Absence of varicella-zoster virus (VZV) glycoprotein V does not alter growth of VZV in vitro or sensitivity to heparin

Jeffrey I. Cohen* and Karen E. Seidel

Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892, U.S.A.

Varicella-zoster virus (VZV) is the aetiologi agent of chickenpox and herpes zoster. The VZV genome contains at least 72 open reading frames (ORFs), which encode several structural proteins including proteins present in the viral nucleocapsid and tegument, and glycoproteins in the lipid envelope.

VZV expresses at least five glycoproteins, gpI, gpII, gpIII, gpIV and gpV, which are encoded by ORF68, ORF31, ORF37, ORF67 and ORF14, respectively (Davison & Scott, 1986). Antibodies to gpII, gpIII and gpV neutralize virus in the absence of complement, whereas antibodies to gpI and gpIV usually require complement for neutralization (Keller et al., 1990; Kinchington et al., 1990b). Humans infected with VZV produce antibodies to gpI, gpII, gpIII and gpV (Grose, 1990; Kinchington et al., 1990b).

VZV ORF14 encodes a 560 amino acid glycoprotein (Davison & Scott, 1986) with a hydrophobic signal sequence at the amino terminus, a hydrophobic carboxy-terminal anchor, and five potential N-glycosylation sites. The gpV sequence contains several 42 bp repeats and the number of repeats is variable between strains of VZV (Kinchington et al., 1986). In addition, the amount of gpV expressed varies between viral isolates and is a reflection of the amount of gpV mRNA transcribed (Kinchington et al., 1990b; Ling et al., 1991). Like its bovine herpesvirus (BHV) homologue, BHV gIII, VZV gpV is homologous to the class II major histo-

compatibility complex antigen constant domain. Thus, gpV may be a distant member of the immunoglobulin superfamily (Fitzpatrick et al., 1989).

VZV gpV is homologous to herpes simplex virus type 1 (HSV-1) glycoprotein C (gC; McGeoch et al., 1988). HSV-1 gpC is dispensable for infection in vitro. HSV-1 gC binds heparin (Herold et al., 1991) and the C3b (Friedman et al., 1984) and iC3b (Tal-Singer et al., 1991) components of complement and prevents virus neutralization by complement (Harris et al., 1990). Neutralizing antibody to HSV-1 gC blocks the binding of HSV-1 to cells (Fuller & Spear, 1985; Svennerholm et al., 1991). HSV-1 unable to express gC has reduced infectivity (lower p.f.u./particle ratio) and impaired absorption to and penetration of cells in vitro compared with wild-type HSV-1 (Herold et al., 1991). HSV-1 gC is apparently required for attachment of the virus to the apical surface of a polarized epithelial cell line (Seas et al., 1991). HSV-1 with gC deleted produces ocular disease in rabbits (Centifanto-Fitzgerald et al., 1982), and HSV-2 with gC deleted spreads in the central nervous system of mice (Johnson et al., 1986) similar to the parental strains.

VZV gpI, gpII, gpIII, gpIV and gpV are homologous to HSV-1 gE, gB, gH, gI and gC, respectively. Unlike HSV-1, VZV does not encode homologues for HSV-1 gG, gJ and gD. In HSV-1 gD, gB and gH are essential for viral growth in cell culture. Since VZV lacks a gD homologue, gpI, gpIV or gpV might assume a role similar to HSV-1 gD and be required for VZV replication in vitro. Here we show that VZV gpV is not essential for
growth in cell culture and that virus that is unable to express gpV grows at a similar rate in vitro as virus that expresses gpV. Furthermore, we show that VZV that does not express gpV is inhibited by heparin to a similar extent as the parental virus.

Methods

Cells and viruses. Human melanoma cells (MeWo) were obtained from C. Grose and whole human fibroblast (WHF) cells were obtained from BioWhittaker. VZV strain Oka, used for generation of recombinant virus, was provided by M. Takahashi (Osaka University, Suita, Osaka, Japan). An additional isolate of attenuated VZV strain Oka (CL304) was provided by J. White (Merck, Sharpe and Dohme), and wild-type VZV strain Ellen was obtained from the American Type Culture Collection.

Plasmids and cosmids. Four cosmids, VZV NotI A, NotI BD, MstII A and MstII B, contain the entire genome of the Oka strain of VZV (Fig. 1; Cohen & Seidel, 1993). Cosmid VZV NotI A was cut with Sse8387I (Takara Biochemicals) and the 12.9 kb fragment (VZV nucleotides 9780 to 22631; Davison & Scott, 1986) was cloned into pUC19 at the PstI site to produce plasmid pUC-Sse (Cohen & Seidel, 1993). Partial digestion of plasmid pUC-Sse was performed with the restriction enzyme Bsi1107I (New England Biolabs) in the presence of ethidium bromide. Bsi1107I cuts pUC-Sse four times at VZV nucleotides 13702, 17547, 19040 and 21070; the latter site is near the 5' end of VZV ORF14, which encodes glycoprotein V. Plasmid cut at one site was separated from uncut plasmid or plasmid cut at multiple sites by pulsed-field gel electrophoresis, and a synthetic double-stranded oligonucleotide (TAGCTAGGCGCGCCTAGCTA) was inserted into the plasmid at the Bsi1107I sites. This oligonucleotide contains stop codons in all three ORFs and an Ascl restriction site. There are no Ascl restriction sites within the VZV genome. Plasmid containing the oligonucleotide inserted into ORF14 was isolated and sequenced to verify that the insertion had the expected sequence. The VZV DNA from the plasmid containing the oligonucleotide was inserted back into cosmid VZV NotI A in place of the wild-type Sse8387I fragment to yield cosmid NotI A-14S (Fig. 1). Two independent clones of NotI A-14S were selected. Plasmid pCMV62 contains the VZV ORF62 gene, a potent trans-activator of VZV genes, driven by the human cytomegalovirus promoter (Perera et al., 1992).

Transfection. Cosmids were linearized with NotI or MstII. One µg of cosmids VZV NotI A-14S, NotI BD and MstII B, 0.5 µg of cosmids VZV MstII A, 0.05 µg of plasmid pCMV62 and 2 µg of sheared salmon sperm DNA were used to transfect human melanoma cells in 60 mm dishes using the calcium phosphate procedure (Moriuchi et al., 1993). Six days after transfection, the cells were passaged into 75 cm² flasks.

Southern blots, immunoprecipitations and growth curves. Southern blots were performed as previously described (Cohen & Seidel, 1993). For immunoprecipitations, VZV-infected cells were radiolabelled with [35S]methionine (200 µCi/ml). After 5 h the cells were washed and lysed in radioimmunoprecipitation assay (RIPA) buffer (10 mM-Tris-HCl pH 8.0, 100 mM-NaCl, 1 mM-EDTA, 1%NP40, 0.5% deoxycholate, 0.1% SDS). After centrifugation, the supernatant was incubated with rabbit antisera to gpV (Kinchington et al., 1990b) for 1 h at 4°C and Protein G-agarose for 30 min. Immune complexes were washed in RIPA buffer, boiled in gel loading buffer and fractionated on an SDS-containing 8% polyacrylamide gel. VZV gpl and ORF62 protein were immunoprecipitated using murine monoclonal antibodies (Chemicon). Growth studies of recombinant VZV (Cohen & Seidel, 1993) were performed as described previously.

Heparin inhibition of VZV growth. Cell-free VZV was prepared using a modification of the procedure of Shirsaki & Hyman (1987). VZV-infected MeWo cells were washed in PBS, scraped from flasks, pelleted and sonicated three times in SPGC medium (PBS, 10% fetal bovine serum, 5% sucrose and 0.1% sodium glutamate). The sonicated cells were pelleted for 15 min at 1500 r.p.m. and the supernatant, containing cell-free VZV, was used immediately for the heparin inhibition assay. Heparin was added to cell-free VZV (10 µl) at a final concentration of 0, 1, 5, 10 or 100 µg/ml and the virus was incubated with human melanoma cells for 2 h at 37 °C. The cells were then washed three times with PBS and Eagle’s MEM supplemented with 2% fetal bovine serum was added. Plaques were counted after 7 days.

Results

VZV gpV is not essential for growth of VZV in cell culture

Cosmid VZV NotI A-14S encodes the VZV glycoprotein V gene (ORF14) with a 20 bp oligonucleotide inserted after the fifteenth codon of the gene (Fig. 1). This oligonucleotide contains stop codons in all three ORFs and an Ascl restriction endonuclease site. VZV containing the mutant gpV gene was constructed by transfecting melanoma cells with cosmids VZV NotI A-14S, NotI BD, MstII A, MstII B and plasmid pCMV62. VZV containing the wild-type gpV gene was obtained by substituting cosmid VZV NotI A, for cosmid VZV NotI A-14S in the transfections. Six days after transfection, when no c.p.e. was apparent, the cells were treated with trypsin and replated into 75 cm² flasks. Eight to 9 days after transfection, typical VZV c.p.e. was seen in cells transfected with the cosmids mixtures containing the mutant or wild-type gpV gene.

To verify that the mutant VZV had the expected genome structure, VZV derived from the transfection was passaged in WHF cells and viral DNA was isolated from nucleocapsids. Viral DNA was cut with BamHI, EcoRI, or EcoRI plus Ascl, and Southern blots were performed using a mixture of the four radiolabelled cosmids DNAs as a probe. Digestion of Oka, recombinant Oka (ROka) and two independent clones of recombinant Oka VZV with a stop codon in ORF14 (ROka14SA and ROka14SB) with BamHI or EcoRI gave identical bands (Fig. 2a, b). Digestion of ROka14S isolates with EcoRI plus Ascl cut the 1.7 kb band corresponding to the EcoRI P fragment of VZV DNA into two bands, owing to the Ascl restriction site inserted into the viral genome (Fig. 2c).

Cells infected with VZV ROka and the two independent clones of ROka14S were radiolabelled with [35S]methionine and immunoprecipitations were performed using rabbit antibody to VZV gpV. Cells infected with VZV ROka expressed a gpV protein of Mr 100K to 110K recognized by the antibody, whereas cells infected with ROka14S did not express gpV (Fig. 3a). The stop
Fig. 1. Generation of recombinant VZV with a stop codon in gpV. The prototype VZV genome is 124848 bp in length (top line) with terminal repeat (TR), unique long (UL), unique short (US), and internal repeat (IR) regions (second line). The BamHI restriction map (third line) and NotI (fourth line) and MstI (fifth line) restriction fragments from VZV Oka used to generate the recombinant VZV are shown. Cosmid VZV NotI A-14S contains a double-stranded oligonucleotide with stop codons in all three ORFs and an Ascl restriction site, inserted after the fifteenth codon of ORF 14.

Fig. 2. Southern blot of DNAs from Oka (lanes 1), recombinant Oka (ROka) (lanes 2), and ROka14SA (lanes 3) and ROka14SB (lanes 4). VZV DNA was cut with BamHI (a), EcoRI (b), or EcoRI and Ascl (c). The BamHI and EcoRI digests showed no variation between the viruses. Digestion of ROka14S with EcoRI and Ascl cuts the 1-7 kb EcoRI P band (open arrow) into two bands of 1.4 kb (closed arrow) and 0.6 kb (not visualized), due to the Ascl site in the inserted oligonucleotide.
Immunoprecipitation of another aliquot of radiolabelled cell lysate with monoclonal antibody to VZV gpI indicated that cells infected with ROka or ROkal4S VZV all expressed gpI (Fig. 3b). Thus, the absence of expression of gpV in cells infected with ROkal4S was not due to lack of VZV gene expression. Although ROkal4S was not plaque purified and the oligonucleotide insertion containing the stop codons could have been deleted during replication in cell culture, immunoprecipitations performed after repeated passage of the virus in culture did not show evidence of gpV expression. In addition, analysis of the viral genome after multiple passages using PCR showed the expected oligonucleotide insertion (data not shown).

Absence of VZV gpV does not markedly alter the sensitivity of VZV to inhibition by heparin in vitro

Heparin has been shown to inhibit the growth of HSV-1, pseudorabies virus (PRV), and VZV in vitro (Herold et al., 1991; Horvath & Hadhazy, 1965; Mettenleiter et al., 1990; R. K. Williams & S. E. Straus, personal communication); however, a PRV mutant devoid of gpIII (the homologue of VZV gpV) was not inhibited by heparin (Mettenleiter et al., 1990), whereas an HSV-1 mutant devoid of gC was inhibited by heparin (Herold et al., 1991). To determine whether the VZV mutant that does not express gpV had a phenotype similar to the corresponding mutant of HSV-1 or PRV, we prepared cell-free ROka and ROkal4S, incubated these viruses with increasing concentrations of heparin, and titrated the viruses on MeWo cells.

VZV that did not express gpV showed a pattern of inhibition with increasing concentrations of heparin similar to that of parental virus (Fig. 5). ROkal4S VZV was slightly less sensitive to inhibition by heparin, especially at lower concentrations; however, the difference was not statistically significant. When ROkal4S VZV was incubated with the lowest dose of heparin tested (1 μg/ml) there was an increase in the number of the cells were trypsinized and serial dilutions of infected cells were titrated on MeWo cells. Oka, ROka, and ROkal4S all grew on MeWo cells with similar growth rates over time (Fig. 4). Therefore, the absence of VZV gpV does not inhibit the growth of VZV in vitro.

Absence of VZV gpV does not affect VZV growth in cell culture

To determine whether the absence of gpV affects the growth of VZV in vitro, we infected MeWo cells using VZV-infected cells containing Oka, ROka, and two independent clones of ROkal4S. At various time points codon, inserted in the gpV gene, should terminate translation of the protein after the fifteenth amino acid.

Immunoprecipitation of another aliquot of radiolabelled cell lysate with monoclonal antibody to VZV gpI indicated that cells infected with ROka or ROkal4S VZV all expressed gpI (Fig. 3b). Thus, the absence of expression of gpV in cells infected with ROkal4S was not due to lack of VZV gene expression. Although ROkal4S was not plaque purified and the oligonucleotide insertion containing the stop codons could have been deleted during replication in cell culture, immunoprecipitations performed after repeated passage of the virus in culture did not show evidence of gpV expression. In addition, analysis of the viral genome after multiple passages using PCR showed the expected oligonucleotide insertion (data not shown).
plaques, compared to incubation without heparin. Enhancement of plaque number by heparin was also observed with an HSV-1 gC-negative mutant in HEP-2 cells (Herold et al., 1991). There was a 50% reduction in the number of plaques for ROka and ROka14S VZV at 8 μg/ml and 9 μg/ml of heparin, respectively. These results suggest that wild-type VZV and VZV that does not express gpV both require heparan sulphate on the cell surface for their attachment to cells.

**Different isolates of attenuated Oka VZV express similar levels of gpV**

Kinchington et al. (1990a) reported that cells infected with attenuated Oka VZV express less gpV than cells infected with the parental wild-type Oka or other wild-type VZV strains. To determine whether cells infected with recombinant-derived Oka VZV made gpV at levels similar to other isolates, we immunoprecipitated gpV from cells infected with different strains of VZV. Immunoprecipitation of gpV from radiolabelled cells infected with these viruses indicated that each of the VZV Oka isolates expressed similar amounts of gpV; however, VZV Ellen tended to express more gpV (Fig. 6a). Immunoprecipitation of another aliquot of radiolabelled cell lysate from the same preparation used for detection of gpV indicated that cells infected with attenuated Oka expressed comparable amounts of VZV gpI and ORF62 protein (Fig. 6b, c).

**Discussion**

We have shown that VZV gpV is dispensable for growth of the virus in cell culture. Whereas ten viral glycoproteins have been identified in cells infected with HSV-1, only five viral glycoproteins have been identified in VZV-infected cells at present. VZV does not encode a homologue of HSV-1 gD, a gene essential for the
replication of HSV-1 in cell culture. Thus gpV, which is dispensable for growth of VZV in cell culture, does not perform the essential function that is supplied by gD in HSV-1.

VZV unable to express gpV grew at the same rate in cell culture as parental virus. Studies with an HSV-1 mutant that does not contain gC indicated that the HSV-1 mutant's infectivity was reduced by approximately 90 to 95%, as determined by a reduced p.f.u./particle ratio, compared with wild-type HSV-1 (Herold et al., 1991). Because of our inability to obtain purified cell-free VZV at high titre we were unable to assess the specific infectivity of our VZV gpV mutant.

Heparan sulphate moieties of cell surface proteoglycans have been shown to be important for the entry of alphaherpesviruses into cells. Addition of heparin to HSV-1, VZV, and PRV results in reduced plaque formation in cell culture (Herold et al., 1991; Horvath & Hadhazy, 1965; Mettenleiter et al., 1990; R. K. Williams & S. E. Straus, personal communication). PRV, like HSV-1, contains a glycoprotein (gIII) that is homologous to VZV gpV (Robbins et al., 1986). Heparin results in reduced plaque formation when added to HSV-1 that lacks gC (Herold et al., 1991). In contrast, studies of heparin inhibition of plaque formation using an assay similar to that used in the HSV-1 study (Herold et al., 1991) and in our study, showed no reduction in plaque number when heparin was added to PRV devoid of gII (Mettenleiter et al., 1990). However, a recent study using an assay that measures radiolabelled PRV found that heparin inhibited the adsorption of PRV devoid of gIII to cells (Karger & Mettenleiter, 1993). We found that heparin reduces plaque formation of both VZV devoid of gpV and parental virus to a similar extent. Thus, using similar assays of plaque reduction by heparin, our VZV gpV mutant more closely resembles the behaviour of the HSV-1 gC mutant than that of the PRV gIII mutant.

Additional herpesvirus proteins have also been shown to be important for interaction with heparan sulphate. HSV-1 gB, the homologue of VZV gpII, binds to heparin-Sepharose (Herold et al., 1991). In contrast, gp50 of PRV (the homologue of HSV-1 gD) appears to be important for interaction with heparan sulphate. PRV devoid of gp50 was severely impaired for heparin-resistant attachment to cells (Karger & Mettenleiter, 1993). Since VZV lacks a PRV gp50 (or HSV-1 gD homologue), but does contain an HSV-1 gB homologue, it will be important to determine whether VZV gpII is also important for the interaction of VZV with heparan sulphate.

Cells infected with different isolates of attenuated VZV Oka expressed similar amounts of gpV, but at a somewhat reduced level compared with wild-type VZV Ellen. In earlier studies attenuated VZV Oka (obtained from the American Type Culture Collection, Smith-Kline R.I.T., or Merck, Sharpe and Dohme) expressed very low levels of gpV compared to wild-type strains Scott or Ellen. Recently, Kinchington et al. (1990a) found that although virus isolated from vaccinees receiving the Oka strain of VZV who had mild symptoms generally expressed a low level of gpV, virus isolated from one vaccinee who also had mild symptoms expressed high levels of gpV. Our results indicate that after molecular cloning and passage in cell culture, the level of gpV expressed in cells infected with recombinant-derived VZV Oka was similar to that for the parental virus (Oka-T) from which ROka VZV was derived.

VZV gpV has been shown to be a target for neutralizing antibodies in humans (Kinchington et al., 1990b). Although gpV is dispensable for virus growth in vitro, the gpV gene has been conserved throughout evolution and may have an important role during infection in vivo. The construction of a VZV mutant that is unable to express gpV should allow us to study the role of this protein in viral pathogenesis in vivo.

Note added in proof. The International Herpesvirus Workshop has decided to give VZV glycoprotein V the alternate name VZV gC.

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References


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