU per dish) is indicated on the *y* axis. Each data point is the average of values from duplicate experiments.

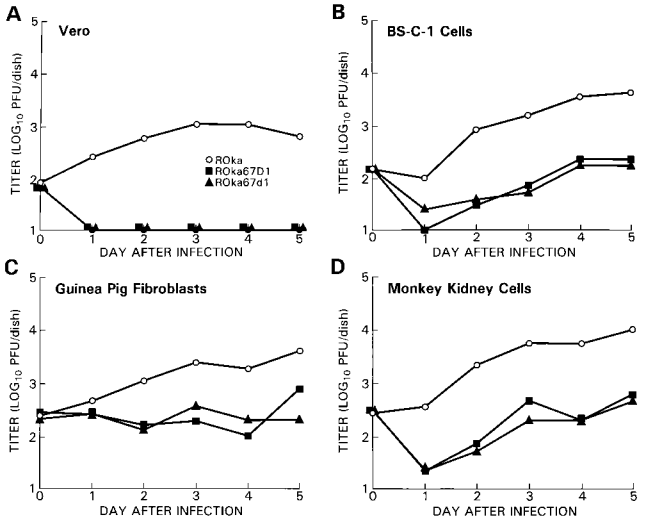

FIG. 5. Growth of VZV gI deletion mutants in nonhuman cells. Vero (A), BS-C-1 (B), guinea pig (C), and African green monkey kidney (D) cells were inoculated with VZV-infected cells, and virus titers were determined as described in the legend to Fig. 4.

were performed with cell-associated virus; infected cells could directly attach to uninfected cells, and the virus could spread in a cell-to-cell fashion. Since VZV gI is on the surface of the virion, we infected melanoma cells with cell-free VZV to study infection by extracellular virus and monitored the titer of virus produced over a 5-day period. While the titer of the inoculum of cell-free VZV deleted for gI was nearly 10-fold higher than that of the cell-free parental virus inoculum (day zero), VZV deleted for gI still grew to lower titers than the parental virus (Fig. 4D). Thus, VZV deleted for gI is also impaired for growth in vitro when infection is initiated by extracellular virus.

VZV deleted for gI does not replicate in Vero cells and replicates to low titers in other nonhuman cells. HSV gI mutants are impaired for growth in several cell lines but grow to normal titers in baby hamster kidney cells (1). VZV has been shown to replicate in monkey and guinea pig cells in vitro, in addition to human cells (for a review, see reference 5). VZV deleted for gI did not show evidence of replication in Vero cells; after inoculation of Vero cells with melanoma cells containing about 100 PFU of VZV, the titer of gI-deleted virus never exceeded 20 PFU during the entire 5-day growth analysis period (Fig. 5A). Infection of Vero cells with 1,000 PFU of VZV deleted for gI, followed by several passages of the infected cells, never resulted in plaque formation. Infection of melanoma cells with aliquots of ROka67D- or ROka67d-infected Vero cells, obtained after three and four passages, did not yield plaques on the melanoma cells. In contrast, a VZV ORF8 deletion mutant, which like the VZV ORF67 deletion mutant also grows to 10-fold-lower titers than parental VZV in MeWo cells, was able to replicate in Vero cells (25).

Since CPE were not detected in Vero cells infected with VZV deleted for gI, an immunofluorescent-focus assay was performed. Melanoma and Vero cells grown on glass coverslips were infected with cell-free VZV ROka or ROka67d. After 48 h, the cells were fixed and stained with monoclonal antibody to the VZV ORF62 protein. Melanoma cells infected with cell-free ROka showed immunofluorescent foci containing about 50 to 100 infected cells (Fig. 6A), while melanoma cells infected with cell-free ROka67d showed foci with about 25 to 50 infected cells (Fig. 6B). Vero cells infected with cell-free ROka showed foci consisting of 25 to 50 infected cells (Fig. 6C). In contrast, Vero cells infected with ROka67d had foci of only one to three infected cells (Fig. 6D). These results indicate that VZV deleted for ORF67 can enter Vero cells; however, entry may be less efficient than for the wild-type virus, and the virus is impaired for spreading to adjacent cells.

To determine whether viral DNA synthesis occurred in Vero cells infected with the VZV ORF67 deletion mutants, total cellular DNA was prepared from cells infected with VZV ROka or ROka67d, applied to a slot blot apparatus, and hybridized with a probe corresponding to the VZV genome. Infection of MeWo cells resulted in increasing viral DNA synthesis over 4 days with VZV ROka, while viral DNA synthesis for ROka67d was maximal 2 days after infection (Fig. 7A). In contrast, infection of Vero cells with VZV ROka showed a peak of viral DNA synthesis at 3 days, while infection with ROka67d resulted in a very low level of viral DNA synthesis at day 3 (Fig. 7B). Taken together, these results indicate that VZV deleted for ORF67 can enter cells but that viral DNA synthesis is impaired.

Additional nonhuman cells were infected with parental (ROka) VZV and the gI deletion mutants. VZV deleted for gI grew to low titers in BS-C-1 cells (Fig. 5B), guinea pig embryo fibroblasts (Fig. 5C), and African green monkey kidney cells (Fig. 5D), with an increase in titer of only 0.1, 0.4, and 0.3 logs over the input inocula, respectively. In contrast, infection of human cells with VZV deleted for gI resulted in an increase in titer of 0.8 to 1.6 logs over the input inoculum (Fig. 4). Thus, VZV gI is important for efficient replication of the virus in nonhuman cells.

To verify that the growth impairment of the VZV gI deletion mutants in MeWo and Vero cells was not due to a mutation elsewhere in the genome, a growth analysis was performed with the gI-rescued viruses in these cell lines. In both cell lines, the growth of the gI-rescued viruses closely paralleled the growth of the parental (ROka) virus (Fig. 8). Thus, the growth impairment of the VZV gI deletion mutants is due to the mutations in gI.

VZV deleted for gI is slightly impaired for adsorption to, but not penetration into, melanoma cells. Cell-free VZV ROka or ROka67d was incubated with melanoma cells for various periods of time; the inoculum was removed, the cells were washed, and plaques were counted after 1 week. Compared with the parental virus, VZV deleted for gI showed a slightly lower percentage of adsorption to melanoma cells at each time point tested after the first 5 min (Fig. 9A).

To test the ability of the virus to penetrate into melanoma cells, wild-type and gI mutant VZV were incubated with cells at 4°C for 2 h to allow the virus to adsorb to cells; the cells were then incubated for various periods of time at 37°C, treated with low-pH buffer to inactivate any virus that had not yet penetrated, and washed, and plaques were counted after 1 week. VZV deleted for gI was able to penetrate into cells nearly as well as the wild-type virus (Fig. 9B). Addition of polyethylene glycol, which has been shown to enhance penetration of HSV gB (26) and gD (16) mutants into cells, to Vero cells which were infected with cell-free VZV deleted for gI did not result in plaque formation. Studies could not be performed in melanoma cells, since polyethylene glycol is toxic to these cells (3). Taken together, these data suggest that VZV deleted for gI is not impaired for penetration into cells.

DISCUSSION

We have shown that VZV gI is not essential for replication of the virus; however, deletion of this glycoprotein impairs the

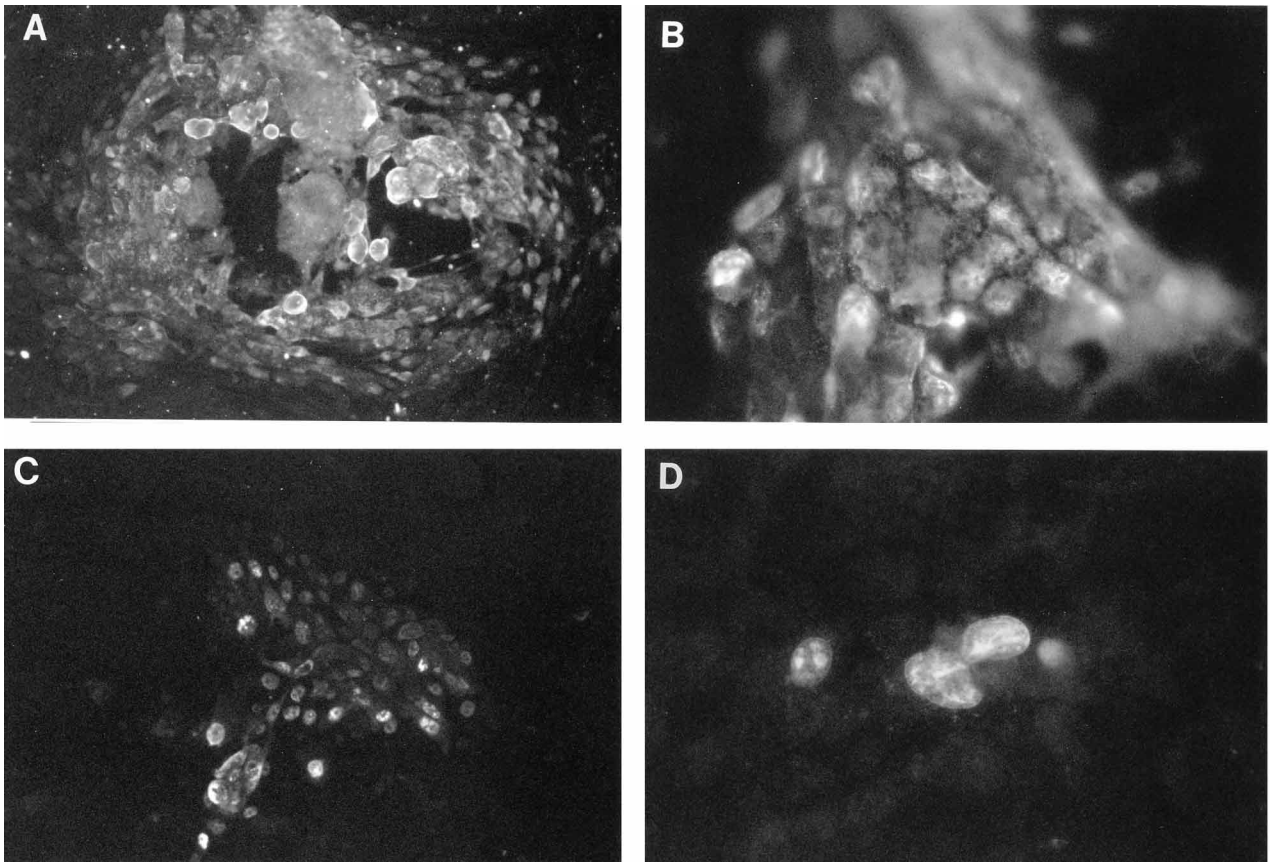

FIG. 6. Immunofluorescence microscopy revealing VZV ORF62 protein in MeWo cells infected with cell-free VZV ROka (A) or ROka67d (B) and in Vero cells infected with cell-free ROka (C) or ROka67d (D). Magnifications, ×15 (A and C) and ×48 (B and D).

growth of VZV in cell culture and results in aberrant processing of gE in virus-infected cells. VZV gI is necessary for efficient replication of the virus in nonhuman cells and is required for replication of the virus in Vero cells. Rescued VZV, in which the gI deletion has been restored, has growth patterns in melanoma and Vero cells similar to those of the parental virus.

VZV gI is homologous to HSV gI. HSV that cannot express gI produces small plaques, and the yield of virus obtained from infected human cells in vitro is about 5- to 10-fold lower than that of wild-type virus when a low multiplicity of infection (MOI; 0.001 PFU per cell) is used (10). In the presence of HSV-neutralizing antibody, infection with an HSV gI mutant results in 100- to 200-fold-lower titers of virus than does infection with wild-type HSV. These results show that HSV gI is important for cell-to-cell transmission of the virus. VZV infects cells exclusively by cell-to-cell spread in vitro, since no infectious virions are present in the medium. Infection of human cells with cell-free VZV gI mutants at a low MOI (0.0001 PFU per cell) resulted in the production of 20- to 30-fold less virus than did infection of these cells with the parental virus (Fig. 4D). Thus, the growth of the VZV gI deletion mutant was similar to that of the corresponding HSV mutant when human cells were infected in vitro.

VZV deleted for gI did not replicate in Vero cells. In contrast to our findings with VZV gI mutants, Dingwell and colleagues noted only a slight diminution in growth of an HSV-1 gI mutant in Vero cells (10). Since HSV gI is important for cell-to-cell spread of the virus, these results imply that VZV and HSV may have different mechanisms for spreading across

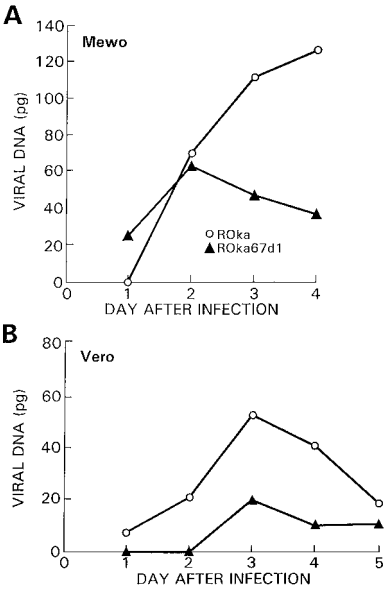

FIG. 7. Viral DNA synthesis in MeWo (A) and Vero (B) cells infected with VZV ROka or ROka67d. Cells were infected with melanoma cells containing about 200 PFU of VZV (MOI, approximately 0.001), and cells were harvested at various times after infection. DNA was extracted from the cells, applied to a slot blot apparatus in parallel with control VZV cosmid DNA representing the entire genome, and hybridized with radiolabeled VZV DNA. The signal was quantitated with a PhosphorImager, and the amount of DNA for each data point was determined by comparison with the signals from dilutions of the control DNA.

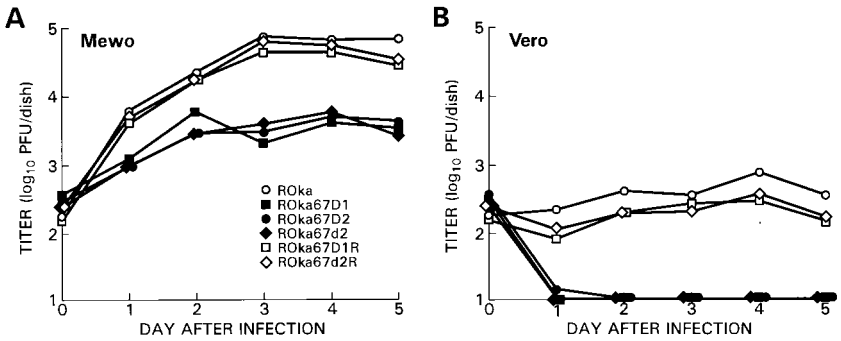

FIG. 8. Growth of rescued VZV gI deletion mutants in melanoma (A) and Vero (B) cells. VZV inoculations and virus titrations were performed as described in the legend to Fig. 4.

cell junctions in Vero cells. HSV and PRV gI promote cell fusion (9, 34). The presence of polyethylene glycol, which promotes membrane fusion and enhances penetration of HSV into cells, did not result in detectable replication of the VZV gI mutant in Vero cells.

VZV deleted for gI grew to much lower titers in nonhuman cells than in human cells. Analysis of the growth of an HSV gI mutant in various cell types showed that the gI mutant grew to titers similar to those seen with the wild-type virus in hamster cells. The HSV gI mutant grew to lower titers than the wild-type virus in Vero or human fibroblast cells, and they grew even less well in human epithelial cells (1). In contrast, we found that VZV deleted for gI grew to higher titers in human fibroblast and melanoma cells than in primary guinea pig or monkey cells and did not replicate in Vero cells. The VZV gI mutants also grew to higher titers in BS-C-1 cells than in Vero cells. While Vero and BS-C-1 cells are both derived from African green monkey (*Cercopithecus aethiops*) kidney cells, Vero cells have a fibroblast-like morphology while BS-C-1 cells have an epithelial-cell-like morphology. Taken together, these results suggest that human cells may have a protein(s) that can partially substitute for VZV gI but is not present, or is present in lower amounts or in a different form, in Vero cells.

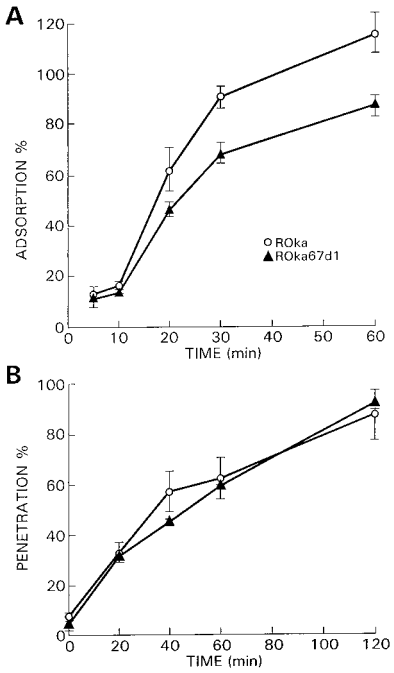

FIG. 9. Adsorption and penetration assays for the parental virus and a VZV gI deletion mutant. Cells were infected with 50 to 100 PFU of cell-free VZV. (A) For the adsorption assay, cells were incubated with VZV for various periods of time and washed with medium, and plaques were counted after 7 days. (B) For the penetration assay, cells were incubated with VZV at 4°C, fresh medium was added, cells were treated with low-pH buffer at various time points and washed with medium, and plaques were counted at 7 days. The bars represent standard error.

VZV deleted for gI was slightly impaired for adsorption to melanoma cells. Similar experiments showed that adsorption of an HSV-1 gI mutant to Vero cells was indistinguishable from adsorption of wild-type HSV-1 to those cells (1). HSV glycoproteins gC and gB are important for attachment to cells by binding to heparan sulfate, while gC and gD participate in attachment by a heparan sulfate-independent step (for a review, see reference 30). Since VZV lacks a gD homolog, gI may contribute to attachment.

VZV gI and gE are present as a complex on the surface of VZV-infected cells. VZV gE migrated as a 98-kDa protein when immunoprecipitated from cells infected with VZV ROka and as a 100-kDa protein when immunoprecipitated from cells infected with ROka67d or ROka67D. Similar findings have been reported previously by Yao et al. (33). Transfection of cells with a plasmid expressing gE alone yielded a 100-kDa gE protein, while cotransfection of cells with plasmids expressing both gE and gI yielded a 98-kDa gE protein. Since the gE-gI complex is formed before posttranslational modifications occur in the Golgi apparatus, the difference in size presumably reflects differences in glycosylation (33).

Infection of mice with an HSV gI mutant by corneal scarification results in small corneal lesions without virus spread, while infection with wild-type HSV results in large dendritic lesions, periocular skin disease, and viral encephalitis (10). Inoculation of mice by ear scarification results in rapid clearance of HSV gI mutant virus from the site of infection and reduced replication in the nervous system when compared to the wild-type virus (1). Intravitreal inoculation of HSV gI mutant virus also results in less viral spread in the retina and central nervous system than wild-type virus (11). Similarly, PRV gI is required for neurovirulence of PRV in rats after intravitreal inoculation of the virus (2). By analogy with other alphaherpesviruses, VZV gI also may be important for neurovirulence and VZV deleted for gI may be useful as a candidate live virus vaccine.

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