Varicella-zoster virus glycoprotein I is essential for spread in dorsal root ganglia and facilitates axonal localization of structural virion components in neuronal cultures

Jenna Christensen¹, Megan Steain¹, Barry Slobedman¹,²* and Allison Abendroth¹,²#

1. Discipline of Infectious Diseases and Immunology, The University of Sydney, Australia;
2. The Centre for Virus Research, Westmead Millennium Institute, Sydney, Australia

#Address for correspondence:

A/Prof Allison Abendroth
Discipline of Infectious Diseases and Immunology
The University of Sydney, New South Wales, Australia 2006.

* Equal contribution

Running Title: Role of VZV gI in neuronal cell infection
Neurons of the sensory ganglia are the major site of varicella-zoster virus (VZV) latency and may undergo productive infection during reactivation. Although the VZV gE/gI complex is known to be critical for neurovirulence, few studies have assessed the roles of these proteins during DRG infection due to the high human specificity of the virus. Here, we show that VZV glycoprotein I is an important neurotropic gene responsible for mediating the spread of virus in neuronal cultures and explanted dorsal root ganglia (DRG). In comparison with the parental strain (VZV rOka), inoculation of differentiated SH-SY5Y neuronal cell cultures with a VZV gI deletion strain (VZV rOkaΔgI) showed a large reduction in the percentage of cells infected and significantly smaller plaque sizes. In contrast VZV rOkaΔgI was not significantly attenuated in fibroblast cultures, demonstrating a cell-type specific role of VZV gI. Analysis of rOkaΔgI protein localization by immunofluorescent staining revealed aberrant localization of viral glycoprotein and capsid proteins, with little or no staining present in the axons of differentiated SH-SY5Y cells infected with rOkaΔgI, yet axonal vesicle trafficking was not impaired. Further studies utilizing explanted human DRG indicated that VZV gI is required for spread of virus within DRG. These data demonstrate a role for VZV gI in cell-to-cell spread of virus during productive replication in neuronal cells and a role in facilitating access of virion components to axons.
INTRODUCTION

Varicella-zoster virus (VZV) is the etiological agent of the human disease varicella (chickenpox) (1). Following primary infection, the virus establishes latency within neurons of the sensory ganglia (2, 3) from where it may reactivate to result in the secondary clinical disease herpes zoster (shingles) (4). Herpes zoster may be followed by a state of debilitating, long-term pain known as post-herpetic neuralgia (PHN) which is commonly resistant to many traditional pain therapies (3, 5, 6).

VZV glycoprotein I (gI) is a type I membrane protein encoded by open reading frame 67 (ORF67) and functions primarily as a heterodimer with the most abundant VZV protein gE (7). Although gI is dispensable in cell culture, deletion results in an impairment of syncytia formation, delayed replication and a decrease in infectious virus yields within melanoma cells (8). In the context of neuronal infection, gI has been shown to be dispensable in a rat model of persistent VZV infection, although this host is non-permissive (9). In a severe combined immunodeficiency human (SCIDhu) mouse model of VZV infection utilizing human xenografted dorsal root ganglia (DRG), infection with a VZV mutant lacking gI (rOkaΔgI) resulted in prolonged replication within the DRG and an inability to transition to a persistent state normally characterized by limited viral transcription (10). In addition, mutation of the cysteine rich region in VZV gE responsible for gE/gI heterodimer formation impaired cell-to-cell spread of virus in DRG (11).

Studies assessing the herpes simplex virus (HSV) and pseudorabiesvirus (PRV) homologs of gI have reported important functions in mediating anterograde axonal egress during neuronal infection. HSV infection of neurons using compartmentalized chamber systems and rat models have demonstrated that gI is essential for anterograde spread of virus (12). Studies of HSV have demonstrated that the gE/gI heterodimer is required for transport of the viral
proteins gB and gD as well as viral capsids (13). PRV gE/gI deletion viruses are also impaired in anterograde spread in rat models of retinal infection, despite no impairment of retrograde transport or replication within retinal ganglia neurons (14, 15). Similarly, deletion of PRV gI results in impairment of anterograde spread using compartmentalized chamber systems (16, 17). However, VZV interactions within neuronal axons have been poorly defined to date due to the high human specificity of the virus, posing limitations on the models available to assess VZV neuronal infection (1, 18).

A number of cell culture models have recently been developed for the assessment of VZV-neuronal interactions. VZV infection of differentiated human neural stem cells (19) and terminally differentiated neurons derived from induced pluripotent stem cells (20, 21) have been reported as persistent models of VZV infection. In contrast, human embryonic stem cell (22) and differentiated human neuroblastoma (23) derived neuronal cells induced productive VZV infection under the study conditions utilized. The differentiated SH-SY5Y neuroblastoma cell culture model previously developed by our laboratory may be particularly useful to assess the neurovirulence of VZV deletion or mutant strains during productive VZV infection (23). Here, we utilize this model to detail the requirements of gI during VZV neuronal infection using parental strain rOka and the gI deletion strain rOkaΔgI (8). Flow cytometry and infectious center assays were used to demonstrate a cell type specific role for VZV gI in mediating spread of virus between neuronal cells. Furthermore, immunofluorescent staining and confocal microscopy identified a role for VZV gI in facilitating access of virion proteins to axons. Experiments utilizing primary human fetal explanted DRG (24) to assess the role of VZV gI in an ex vivo setting where the natural architecture of the ganglia is preserved support the hypothesis that gI is an important VZV protein required for cell-to-cell spread of virus between ganglionic cells.
MATERIALS AND METHODS

Cell and virus culture

SH-SY5Y cells were differentiated as previously described and cultures established by inoculating $1 \times 10^5$ cells/coverslip in 24-well culture plates (23). In parallel, cultures of human foreskin fibroblasts (HFFs) were similarly established by inoculating $1 \times 10^5$ cells/coverslip in 24-well culture plates. HFFs were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco, Australia) containing fetal calf serum (FCS, 10% v/v; In Vitro Technologies, Australia) and penicillin-streptomycin (50 IU/mL; Gibco, Australia) and were allowed to adhere overnight prior to infection.

VZV rOka and rOkaΔgI (8) were propagated in HFFs in DMEM containing FCS (10% v/v) and penicillin-streptomycin (50 IU/mL) and were harvested for infection experiments when 80-90% of the monolayers were exhibiting cytopathic effect (CPE). Infected or uninfected (mock) cultures were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Sigma-Aldrich, Australia) and then inoculated onto either differentiated SH-SY5Y or HFF cultures at a density of $1 \times 10^5$ cells/well, or a final 1:1 ratio of infected cells to uninfected cells. Coverslips were harvested at various times p.i. for analysis.

Immunostaining and flow cytometry

Triplicate wells containing differentiated SH-SY5Y cells or HFFs inoculated with CFSE labeled mock, rOka or rOkaΔgI infected HFFs were pooled together at either 24, 48 or 72 hours p.i. and fixed in 1% paraformaldehyde (PFA; BD Biosciences, Australia) for 20 minutes. Prior to immunostaining, cells were permeabilized in saponin buffer (PBS containing 1% FCS, 0.2% sodium azide (Sigma-Aldrich, Australia), 10 mM EDTA (Gibco, Australia) and 0.3% saponin (Sigma-Aldrich, Australia)) for 10 minutes. To quantify the
proportion of VZV antigen positive cells, samples were separately stained in saponin buffer with a mouse anti-VZV primary antibody specific to the immediate-early IE62 protein (clone IE(62) 1:50; Meridian Life Science, USA) or a mouse anti-VZV primary antibody specific to late gEgI proteins (clone SG1 1:200, Meridian Life Science, USA). The gEgI specific antibody used in this study is documented to individually react with both the precursor and mature forms of gE and gI. Cells were incubated at 4°C for an hour, washed in PBS and then incubated with a goat anti-mouse Alexa Fluor 647 conjugated antibody (1:200; Life Technologies, Australia) in saponin buffer at 4°C for an hour. Cells were washed in PBS, filtered and processed using a FACS Canto cytometer and analyzed using FACS Diva software. Specificity of staining was confirmed by assessing isotype antibody treated cultures at corresponding concentrations. All statistical analysis was performed using a paired 2-tailed student’s t-test.

**Infectious Center Assay**

Coverslips containing cultures of either differentiated SH-SY5Y cells or HFFs were inoculated with serial 1 in 10 dilutions of mock, rOka or rOka\(\Delta\)gI infected HFFs. Cultures were left to incubate for 96 hours and were then fixed in 4% PFA for 20 minutes. Single immunofluorescent staining was performed using a primary antibody against VZV gEgI (1:600; Meridian Life Sciences, USA) followed by a donkey anti-mouse Alexa Fluor 594 conjugated secondary antibody (1:200; Life Technologies, Australia). Specificity of staining was confirmed by assessing isotype antibody treated cultures at corresponding concentrations. Coverslips were mounted with Prolong Gold antifade reagent containing 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; Life Technologies, Australia). Infectious centers were imaged and counted using a Zeiss LSM 510 Meta confocal microscope. Approximately 10 infectious centers per sample were randomly selected and area quantified using Zeiss
Axiovision software. All statistical analysis was performed using a paired 2-tailed student’s t-

test.

**Immunofluorescence staining and confocal microscopy**

Coverslips containing differentiated SH-SY5Y cells inoculated with CFSE labeled mock, 
rOka or rOkaΔgI infected HFFs were fixed in 4% PFA for 20 minutes. Cells were washed in 
PBS, permeabilized and blocked in PBS containing 0.1% Triton X and 20% normal donkey 
serum (NDS) for 30 minutes at room temperature. For single immunofluorescent staining, 
cells were incubated with mouse anti-VZV primary antibodies against gH (clone SG3 1:500; 
Meridian Life Science, USA), gB (clone SG2-E6, 1:50; Meridian Life Science, USA), gEgI 
(1:600, Meridian Life Science, USA) or pORF40 (clone NCP-1 1:500; Meridian Life Science, 
USA) at 37°C for an hour under humidified conditions. VZV antibodies were detected by 
incubation with a donkey anti-mouse Alexa Fluor 546 conjugated secondary antibody (1:200; 
Life Technologies, Australia) for a further 30 minutes at 37°C. Specificity of staining was 
confirmed by assessing isotype control antibody treated cultures at corresponding 
concentrations. Coverslips were mounted with Prolong Gold antifade reagent containing 
DAPI.

Dual immunofluorescent staining was performed using the VZV antibodies utilized in single 
immunofluorescent experiments in combination with rabbit antibodies specific to syntaxin-6 
(clone C34B2 1:100; Cell signaling, USA), synaptophysin (clone Z66 1:10; Invitrogen, 
Australia) or chromogranin A (N-terminus 1:200; Abcam, UK). Coverslips incubated with 
syntaxin-6 antibody were incubated overnight at 4°C or coverslips incubated with 
synaptophysin or chromogranin A were incubated for an hour at room temperature, under 
humidified conditions. Primary antibodies were detected with donkey anti-rabbit Alexa Fluor 
647 conjugated (1:200; Life Technologies, Australia) and donkey anti-mouse Alexa Fluor
546 conjugated secondary antibodies (1:200; Life Technologies, Australia) applied for 30 minutes at 37°C. Specificity of staining was confirmed by using isotype antibody treated cultures at corresponding concentrations. All coverslips were mounted with Prolong Gold antifade reagent containing DAPI.

Fluorescence images were acquired using a Zeiss LSM 510 Meta confocal microscope. Areas containing abundant axonal processes were identified by transmitted light imaging with DIC and were chosen for further analysis. Image analysis was performed using Zeiss Axiovision software.

**Explant DRG culture and immunofluorescence staining**

Human fetal spinal tissue was obtained at 15-20 weeks gestational period after informed consent and approval by the University of Sydney Human Ethics Committee. DRG were dissected and explanted as previously described (24). 4 days post-plating, DRG exhibiting extensive axonal outgrowth were infected with either mock, rOka or rOkaΔgI infected HFFs at a density of 1 x 10^5 HFFs/well. DRG were formalin fixed at 96 hours p.i., paraffin embedded and 5µm sections microtomed then collected onto microscope slides.

Immediately preceding immunofluorescent staining of DRG, sections were dewaxed in histolene and rehydrated in a gradient of decreasing ethanol dilutions. Unmasking was performed in citrate buffer (pH 6) at 95°C. Sections were blocked in 20% NDS in tris-buffered saline (TBS) for an hour at room temperature. VZV infection was detected using a rabbit anti-VZV pORF62 specific antibody (1:200; kindly provided by P. Kinchington, University of Pittsburgh) or a mouse anti-VZV gE specific antibody (1:10; Millipore, Australia). In conjunction, a mouse anti-NCAM specific antibody (clone 123C3, 1:20; Cell Signaling, Australia) or a rabbit anti-S100B specific polyclonal antibody (predilute; Dako,
Denmark) were utilized. Primary antibodies were applied for 2 hours at room temperature under humidified conditions. Donkey anti-mouse Alexa Fluor 488 conjugated and donkey anti-rabbit Alexa Fluor 594 conjugated secondary antibodies (1:200; Life Technologies, Australia) were used for detection by incubation at room temperature for 30 minutes. Specificity of staining was confirmed by using isotype antibody treated sections at corresponding concentrations. Sections were mounted with Prolong Gold antifade reagent containing DAPI and sealed with coverslips. Images were acquired using a Zeiss LSM 510 Meta confocal microscope and were analyzed using Zeiss Axiovision software.

RESULTS

VZV infection of neuronal cells is significantly impaired in the absence of gI.

To assess the kinetics of VZV infection in neuronal cultures, HFFs infected with the VZV gI deletion virus rOkaΔgI (8), parental virus rOka or mock HFFs were CFSE labeled and inoculated onto differentiated SH-SY5Y neuronal cultures as previously described (23). The percentage of infected cells in inoculating cultures ranged from 77-91%, with viral strains within 5% infection for each replicate experiment. In parallel, cultures of HFFs were inoculated at the same ratio of infection. Immunostaining was performed for cultures at 24, 48 and 72 hours p.i. using antibodies for Immediate Early (IE62) and Late (gEgI) class VZV proteins and the proportion of VZV antigen positive (CFSE negative) cells were quantified by flow cytometry. Whilst the gEgI antibody reacts with both gE and gI, we did not observe any difference in staining efficiency of cells infected with parental or gI deletion viruses. Results are the collation of data from three independent experiments, with the same inoculating cultures used to infect both HFF and SH-SY5Y cells in parallel experiments.
In HFF cultures, very little difference in the percentage of cells infected with rOka and rOkaΔgI was observed at 24 hours p.i. However, a slight reduction in VZV infection was observed in cultures inoculated with rOkaΔgI when compared with rOka for both antibodies tested at 48 and 72 hours p.i. Representative flow cytometry scatter plots are shown for VZV IE62 staining of HFFs at 72 hours p.i. inoculated with mock, rOka and rOkaΔgI infected HFFs (Fig. 1A). By 72 hours p.i., the average percentage of VZV IE62 positive cells in rOka inoculated cultures was 35% (Fig. 1B), with the gEgI specific antibody detecting 48% HFFs VZV positive (Fig. 1C). In rOkaΔgI inoculated cultures, the average proportion of VZV IE62 positive cells at 72 hours p.i. was 23% (Fig. 1B), with 34% cells VZV gEgI positive (Fig. 1C). No VZV specific staining was observed in mock infected cultures (Fig. 1A).

In neuronal cell cultures, a statistically significant difference in the percentage of cells infected was observed when infection was compared between the two VZV strains rOka and rOkaΔgI. Representative flow cytometry scatter plots are shown for VZV IE62 staining of SH-SY5Ys at 72 hours p.i. inoculated with CFSE labeled mock, rOka and rOkaΔgI infected HFFs (Fig. 1D). By 72 hours p.i., an average of 48% SH-SY5Y cells inoculated with rOka were VZV IE62 positive (Fig. 1E), with 69% cells VZV gEgI positive (Fig. 1F). Conversely, only 12% of SH-SY5Y cells inoculated with rOkaΔgI were VZV IE62 positive (Fig. 1E) and only 20% of SH-SY5Y cells were VZV gEgI positive (Fig. 1F). No VZV specific staining was observed in mock infected cultures (Fig. 1D). Overall, the reduction in the percentage of VZV IE62 antigen positive cells infected with rOkaΔgI compared with rOka at 48 and 72 hours p.i. correlated to a significant 3-fold and 4-fold decrease, respectively. Similarly, the percentage of VZV gEgI positive cells in rOkaΔgI inoculated cultures was found to amount to a 3.5-fold significant difference at 72 hours p.i. when compared with rOka infected cells.
The detection of both the immediate early VZV protein IE62 and late proteins gEgI by flow cytometry in neuronal cell cultures infected with rOkaΔgI was indicative of a complete, productive VZV infection in these cells. Thus it was hypothesized that the reduction of infection in neuronal cell cultures inoculated with VZV rOkaΔgI observed by flow cytometry was not due to an impediment of virus entry and/or replication.

**VZV spread in neuronal cultures is significantly impaired in the absence of gI**

To assess the ability of VZV rOkaΔgI to spread in neuronal cultures, we performed a series of infectious center assays by inoculating HFFs infected with either rOka or rOkaΔgI onto monolayers of both HFFs and SH-SY5Y cells. Cultures were assessed at 96 hours p.i. by immunofluorescent staining using a VZV gEgI specific primary antibody. Approximately 10 infectious centers per culture were randomly selected and plaque areas quantified using Zeiss Axiovision software. Results were averaged for three independent experiments. HFFs inoculated with rOkaΔgI (Fig. 2B) exhibited a 1.9-fold average reduction in the size of infectious centers compared with rOka inoculated cultures, although this decrease was not statistically significant (Fig. 2A and E). However, SH-SY5Y cells were found to support spread of rOka (Fig. 2C) much more efficiently than rOkaΔgI (Fig. 2D) with a 6.8-fold average decrease in infectious center area observed in rOkaΔgI inoculated cultures when compared with rOka (Fig. 2E). Thus, it was concluded from these experiments that gI may play an important role in facilitating virus spread between neuronal cells.

**VZV gI is required for viral glycoprotein and capsid protein localization to neuronal axons**

As neuronal cells form extensive networks of axonal projections to facilitate cellular contact, we hypothesized that axons in these cultures might be important for efficient neuron-to-
neuron spread of infection. We therefore sought to assess the axonal localization of structural virion proteins by performing immunofluorescent staining of differentiated neuronal cultures inoculated with either mock, rOka or rOkaΔgI infected HFFs. At 48 hours p.i. coverslips containing cells were incubated with antibodies specific to VZV gB, gH, gEgI and the major capsid protein pORF40 and were analyzed by confocal microscopy. In order to confirm that the regions assessed within these cultures contained abundant axons, transmitted light/DIC imaging was utilized to identify regions abundant in axonal processes and cells containing visible axons were randomly selected for analysis.

In rOka infected neuronal cells, staining for VZV gB (Fig. 3A), gH (Fig. 3B) and gEgI (Fig. 3C) was readily observed with antigen localized predominantly to the cell surface and cytoplasm of the cell body. Clear punctate staining for these VZV proteins were also visible within the neuronal axons. pORF40 staining was predominately nuclear, with some cytoplasmic staining and axonal puncta also clearly visible (Fig. 3D). In rOkaΔgI infected neuronal cells, staining for VZV gB (Fig. 3E), gH (Fig. 3F) and gEgI (Fig. 3G) showed an aberrant localization of viral antigen within the cell cytoplasm. Specifically, VZV glycoproteins were found to accumulate in a nuclear adjacent site, with limited expression on the neuronal surface. Although VZV glycoproteins were detected as a puncta within a small number of neuronal axons, many axons projecting from infected cells contained little or no VZV glycoprotein specific staining. Similarly, neuronal cells infected with rOkaΔgI were found to exhibit pORF40 antigen within only the nucleus of infected cells, with little or no antigen detected within either the cytoplasm or axons of infected neurons (Fig. 3H).

To further examine the aberrant sub-cellular localization of VZV glycoprotein retention within the cytoplasm of neuronal cells infected with rOkaΔgI, dual immunofluorescent staining was performed using antibodies specific for VZV gB, gH and gEgI in conjunction.
with the trans-Golgi network (TGN) marker syntaxin-6. Confocal microscopy confirmed that there was abundant syntaxin-6 expression in mock infected differentiated neuronal cells adjacent to the nucleus, indicative of a TGN specific localization (Fig. 3L). An identical localization of staining was observed for syntaxin-6 in cells infected with rOkaΔgI (Fig. 3I to K). Furthermore, co-localization of syntaxin-6 was observed with each of the VZV glycoprotein specific antibodies gB (Fig. 3I), gH (Fig. 3J) and gEgI (Fig. 3K) within infected cells. No VZV glycoprotein staining was observed in mock infected cultures (Fig. 3L).

The combined data from three independent experiments demonstrated that gI is required for axonal localization of VZV glycoprotein and capsid proteins within infected neuronal cells. Furthermore, VZV glycoproteins localized aberrantly to the TGN of cells infected with VZV rOkaΔgI.

**Axonal vesicle trafficking is not impaired in VZV rOkaΔgI infected neuronal cells**

To establish whether VZV glycoprotein retention within the TGN of rOkaΔgI infected neuronal cells was imparting a global effect on cellular TGN vesicle-derived trafficking pathways, we sought to identify whether cellular vesicle proteins could be detected within the axons of neuronal cells. Mock, rOka or rOkaΔgI infected SH-SY5Y cultures were assessed at 48 hours p.i. by dual immunofluorescent staining using antibodies against VZV gH in conjunction with the large dense-core vesicle marker chromogranin A (25) or the synaptic vesicle marker synaptophysin (26).

Mock infected neuronal cells exhibited punctate staining within the axons for both chromogranin A (Fig. 4E) and synaptophysin (Fig. 4F). Similar localization of staining was observed in the axons of neuronal cells infected with VZV rOka (Fig. 4A and B). As expected, VZV rOka infected cells additionally contained punctate gH staining along the
length of the axons. As determined previously neuronal cells infected with VZV rOka∆gI contained little or no gH specific staining within the axons of infected cells (Figure 3F).

However, chromogranin A (Fig. 4C) and synaptophysin (Fig. 4D) were detectable as puncta within the axons of infected cells, with a distribution and intensity of staining indistinguishable from VZV rOka infected cells (Fig. 4A and B). The results from three independent experiments indicated that VZV glycoprotein retention within the TGN of neuronal cells infected with rOka∆gI does not appear to impair normal cellular vesicle trafficking pathways.

VZV cell-to-cell spread within human DRG is impaired in the absence of gI

To examine the role of gI in facilitating infection in the context of intact human DRG, we utilized a model of human fetal DRG explanted *in vitro*. Briefly, fetal DRG were harvested, explanted and infected with either VZV rOka or rOka∆gI as previously described (24). DRG were formalin fixed and paraffin embedded at 96 hours p.i. for assessment by immunofluorescent staining and confocal microscopy.

To assess the localization of VZV antigen within the DRG at 96 hours p.i., sections were immunostained with a VZV pORF62 specific antibody in conjunction with a neural cell adhesion molecule (NCAM) antibody. DRG infected with rOka exhibited areas of extensive VZV antigen positivity, suggesting that VZV was readily undergoing cell-to-cell spread (Fig. 5A). In contrast, DRG infected with rOka∆gI revealed limited pORF62 staining, which was consistently restricted to isolated cells within the DRG (Fig. 5B). Staining and imaging of multiple consecutive sections verified that these isolated pORF62 antigen positive cells were not in contact with any clusters of VZV positive cells which may have undergone infection via cell-to-cell contact with inoculating fibroblasts at the DRG periphery. No VZV specific staining was observed within mock infected DRG (Fig. 5C). To determine which cell types
within the DRG center were undergoing productive VZV infection, DRG were immunostained at 96 hours p.i. for VZV gE in conjunction with a S100B satellite cell marker. DRG infected with rOka showed localization of gE to both satellite cells and adjacent neurons (Fig. 5D). In contrast, in DRG infected with rOkaΔgI gE staining was observed almost exclusively in neurons, with satellite cells being almost universally gE negative (Fig. 5E). No VZV specific staining was observed within mock infected DRG (Fig. 5F). These results indicate rOkaΔgI is impaired in cell-to-cell spread of VZV within intact DRG.

NCAM expression is disrupted during VZV infection and is dependent on gI

As disruption of the gE/gI complex has previously been shown to inhibit VZV induced membrane fusion of neuron-satellite cell complexes using the SCIDhu model of infection (11), we sought to determine whether a similar phenotype could be observed in a DRG explant model of VZV infection. Dual immunofluorescent staining of DRG inoculated with mock, rOka or rOkaΔgI infected HFFs was performed at 96 hours p.i. using antibodies specific to VZV gE in conjunction with NCAM. In mock infected cells, continuous NCAM staining was readily observed along the membrane of neurons, demarcating the neuron-satellite cell boundary (Fig. 6C). Quantification of mock infected neurons revealed that intact NCAM staining was evident surrounding approximately 96% of neurons. In contrast, 53% of rOka infected neurons exhibited discontinuous or dim NCAM staining, although adjacent VZV gE negative neurons exhibited staining with comparable intensity and localization to mock (Fig. 6A). Discontinuous NCAM staining was predominately observed when VZV gE positive neurons were adjacent to infected satellite cells, as has been previously reported (11, 27). Neurons infected with rOkaΔgI however, showed a reduced alteration in NCAM localization and intensity when compared with rOka, with only 20% of cells exhibiting aberrant NCAM staining (Fig. 6B). No specific staining was observed in isotype control
antibody treated cultures (Fig. 6D). These data illustrate that gI is required for VZV induced neuron-to-satellite cell fusion during VZV infection of human explant DRG.

DISCUSSION

We assessed the role of VZV gI in mediating spread of infection in cultures of synaptically linked neuronal cells and human fetal DRG explanted in vitro. A significant impairment of rOkaΔgI spread was observed in both of these models, despite only a conservative reduction in cell-to-cell spread within cultures of permissive HFFs. This report provides evidence that gI is required for axonal localization of VZV virion proteins and suggests a role for gI in facilitating axonal transmission of virus.

We observed that VZV glycoproteins localized aberrantly to the TGN in the absence of gI, in agreement with a number of previous studies. Electron microscopy studies of infected human embryonic lung fibroblasts showed that gI is required for VZV envelopment in the TGN, with abnormal membranous TGN stacks formed during rOkaΔgI infection (28). Experiments using SCIDhu xenografted DRG similarly confirmed this phenotype within rOkaΔgI infected neurons, with distorted Golgi-like cisternae and disruption of gE trafficking observed (10).

We expanded on these findings to also show that the virion components gE, gH, gB and pORF40 are retained within the TGN and trafficking of these protein to axons is disrupted during rOkaΔgI infection of cultured neuronal cells.

Although a chamber system was not employed in this study to separate inoculating cells from newly infected cells, these results indicate that there may be some impairment of VZV axonal transport in the absence of gI. As neurons grown in culture have limited cell-to-cell body contact, it is likely that viral infection is mediated at least in part by axonal transmission of virus. Impairment of virion envelopment resulting from gI deletion suggests that the major
impediment in rOkaΔgI transport may occur during anterograde viral spread, consistent with the identified roles of gI during HSV and PRV infection (12, 13, 15-17, 29, 30). Additionally, infection of explanted DRG with rOkaΔgI showed that the ability to infect discrete individual neurons within the ganglia center was retained. Although the possibility that infection within these cells is initiated by infiltrating inoculating cells cannot be completely excluded, it is likely that the large size of fibroblasts would prevent their entry into the DRG. It is therefore probable that these cells become infected by axonal transport of virus to the DRG center.

The obstruction of virion protein trafficking to the axons of infected neuronal cells observed is likely to be a consequence of the morphological changes to the TGN resulting from rOkaΔgI infection (10, 28), although it remains possible that VZV gI is directly required to facilitate virion axonal egress via interactions with vesicles or their molecular motor machinery as is hypothesized for HSV (13, 31). Synaptophysin and chromogranin A puncta were still readily observed along the length of the axons within our study, illustrating that the functionality of TGN secretory pathways was at least partly retained. Although a model of VZV retrograde transport has recently been reported (22, 32), a model assessing anterograde spread of virus still remains in developmental stages. Thus, development of more suitable models will be required before these fundamental questions can be directly addressed.

Efficient neuron-to-satellite cell spread of virus within the DRG was dependent on gI. Inspection of rOka infected DRG at 96 hours p.i. revealed that extensive areas of pORF62 staining were visible. Extensive cell-to-cell spread was observed throughout the DRG, with both neurons and S100B antigen positive satellite cells infected with VZV. In contrast, pORF62 staining within rOkaΔgI infected DRG was mainly restricted to isolated neurons within the DRG center, with limited evidence of any viral spread at 96 hours p.i. The impairment of VZV spread in the absence of gI supports the findings of a previous study.
using SCIDhu xenografted DRG, whereby single rOkaΔgI infected neurons were observed at 42 days p.i., similarly implicating impairment of virus spread to neighboring satellite cells (10). Infection of these DRG with both rOkaΔgI and a virus lacking the cysteine rich gE/gI binding domain resulted in impaired replication of VZV and an inability to establish persistence for extended timepoints p.i. (10, 11). Thus the neurotropic role of gI characterized in our study is likely to be integrally linked to its ability to heterodimerize with gE.

The SCIDhu model has been used to demonstrate that polykaryon formation between neurons and satellite cell membranes is disrupted during VZV infection, with reduced NCAM expression on the membranes of infected cells observed (11, 27). It was also shown that deletion of gI or the cysteine rich region facilitating gE/gI heterodimer formation was able to restore NCAM expression (11). In our study, we also found that rOka infected neurons exhibited a downregulation of NCAM expression by IFA. In contrast, mock or rOkaΔgI infected DRG retained strong NCAM staining localized to the membrane of neuron-satellite cell junctions, similarly indicating that rOkaΔgI may be impaired for polykaryon formation.

The restriction of NCAM to neural tissue may assist in explaining the cell-type specific impairment of infection resulting from rOkaΔgI infection. Although a small reduction in VZV rOkaΔgI infection was observed in cultures of HFFs over a 72 hour timecourse when compared with the parental virus, it has been shown that free nucleocapsids within the cytosol are able to fuse with adjacent cells to potentiate infection (28). It is possible that the gI mediated inhibition of NCAM downregulation inhibits cell fusion and therefore prevents the transmission of these free cytosolic nucleocapsids to neighboring cells.

In summary, this report details a neurotropic role of VZV glycoprotein I in facilitating axonal localization of virion proteins and virus spread between cultured neuronal cells. Additional experiments performed using an explant DRG model of productive VZV infection confirmed
that neuron-to-satellite cell spread of virus and NCAM expression was impaired in the absence of gI. These results will aid in our understanding of VZV neuropathogenesis and the requirements for productive neuronal infection within the DRG characteristic of reactivation. They also validate the combined use of SH-SY5Y and primary DRG models of infection to more rapidly facilitate definition of the roles of VZV gene products in neuronal infection.

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Figure 1. Kinetic analysis of rOka and rOkaΔgI infection in HFFs and neuronal cells by flow cytometry. Either mock, rOka or rOkaΔgI infected HFFs were labeled with CFSE and inoculated onto neuronal cells. Cultures were harvested for immunostaining and flow cytometry at 24, 48 and 72 hours p.i. In parallel, cultures of HFFs were infected and analyzed using the same method. (A) Flow cytometry scatter plots of HFFs inoculated with mock, rOka or rOkaΔgI infected HFFs and stained with a VZV IE62 specific antibody at 72 hours p.i. Red boxes show the total non-inoculum (CFSE negative) population, with the percentage of VZV positive cells within this population given. (B) The average percentage of VZV IE62 positive HFFs from three independent experiments are shown over a timecourse of infection comparing rOka (blue) and rOkaΔgI (green). (C) The average percentage of VZV gEgI positive HFFs from three independent experiments are shown over a timecourse of infection comparing rOka (blue) and rOkaΔgI (green). (D) Flow cytometry scatter plots of neuronal cells inoculated with mock, rOka or rOkaΔgI infected HFFs and stained with a VZV IE62 specific antibody at 72 hours p.i. Red boxes show the total non-inoculum (CFSE negative) population, with the percentage of VZV positive cells within this population given. (E) The average percentage of VZV IE62 positive neuronal cells from three independent experiments are shown over a timecourse of infection comparing rOka (blue) and rOkaΔgI (green). (F) The average percentage of VZV gEgI positive neuronal cells from three independent experiments are shown over a timecourse of infection comparing rOka (blue) and rOkaΔgI (green). Values are means, with error bars showing the standard error of the mean from three independent experiments. Significant difference was determined using a paired two-tailed student’s t-test, (*P, < 0.05, **P, < 0.01).
Figure 2. Assessment of rOka and rOka∆gI spread in HFFs and neuronal cells by infectious center assay. Serial dilutions of either rOka or rOka∆gI infected HFFs were inoculated onto monolayers of either HFFs or differentiated neuronal cells. At 96 hours p.i. coverslips were harvested and immunofluorescent staining was performed using an antibody specific to VZV gEgI (red) and a DAPI counterstain (blue). Representative infectious centers are shown following infection of HFFs with either (A) rOka or (B) rOka∆gI, or following infection of neuronal cells with either (C) rOka or (D) rOka∆gI. (E) Infectious center areas were quantified using Zeiss Axiovision software and the mean values from three independent experiments determined. Error bars show the standard error of the mean and significant difference was determined using a paired two-tailed student’s t-test, (*P, < 0.05).

Figure 3. Assessment of viral capsid and glycoprotein localization in neuronal cells infected with rOka and rOka∆gI. Either (A-D) rOka, (E-K) rOka∆gI or (L) mock infected HFFs were labeled with CFSE (pseudocoloured purple) and inoculated onto cultures of neuronal cells. (A-H) Cultures were harvested at 48 hours p.i. and immunostained using an antibody against (A,E) VZV gB, (B,F) gH, (C,G) gEgI, or (D,H) pORF40 (pseudocoloured green) and were counterstained with DAPI (blue). Areas containing abundant axons were selected for imaging using transmitted light/DIC overlay (data not shown). (I-L) Cultures were immunostained for syntaxin-6 (pseudocoloured green), with dual immunofluorescent staining performed using antibodies specific for (I) VZV gB, (J) gH or (K) gEgI (red). Cell nuclei were visualized using a DAPI counterstain (blue). (L) No VZV specific staining was observed in mock infected cultures.

Figure 4. Analysis of axonal secretory vesicle proteins in rOka and rOka∆gI infected neuronal cells. (A-B) rOka, (C-D) rOka∆gI or (E-F) mock infected HFFs were CFSE labeled (pseudocoloured purple) and inoculated onto cultures of neuronal cells. Cultures were
harvested at 48 hours p.i. and immunostained for either (A,C,E) chromogranin A or (B,D,F) synaptophysin (pseudocoloured green). VZV antigen was detected with a gH specific antibody (red). Cells were counterstained with DAPI (blue).

Figure 5. Assessment of viral spread within explant dorsal root ganglia. (A,D) rOka, (B,E) rOkaΔgl or (C,F) mock infected DRG were harvested at 96 hours p.i. and assessed by dual immunofluorescent staining. (A-C) DRG were stained for NCAM (green) in conjunction with a VZV pORF62 specific antibody (red). (D-F) DRG were stained for S100B (green) in conjunction with a VZV gE specific antibody (red). Cells were counterstained with DAPI (blue).

Figure 6. NCAM and VZV antigen detection within explant dorsal root ganglia. (A,D) rOka, (B) rOkaΔgl or (C) mock infected DRG were harvested at 96 hours p.i. and assessed by dual immunofluorescent staining. (A-C) DRG were stained for NCAM (green) in conjunction with a VZV pORF62 specific antibody (red) or with (D) isotype control antibodies. Cells were counterstained with DAPI (blue). Arrows depict discontinuous NCAM staining on the membranes of VZV infected neurons adjacent to infected satellite cells.