Analysis of the Glycoproteins I and E of Varicella-Zoster Virus (VZV) using Deletional Mutations of VZV Cosmids

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The contributions of the glycoproteins gl (ORF67) and gE (ORF68) to varicella-zoster virus (VZV) replication were investigated in deletion mutants made by using cosmids with VZV DNA derived from the Oka strain. These experiments demonstrated that gl was not required for VZV replication in vitro but gE appeared to be. Although VZV gl was not required, its deletion or mutation resulted in a significant decrease in infectious virus yields, and it disrupted syncytial formation and altered the conformation and distribution of gE in infected cells. Normal cell-to-cell spread and replication kinetics were restored when gl was expressed from a non-native locus in the VZV genome. The expression of intact gl, the ORF67 gene product, is required for efficient VZV replication.

Methods and Results

The functions of varicella-zoster virus (VZV) glycoproteins E (gE) and I (gl) in viral pathogenesis in vivo have not been defined, although both glycoproteins are known to be immunogenic in the human host and in the guinea pig model of VZV infection [1–6]. This summary describes our experience with the analysis of the role of gl and gE in VZV infectivity in vitro, using deletional mutation of VZV cosmids, as previously reported by Mallory et al. [7]. Within the unique short (US) region, VZV has only two glycoprotein genes, ORFS 67 and 68, which encode gl and gE, while other alphaherpesviruses have at least four, including gD, gE, gG, and gl. VZV gE and gl may have functions in viral infection that are essential for infectivity, whereas counterparts in the other alphaherpesviruses are dispensable. When gE (glycoproteins I) and gl (glycoproteins IV) are produced in VZV-infected cells or from plasmid, vaccinia, or baculovirus vectors, the two glycoproteins form a noncovalently linked complex after their synthesis in the endoplasmic reticulum. A site of interaction between gE and gl has been mapped to the N-terminus of gl [8]. This complex is expressed on the cell surface [9]. The mature forms of gE and gl are glycosylated and phosphorylated [10–13].

Our objective was to investigate the functions of gl and gE in VZV by mutational analysis of ORF67 and ORF68, using a cosmid system. VZV replicates as a cell-associated virus in tissue culture, which makes it difficult to recover pure populations of mutant viruses by homologous recombination methods. With cosmids, recombinants can be made by creating mutations in one or more of the overlapping fragments, which represent the full-length genome of VZV and transfecting permissive cells [14]. The cosmid used in our experiments were provided by George Kemble (Aviron, Mountain View, CA). As previously described in [7], we subcloned ORF67 and ORF68 into plasmid vectors from the cosmid containing the US region of the genome, gl and/or gE gene sequences were altered and ligated back into the cosmid, and the modified cosmid was cotransfected into permissive cells along with the three intact cosmids. This approach permitted an investigation of the effects of genetic mutations in the gl and gE on VZV replication.

Next, a complete deletion of ORF67 was made to ensure that after the dual deletion of gE and gl, experiments were done to determine whether only gE was dispensable and if so, to localize the regions of gl that were important for viral replication in the presence of intact gE (figure 1). The first mutation procedure was designed to remove ~60% of the N-terminal region of gl (figure 1, line 6). Infectious virus was recovered after transfection of the cosmid with the partial gl deletion along with the three intact cosmids. However, several differences were observed between the mutants and intact virus, consisting of delays in the interval to plaque formation, lower peak titers of infectious virus, and smaller plaques (figure 2). Two separate clones of the N-terminal deletion mutant were made, and they exhibited the same altered phenotype. The deletion in gl was verified by Southern blot analysis of VZV DNA from infected cells, polymerase chain reaction (PCR) amplification across the mutated region, and sequencing of the PCR products from the mutated viral DNA.

Next, a complete deletion of ORF67 was made to ensure that the altered phenotype observed in melanoma cells infected with the N-terminal deletion mutant was due to a loss of gl function.
Figure 1. Construction of cosmid vectors with deletions of varicella-zoster virus (VZV) open-reading frame (ORF)67 (gI) and ORF68 (gE). Line 1 is schematic diagram of VZV genome with location of genes that encode gI and gE in unique short (US) region. Line 2 depicts overlapping segments of VZV genome used to construct VZV cosmids. Line 3 shows subcloned SacI fragment that includes ORF67 and ORF68 (black boxes) with restriction sites used to construct deletions in ORF67 and ORF68 and SgrAI sites used to insert mutated fragments back into cosmid. Lines 4–7 depict deleted regions (white boxes) resulting in cosmids pVSpe21EID (ΔgI/gE), pVSpe21ΔgI, pVSpe21ΔgI-N, and pVSpe21ΔgI-C, respectively. To restore gI, ORF67 was ligated into unique AvrII site of pVSpe21ΔgI in each orientation, resulting in pVSpe21gIAvrII (left to right orientation) and pVSpe21gI AvrI9 (right to left orientation) (line 8). TRL = terminal repeats long; UL = unique long; IRL = internal repeats long; TRS = terminal repeats short. Reproduced from [7] with permission.

Rather than to the possible synthesis of a partial gene product containing the C-terminal region of gI (Figure 1), infectious virus was recovered after transfection of the cosmid with the complete gI deletion along with the three intact cosmids. The patterns of initial plaque formation and morphology, delayed interval to peak titer, and lower peak titer were similar to the N-terminal deletion mutant (Figure 2). The complete deletion mutants were evaluated to confirm that the anticipated changes in viral DNA sequence had occurred.

A peptide sequence of gI that binds to gE has been identified in the N-terminal region of gI [8]. To determine whether the presence of the N-terminus of gI was sufficient to restore the replication and plaque size characteristics of intact VZV, a C-terminal deletion mutant was made (Figure 1). This mutation left the N-terminal sequence of gI, including the sequence encoding the gE-binding peptide region, intact. Despite the presence of this domain, the phenotype of the C-terminal deletion resembled the N-terminal and complete gI deletion mutants. The initial plaque phenotype, interval to plaque formation, peak titer, and mean size of plaques were similar for all gI mutants; however, the complete deletion of gI resulted in the most delayed growth rate and the lowest yield of infectious virus (Figure 2). The preservation of the N-terminus was associated with slightly better replication.

To show that the altered replication characteristics of the gI deletion mutants were caused by the absence of the ORF67 gene product, we inserted the ORF67, including regions spanning the upstream and downstream regions, into a unique AvrII restriction site in one of the cosmids. The ORF67 coding sequence was cloned into the cosmid vector at this site between ORF65 and ORF66 in both orientations. Infectious virus was recovered in all transfec-
tions, regardless of the orientation of gI. The initial plaque morphology and replication kinetics as well as the plaque size and peak virus titers were indistinguishable from those for virus made using intact cosmids. As in the case of the deletion mutants, the expected changes in the DNA sequence of the repaired viruses were confirmed by Southern blot, PCR, and sequencing.

The effects of gI mutations, melanoma cells infected with these viruses, and intact VZV were further analyzed [7] by use of confocal microscopy. Syncytia characteristic of VZV infection, with extensive formation of polykaryocytes, were observed in cells infected with intact virus or with the repaired mutants. In these multicucleated cells, the nuclei were organized in a regular pattern encircling centralized Golgi. Recombinant strains, in which gI production was intact, also induced the formation of viral "highways" between cells [21]. In contrast, infection with all of the gI deletion mutants resulted in disrupted syncytia. Cells had multiple nuclei, but their arrangement was disorganized, and viral highways were not detected.

Because of the change in growth characteristics of the gI deletion mutants, we used confocal microscopy to examine the effects of removing gI on the maturation and cellular location of gE. By 56 h after infection, gE was present in a diffuse distribution in infected cell membranes when intact gI was present. In contrast, gE expression was altered significantly when cells were infected with each of the gI deletion mutants. The gE trafficked to the cell surface, but its distribution in the absence of gI was in a localized, punctate pattern. The abnormal pattern of gE localization was noted even when the N-terminus of gI was preserved. In these mutants, a truncated form of gI, which contained the peptide sequence in the extracellular domain that is known to bind gE, was expressed [8].

In cells infected with intact VZV, the expression of gI was diffuse, while the truncated form of gI made by the C-terminal deletion mutant was detected in a localized, punctate pattern.

Experiments using fluorescence-activated cell sorter analysis, immunohistochemistry, and Western blot were done using antibody reagents specific for VZV gE. These experiments showed changes in antibody binding consistent with altered gE conformation when gE was made in the absence of gI. For example, binding to the underglycosylated 73-kDa form of gE of one gE monoclonal antibody was disrupted in the absence of gI and when truncated gI was made.

**Discussion**

Herein, we have summarized our experiments using VZV cosmids as described previously by Mallory et al. [7]. This work demonstrated that gI was not required and gE appeared to be necessary for viral replication. Nevertheless, the deletion or mutation of gI inhibited and altered the formation of syncytia, which is a defining characteristic of VZV infection in tissue culture cells. VZV recombinants with complete or partial deletions of gI also consistently exhibited a longer replication cycle and lower yields of infectious virus. The characteristic plaque morphology and replication pattern were restored when the gI gene, ORF67, was inserted into a non-native site in the VZV genome. These experiments with repaired VZV indicated that the changes in plaque morphology and replication patterns were due to gI mutations rather than to disruption of promoter sequences or other regulatory
regions affecting adjacent genes in the US segment or to random mutations at other sites in the genome.

The small plaque phenotype of the VZV gI deletion mutants suggests that gI gene products contribute to fusion between infected cells and adjacent, uninfected cells. Since VZV is not released from infected cells in vitro, interference with cell-to-cell spread was predicted to have the direct effect on infectious virus production that we observed. The effects of VZV gI deletion or mutation on viral replication were similar to those described after disruptions of homologous genes in other alphaherpesviruses [15, 16, 18–20, 22, 23].

The abnormal membrane fusion that occurred in cells infected with the VZV gI mutants could reflect loss of a function that is mediated by gI only. Alternatively, it may represent disruption of the formation of the normal gE:gl complexes [9], or the disruption of syncytia in infected cells with gI mutants could be due to changes in gE conformation and trafficking that occurred in the absence of a necessary gI chaperon function. A requirement for gI to achieve maturation or transport of gE has been observed in other alphaherpesviruses [16, 24].

In our experiments, VZV gE reached the cell surface despite deletion of gI from infectious virus. This trafficking of VZV gE to the cell membrane was expected even though gI was not made, because surface localization has been observed after expression of gE from plasmid constructs [9, 10, 25]. In addition, Zhu et al. [26] showed that VZV gE has a targeting sequence for the trans-Golgi network that was sufficient for independent transport of the protein. Nevertheless, examination by use of confocal microscopy revealed that cell surface expression of gE was changed to a clumped, punctate pattern that differed dramatically from the diffuse distribution of gE seen in cells infected with intact VZV or the gI-repaired recombinants.

In our experiments, posttranslational modifications of gE appear to require the presence of intact gI within virus-infected cells, and transfection experiments have shown that the forms of gE differed from those detected when gE was coexpressed with gI [9, 11]. Alterations in VZV gE folding when it is made in the absence of gI may diminish its capacity to enhance membrane fusion at the cell surface during viral replication. The patched localization of gE on the membrane of cells infected with the gI deletion mutants further suggested that the process of gE endocytosis and recycling may be disrupted in the absence of gI [27]. Phosphorylation and dephosphorylation of gE in VZV affects trafficking events involved in cell-to-cell spread and viral egress [21]. VZV gE may require intact gI to act as a cofactor for phosphorylation and to facilitate the correct folding of gE. Altered phosphorylation could also explain the fact that deletion of the C-terminal sequences of gI was associated with an altered pattern of VZV replication in tissue culture cells, even though the gE:gl binding site in the N-terminal segment was preserved.

Our experiments suggested that gE was necessary for VZV replication since mutant virus could not be generated from cosmids when the ORF68 was also removed. In other alphaherpesviruses, the gE homologue is dispensable for replication of other alphaherpesviruses in tissue culture [15–20, 22]. However, a requirement for VZV gE is not unexpected because it is the most abundant glycoprotein in the virion envelope and in infected cells [28, 29]. Since VZV is the only alphaherpesvirus that lacks a gene encoding gD in the US region, VZV gE may mediate necessary functions that are usually associated with gD, which is indispensable in most alphaherpesviruses. In addition, gE has targeting sequences for the trans-Golgi network within the cytoplasmic domain that are involved in the virion envelopment process [26, 30].

The Oka strain of VZV is being used to produce the newly licensed varicella vaccine. Extensive clinical studies have proven that the live attenuated varicella vaccine is safe, effective, and clinically beneficial [31]. However, the Oka strain retains the capacity to establish latency and to reactivate from dorsal root ganglia. Experiments are in progress to assess the effect of gI deletion on neurovirulence using the rat model of VZV neurotropism [32]. If gl is required for neurotropism (i.e., in dorsal root ganglion cells), then the vaccine strain might be further attenuated by the targeted deletion of gI.

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References


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