A Variant allele in Varicella-Zoster Virus Glycoprotein B Selected during Production of the Varicella Vaccine, Contributes to its Attenuation

Running title

VZV attenuation associated with a variant allele in gB

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Abstract

Attenuation of the live varicella Oka vaccine (vOka) has been attributed to mutations in the genome acquired during cell culture passage of pOka (parent strain); however, the precise mechanisms of attenuation remain unknown. Comparative sequence analyses of several vaccine batches showed that over 100 single-nucleotide polymorphisms (SNPs) are conserved across all vaccine batches; six SNPs are nearly fixed, suggesting that these SNPs are responsible for attenuation. By contrast, prior analysis of chimeric vOka and pOka recombinants indicates that loci other than these six SNPs contribute to attenuation. Here, we report that pOka consists of a heterogenous population of virus sequences with two nearly equally represented bases, G or A, at nucleotide 2096 of the ORF31 coding sequence which encodes glycoprotein B (gB) resulting in arginine (R) or glutamine (Q), respectively, at amino acid 699 of gB. By contrast, 2096A/699Q is dominant in vOka (>99.98%). gB699Q/gH/gL showed significantly less fusion activity than gB699R/gH/gL in a cell-based fusion assay. Recombinant pOka with gB669Q (rpOka_gB699Q) had a similar growth phenotype as vOka during lytic infection in cell culture including human primary skin cells; however, rpOka_gB699R showed a growth phenotype similar to pOka. rpOka_gB699R entered neurons from axonal terminals more efficiently than rpOka_gB699Q in the presence of cell membrane-derived vesicles containing gB. Strikingly, when a mixture of pOka with both alleles equally represented was used to infect human neurons from axon terminals, pOka with gB699R was dominant for virus entry. These results identify a variant allele in gB that contributes to attenuation of vOka.

Importance

The live-attenuated varicella vaccine has reduced the burden of chickenpox. Despite its development in 1974, the molecular basis for its attenuation is still not well understood. Since the live-attenuated varicella vaccine is the only licensed human herpesvirus vaccine that prevents primary disease, it is important to understand the mechanism for its attenuation. Here we identify that a variant allele in glycoprotein B (gB) selected during generation of the varicella vaccine contributes to its attenuation. This variant is impaired for fusion, virus entry into neurons from nerve terminals, and replication in human skin cells. Identification of a variant allele in gB, one of the essential herpesvirus core genes, that contributes to its attenuation may provide insights that assist in the development of other herpesvirus vaccines.

Introduction

The live-attenuated varicella-zoster virus (VZV) vaccine Oka strain (vOka), is the first and only licensed vaccine to protect against varicella. The vaccine was developed by serial passage of the parental Oka strain (pOka) in human embryonic lung cells, guinea pig embryo fibroblasts (GPEFs), and human fibroblasts (1). Adaptation of the virus to growth in GPEFs is thought to be primarily responsible for its attenuation in humans. While the vaccine is well tolerated and effectively reduces the burden of varicella (2), vOka establishes latency and can reactivate in otherwise healthy individuals, albeit to a significantly lesser extent than wild-type VZV (3). However, herpes zoster (HZ) associated with vOka can be indistinguishable from wild-type VZV in healthy or in immunocompromised individuals (4–7). In very rare cases, the vaccine has caused fatal disease in severely immunocompromised persons (8, 9). While an adjuvanted recombinant zoster vaccine (RZV) is licensed to prevent HZ and is more effective than the live-attenuated zoster vaccine (10, 11), RZV has not been tested to prevent varicella. Studies of RZV suggest that priming by natural infection or administration of the live-attenuated vaccine may be essential for the ability of RZV to recall VZV-specific T-cell immunity (12, 13), which is considered to be the correlate of protection to prevent HZ (14–16).

Due to the lack of a small animal model that results in varicella, the mechanisms of attenuation of vOka are poorly understood (17). Recent advances in sequencing technology have been informative for analysis of virus variants in vaccine preparations and in humans. During production of vOka by cell culture, multiple single nucleotide

polymorphisms (SNPs) accumulated in multiple viral genomes (18) and the sequence of vOka is highly heterogenous with 150 to 466 SNPs in the three available commercial vOka preparations (19–21). Of these SNPs, 137 are consistently observed across the different preparations and are defined as core SNPs. Six of the core SNPs are near fixation (> 90%) in all the preparations indicating that these six core SNPs (five of the six are in ORF62 and the other in ORF0) are highly likely to be responsible for attenuation of vOka (19). Strikingly, another live-attenuated vaccine, strain SuduVax, shares all six core SNPs reinforcing the contributions of the six core SNPs in attenuation of vOka (22, 23). However, pOka/vOka recombinants generated using cosmids or other mutant viruses produced using bacterial artificial chromosome (BAC) systems have not identified a relationship between these six core SNPs and attenuation of vOka (24, 25).

We hypothesized that the discrepancy between the comparative genomics and mutagenesis approaches regarding the role of SNPs in attenuation of vOka may be due to the lack of information about the population diversity within the original pOka isolate. Originally, SNPs in vOka were identified by comparing sequences with pOka obtained by traditional Sanger sequencing – an inefficient method for identifying heterogenous populations of viruses in which two or more alleles are present. Therefore, we reanalyzed the population diversity of pOka using data we previously obtained by Illumina deep sequencing in combination with targeted enrichment technology (21).

Results

A SNP at nucleotide position 2,096 in ORF31, encoding gB, is maintained in heterogenous populations of pOka.

We received pOka directly from Dr. Michiaki Takahashi at passage 6 following the original virus isolation and passaged the virus three additional times in MRC-5 cells prior to sequencing to generate pOka_P9 (21). Reanalysis of Illumina sequencing data derived from pOka_P9 genomic DNA (21) identified 61 sites, of which 15 are located in the duplicate loci (internal repeat short [IR_S] and terminal repeat region [TR_S]) with variant allele frequencies > 5% at which variant alleles were present with the pOka reference sequence (pOka_AB097933.1) which was previously generated by Sanger sequencing (18). As the current study focuses on pOka and vOka, we use nucleotide (nt) position numbers based on pOka_AB097933.1 while Dumas__NC001348.1 is simultaneously listed in **Table 1** for better comparison with previous genetic analyses of VZV. Of 61 polymorphic sites, 35 sites had 10% or more variant frequencies (either pOka_P9 or pOka_R5; see below) (**Table 1**). In total, 28 single nucleotide polymorphism (SNPs), two insertions of 3-4 nt, three deletions of 1 nt, one deletion of 1-2 nt, and one deletion of 2 nt were identified. Six insertions or deletions (indels) were located in non-coding regions and one deletion at position 40,407 in the ORF22 gene caused a frame-shift in about 30% of genomes. Of the 28 SNPs, 6 were located within non-coding regions, 6 produced synonymous changes, and 16 produced non-synonymous changes (highlighted in grey in **Table 1**).

Variant frequencies for most of the SNPs causing non-synonymous changes were less than 15%, however, two alleles (A and G) at nt position 58,777 corresponding to nt

2,096 in the ORF31 coding sequence were present at nearly equal frequencies in pOka_P9 (51.5% for A and 48.5% for G) (**Table 1**). The allele, 2096G in the ORF31 gene (ORF31_2096G) was not reported in pOka_AB097933.1 (18), but was described in another pOka genome sequence, pOka_JN704698.1 which did not examine the population diversity within the pOka sample that was used (26). While exact passage numbers of pOka_AB097933.1 were not reported (18), pOka_JN704698.1 was reported to be passaged six times in HELF (human embryonic lung fibroblast) cells after receipt before analysis by 454 sequencing technology (26). ORF31 is one of the core genes conserved in all herpesviruses and encodes glycoprotein B (gB) which functions as a fusogen and is essential for entry of herpesviruses into host cells (27). The 2096G allele results in an arginine at amino acid (aa) position 699 (699R) within domain V of gB (28, 29), while the 2096A allele results in glutamine (699Q). In all the preparations of vOka (OkaVax [Biken], VarilRix [GSK] and VariVax/ZostaVax [Merck]) available in 2016, the 2096G allele was never reported (19), whereas only 0.01% of genomes contained 2096G in the live-attenuated zoster vaccine (Zostavax, derived from vOka) previously obtained from Merck (21). Importantly, a pOkaBAC genome, established from virus passaged multiple times in HELF cells, before cloning into a BAC vector (30) contains ORF31_2096G (**Table 1**). These results demonstrate that an early passage of pOka contained a heterogenous population of viral genomes and suggest that the virus population with ORF31_2096G was eliminated during production of vOka.

pOka with the 2096G allele in ORF31 is selected over the 2096A allele for axonal infection of human embryonic stem cell-derived neurons in a microfluidic device.

An *in vitro* VZV latency system using human embryonic stem cell (hESC)-derived neurons in combination with a microfluidic device (21, 31) provided a unique opportunity to analyze viral entry from peripheral axon termini. hESC-derived neurons are highly permissive for VZV lytic infection by direct infection of the neuronal soma (cell body) either using cell-free or cell-associated virus (32) or by axonal infection of cell-associated virus (33); however, VZV infection of axon terminals with cell-free virus in this system results in viral genome transfer to the neuronal soma and establishment of latency (21, 31, 34).

Axonal infection with pOka_P9 cell-free virus, which contained similar frequencies of A (51.5%) and G (48.5%) at nt 2,096 of ORF31, followed by Illumina sequencing of viral genomes isolated from neuronal soma at 14 days post infection (dpi) showed that the 2096G allele increased from 48.5% (input) to 90.37 ± 0.47% (mean ± SEM [standard error of the mean] of three biological replicates) (**Figure 1A**, 58777 labeled in red triangle) (21). Consistent with the increased variant frequency of 2096G at 14 dpi by the axonal route, 90.0% of the pOka genomes in neuronal soma at 24 hours post infection (hpi) by the axonal route had the 2096G allele in ORF31. Other than the SNP at nt 2,096 in ORF31, the only other alleles that had an appreciable change in frequency after axonal infection were the 2872G allele in ORF62/ORF71 (duplicate gene) which increased from 23.2/23.4% to 53.0/56.3% at 24 hpi and was maintained at 55.2 ± 0.35/59.6 ± 2.42% at 14 dpi (**Figure 1A**, 106227/123880 labeled in teal/cyan square), and the

109246/120861G allele in the non-coding region between ORF62 and ORF63 (duplicate loci) which increased from 26.5/25.0% to 49.4/50.4% at 24 hpi and was maintained at 50.6 ± 0.76/48.7 ± 1.19% at 14 dpi (**Figure 1A**, 109246/120861 labeled in violet/magenta diamond).

In addition to pOka_P9, pOka_R5 passaged in HELF and MRC-5 cells (<10 times) was also examined. pOka_R5 had 77.3% of ORF31 with the_2096G allele (**Table 1**). Axonal infection of the pOka_R5 resulted in enrichment of ORF31_2096G from 77.3% to 95.6 ± 2.60% at 24 hpi (three biological replicates) and 89.7 ± 2.89% at 14 dpi (four biological replicates) (**Figure 1B**, 58777 labeled in red triangle). An increase of ORF62/71_2872G from 21.5/20.7% to 31.2 ± 2.30/30.6 ± 2.16% (24 hpi) and 35.3 ± 1.95/36.4 ± 1.33% (14 dpi) was also observed (**Figure 1B**, 106227/123880 labeled in teal/cyan square), whereas the 109246/120861G in the non-coding region between ORF62 and ORF63 was decreased from 77.9/78.1% to 61.3 ± 3.60/60.8 ± 3.90% (24 hpi) and 58.3 ± 0.49/58.9 ± 0.78% (14 dpi) (**Figure 1B**, 109246 labeled in violet diamond). Because neither viral replication nor production of infectious progeny virus is observed in this system up to 70 dpi in the absence of reactivation stimuli (21), the consistent selection of the 2096G allele in ORF31 encoding gB indicates that pOka with gB 699R (2096G) is dominant over gB 699Q (2096A) in a mixed population of viruses for entry into axon termini of human ESC-derived neurons.

The amino acid difference at 699 of gB does not affect the level of gB when expressed alone or in the context of VZV lytic infection.

198 To test whether the SNP at 2,096 in ORF31 affects levels of gB, human retinal pigmented
199 epithelial (ARPE-19) cells which support VZV lytic infection were either transfected with
200 plasmid CAG_gB699R or CAG_gB699Q (which express gB with an R or G at aa 699,
201 respectively), or infected with recombinant pOka virus with gB699R (rpOka_gB699R) or
202 gB699Q (rpOka_gB699Q) (**Figure 2A**). VZV gB is cleaved into two portions by the cellular
203 furin protease or other subtilisin-like pro-protein convertases via an RSRR motif located
204 at aa positions 491-494 of gB and generates a heterodimer consisting of the N-terminal
205 (494 aa) and C-terminal (437 aa) portions of the glycoprotein held together by disulfide
206 bonds (35). Transfection of cells with plasmids CAG_gB699R or CAG_gB699Q, resulted
207 in equal levels of gB whether the cell lysates were treated with dithiothreitol (DTT)
208 (yielding the two cleavage products of gB; only the C-terminal portion is recognized by
209 anti-gB polyclonal antibody) or not treated with DTT (showing the heterodimeric form
210 of gB) (**Figure 2B, left panels**). A dominant band of 20-kDa was detected with anti-gB
211 polyclonal antibody in ARPE-19 cells transfected with the plasmid expressing gB699R/Q,
212 but not in cells transfected with empty vector, regardless of whether or not the cell
213 lysates were treated with DTT. A 20-kDa band was also observed in membrane protein-
214 enriched extracellular vesicles containing gB produced in HEK-293T cells using the anti-
215 gB antibody (see below) and a 26-kDa band was seen in ARPE-19 cells after infection
216 with VZV (see below). We speculate that these bands are caused by further cleavage of
217 the C-terminal portion of gB as the antibody recognizes the C-terminus of gB. The
218 variability of detection of α-tubulin, used as a loading control for the samples treated
219 with and without DTT indicates that recognition of the anti-α-tubulin antibody is weaker

220 in the absence of DTT (**Figure 2B, lower panels**). Confocal microscopy did not show any
221 differences in cellular localization between gB699R and gB699Q; both variants formed
222 cytoplasmic vesicle-like structures, but rarely co-localized with *trans*-Golgi network
223 (TGN) if expressed in the absence of VZV infection (**Figure 2C**). In the context of VZV
224 infection, there was again no difference between gB699R and gB699Q either in the size
225 in the presence or absence of DTT (**Figure 2B, right panels**) or in the cellular localization
226 both at the cell surface or with the TGN (**Figure 2D**). Thus, gB expression, cleavage,
227 heterodimer formation, and localization were not affected by the difference at amino
228 acid position 699 of gB.
229
230 ***rpOka_gB699Q shows a similar phenotype to vOka, while rpOka_gB699R resembles***
231 ***pOka for cell-to-cell spread during lytic infection in cell culture.***
232 To test whether the allele selection at 2,096 of ORF31 of vOka contributes to its
233 attenuation during lytic replication in cell culture and spread between cells, different
234 cell lines were infected with pOka (pOka_R5), rpOka_gB699R, rpOka_gB699Q, or vOka.
235 Cell-free virus inocula were prepared in and titrated in MRC-5 cells, which are used for
236 production of vOka vaccine, and the same titer of each virus was used for viral growth
237 assays.
238 In MRC-5 cells, no significant differences were observed in size of infectious foci
239 or virus replication among all the various viruses tested (**Figure 3A** and **B**). By contrast,
240 in ARPE-19 cells rpOka_gB699Q formed foci comparable in size to those observed with
241 vOka, but significantly smaller foci than those seen with rpOka_gB699R (**Figure 3C**)

despite similar levels of replication of all the viruses (**Figure 3D**). In MeWo cells, like ARPE-19 cells, the size of infectious foci of rpOka_gB699Q was comparable with those observed with vOka, but significantly smaller than those with rpOka_gB699R (**Figure 3E**). Despite the use of the same amount of cell-free virus, based on virus titrations performed in MRC-5 cells, vOka and rpOka_gB699Q produced fewer infectious foci than rpOka_gB699R and pOka in MeWo cells throughout infection; however, the growth curve pattern of all the viruses were parallel, indicating that once lytic infection is established in MeWo cells, levels of replication of vOka and rpOka_gB699Q in MeWo cells were nearly identical to rpOka_gB699R and pOka (**Figure 3F**). This is consistent with the previously reported identical growth curves of wild type VZVs and vOka in MeWo cells (24, 36). Taken together, rpOka_gB699Q was indistinguishable from vOka for virus replication, and both viruses show reduced cell-to-cell spread in ARPE-19 cells and MeWo cells compared with pOka and rpOka_gB699R.

Amino acid 699 of VZV gB is important for its fusion activity.

Since VZV gB is part of the core fusion machinery along with gH/gL (37, 38), we compared the fusion activity of gB699R/gH/gL with gB699Q/gH/gL in a luciferase-based VZV glycoprotein-mediated cell fusion assay. Consistent with the results of transient expression of gB699R and gB699Q in ARPE-19 cells (**Figure 2**), total cell levels (**Figure 4A**) and cellular localization (**Figure 4B**) of gB699R and gB699Q were comparable in HEK-293T cells. Cell surface levels of gB699R and gB699Q were also similar in transfected HEK-293T cells (**Figure 4C**). When co-expressed with gH/gL, total cell levels, cellular

localization, and cell surface levels of gB699R and gB699Q were similar (**Figure 4A, 4B,** and **4C**, respectively). Co-expression of gB with gH/gL reduced total and cell surface levels of gB compared with expression of gB alone (**Figure 4A** and **4C**).

gB699R/gH/gL showed significantly higher fusion activity than gB699Q/gH/gL at 48 hr after mixing ARPE-19 target cells expressing T7 polymerase with HEK-293T cells expressing gH/gL, gB, and luciferase driven by the T7 promoter (p = 0.00003, **Figure 4D**). However, gB699R/gH/gL and gB699Q/gH/gL showed similar levels of fusion activity when fusion was measured at 24 hr after cell mixing (p = 0.35134, **Figure 4D**). Fusion activity of gB699R/gH/gL was significantly higher than gB699Q/gH/gL at both 24 hr and 48 hr after mixing when MeWo cells were used as target cells instead of ARPE-19 cells (p = 0.00003 or p = 0.00007, respectively, **Figure 4D**). Fusion activity was negligible when either ARPE-19 or MeWo target cells expressing T7 polymerase were mixed with HEK-293T cells expressing luciferase driven by the T7 promoter and gB699R or gB699Q without gH/gL, or gH/gL without gB (**Figure 4D**). Taken together, we found that both gB699R and gB699Q induce membrane fusion along with gH/gL; however, a single amino acid substitution (R to Q) at position 699 in gB significantly reduced glycoprotein-mediated membrane fusion activity for gB/gH/gL.

Membrane protein-enriched extracellular vesicles containing gB reduce axonal infection of neurons with rpOka_gB699Q.

To further analyze how the difference in gB sequences affect entry of VZV, we used membrane protein-enriched extracellular vesicles (MPEEVs) (39) to isolate vesicles that

286 could deliver gB699R or gB699Q to cells prior to infection with VZV. MPEEVs contain
287 intact membrane proteins with their correct topology on the surface of virus-like
288 vesicles and have been shown to be useful for studying herpes simplex virus type 1 (HSV-
289 1) gB (40). We transfected HEK-293T cells with CAG_gB plasmids to maximize their
290 expression and purified MPEEV expressing gB from cell culture supernatants. gB on
291 purified MPEEV_gB699R or MPEEV_gB699Q was shown to be cleaved and only the C-
292 terminal portion was detected by anti-gB polyclonal antibody in the presence of DTT
293 (**Figure 5A, left panel, DTT [+]**). In the absence of DTT, gB formed a heterodimer through
294 disulfide bonding (**Figure 5A, left panel, DTT [-]**) similar to the gB forms observed in
295 rpOka_gB699R or rpOka_gB699Q cell-free virus (**Figure 5A, middle panel**). VZV gB
296 MPEEVs were readily purified, similar to those reported for HSV-1 gB; by contrast, when
297 co-expressed with gH/gL in HEK-293T cells, no gB/gH/gL MPEEVs were detected despite
298 apparent expression of the glycoproteins in the cells (**Figure 4A**).

299 To determine whether MPEEVs expressing gB can compete with cell-free VZV for
300 entry into cells, ARPE-19 cells were incubated with MPEEV_empty (i.e. not expressing
301 gB), MPEEV_gB699R, or MPEEV_gB699Q (50 µL/well; 10 µL [5 µg]/lane in **Figure 5A, left**
302 **panel**) for 30 min at 37°C, and then infected with VZV cell-free virus (20 PFU [1.5-2.0 x
303 10^7 genomes based on qPCR]/well) in the presence of MPEEVs for 1 hr at 37°C. Viruses
304 with equal infectious titer contain comparable level of gB (**Figure 5A, middle panel**) and
305 other virion components, gH, pORF63, and pORF49 whereas their recognition by each
306 antibody were variable between the samples of DTT (+) and DTT (-) (**Figure 5A, right**
307 **panel**). The cells were then treated with low-pH buffer for 30 sec to inactivate virus still

on the surface of the cells and cultured for 6 days. Neither MPEEV_gB699R nor MPEEV_gB699Q significantly reduced the infectious focus number when compared to MPEEV_empty treated ARPE-19 cells infected with either rpOka_gB699R or rpOka_gB699Q (**Figure 5B**). Similar results were seen in MeWo cells (T. Sadaoka and J. I. Cohen unpublished data). Thus, MPEEV_gB699R or MPEEV_gB699Q does not compete with rpOka_gB699Q or rpOka_gB699R for entry into ARPE-19 (or MeWo) cells.

Since gB699R is dominant to gB699Q for pOka infection of terminal axons of neurons (**Figure 1A and B**), we measured the ability of MPEEVs expressing gB to inhibit infection of rpOka_gB699R or rpOka_gB699Q at neuronal axons by quantifying viral genomes in neuronal soma at 24 hpi (four replicates performed for per each combination of MPEEVs and rpOka_gBs). rpOka_gB699R and rpOka_gB699Q reached the soma equally well in the presence of MPEEV_empty. While MPEEV_gB699R did not reduce infection of rpOka_gB699R (p = 0.120), it significantly reduced infection of rpOka_gB699Q (p = 0.00479). Even in the presence of MPEEV_gB699Q, infection of the neuronal soma of rpOka_gB699Q via the axon termini was significantly reduced (p = 0.0111), but infection of rpOka_gB699R was not inhibited (p = 0.539) (**Figure 5C**). In summary, infection of the neuronal soma via the axon termini with rpOk_gB699Q was less efficient compared to rpOka_gB699R only in the presence of MPEEV_gB699R or MPEEV_gB699Q.

Selection of pOka with gB699Q contributes to vOka attenuation in human skin cells.

329 Skin tropism is a key feature in the pathogenesis of VZV (41). Reduced replication of
330 vOka compared with pOka in skin, based on the SCID-hu mouse skin xenograft model,
331 has been proposed as an important factor in attenuation of the vaccine virus (24, 42).
332 Deep sequencing of pOka_W (pOka passaged fewer than 10 times in MeWo cells
333 resulted in enrichment of 2096G/699R of ORF31/gB from 77.3% (pOka_R5) to 91.0%
334 (pOka_W) (**Table 1**), suggesting that gB699Q in vOka contributes to attenuation of the
335 virus in human skin.

336 Human neonatal epidermal keratinocytes (HEKn) were infected with cell-free
337 pOka (pOka_R5), rpOka_gB699R, rpOka_gB699Q, or vOka, and the size of infectious foci
338 were compared (**Figure 6A**). HEKn are primary cells and have limited cell divisions. We
339 were able to passage HEKn cells more than 10 times, but observed that about 10% of
340 the cells have a fibroblast-like morphology at passage number 6. Therefore, we
341 performed experiments only in HEKn cells that had been passaged three times
342 (HEKn_P3). Unlike MRC-5, ARPE-19, and MeWo cells, HEKn cells are difficult to detach
343 and disperse as single cells, an essential step for a viral growth assay. The cells required
344 about 10 minutes of treatment with trypsin to detach them, resulting in inactivation of
345 virus on the cell surface; therefore, only infectious focus formation using infection with
346 cell-free virus was compared. This assay measures cell-to-cell spread of virus.

347 All the VZV isolates gave comparable results in infectious foci assays using
348 HEKn_P3 cells (**Figure 6A**). Since rpOka_gB699Q and vOka showed reduced replication
349 in MeWo cells compared with rpOka_gB699R and pOka (**Figure 3E** and **F**), infectious
350 focus formation was compared in normal human skin fibroblasts (Hs68 cells). In Hs68

cells, rpOka_gB699Q and vOka formed comparable infectious foci, and significantly smaller foci than those formed by rpOka_gB699R and pOka_R5 (**Figure 6B**). These results indicate that gB699Q contributes to vOka attenuation in human skin and suggest that vOka is impaired for replication and/or spreading in skin fibroblasts compared with keratinocytes.

To test this hypothesis, we examined infectious focus formation in HEKn_P6 cells, which, as noted above, are a mixture of cells with keratinocyte and fibroblast-like morphologies. rpOka_gB699Q and vOka formed significantly smaller infectious foci than those formed by rpOka_gB699R and pOka_R5 in HEKn_P6 cells (**Figure 6C**). These results were similar to those in Hs68 cells, but different from those in HEKn_P3 cells. In addition, all the VZV isolates formed significantly larger infectious foci in HEKn_P6 than in HEKn_P3 cells (**Figure 6D**). Thus, an amino acid change at position 699 in gB from R to Q is a determinant for vOka attenuation in human skin, and impaired replication in human skin fibroblasts rather than keratinocytes may contribute to attenuation of vOka.

Discussion

By analyzing the genome sequence heterogeneity of pOka and comparing it with that of vOka, we identified a SNP within the VZV ORF31 gene at which two alleles (G and A) exist at similar frequencies in low passage pOka (pOka_P9) at position 2096; however, one allele, 2096G, was absent in vOka. The SNP located at nt position 2096 (G [absent in vOka] and A [present in vOka]) in the ORF31 gene caused an amino acid change at position 699 (R and Q, respectively) in gB. gB together with gH/gL (37, 38) makes up the

core fusion machinery of VZV (43), is essential for VZV entry into cells (44), and is conserved among all herpesviruses (45). The amino acid change at 699 in gB from arginine (R), a basic amino acid, to glutamine (Q), an acidic amino acid results in reduced fusion activity of gB/gH/gL. rpOka_gB699Q showed a similar phenotype as vOka during lytic infection in cell culture including primary human skin cells, while rpOka_gB699R had a similar phenotype in cells as pOka. When these recombinant viruses were used to infect human ESC-derived neurons via axon terminals in a microfluidic device, both viruses could transfer their genomes equally well to neuronal soma as shown previously for pOka and vOka (21). By contrast, when virus infections were performed in the presence of MPEEVs expressing either gB699R or gB699Q, infection of neurons by rpOka_gB699Q was significantly reduced compared with that of rpOka_gB699R, and similar to the reduction of infection of pOka with ORF31_2096A/gB699Q compared with pOka with ORF31_2096G/gB699R. Thus, the current study demonstrates that the presence of the 2096A allele (and absence of the 2096G allele) in ORF31 in vOka is one of the determinants of its attenuation.

The use of nucleotide sequencing has suggested possible mechanisms of attenuation for vOka with the finding of six core SNPs present in ORF62 and ORF0 genes those are nearly fixed in all vOka preparations based on Sanger sequencing or deep sequencing (18–20, 22, 23, 46, 47). However, these studies have all been conducted using sequence information of pOka_AB09733.1 which was obtained by Sanger sequencing with no information on variant alleles present. Thus, the contribution of allele selection at nt position 2,096 in ORF31 for attenuation of vOka has not been

395 recognized. Using 454 sequencing, the 2096G allele, but not 2096A was included in the
396 data set of another pOka genome sequence, pOka_JN704698.1 (26). Analysis of
397 attenuation of vOka by generating chimeric viruses containing portions of pOka and
398 vOka using the cosmid system (48) and analyzing replication of the viruses in human skin
399 xenografts in the SCID-hu mice (49) showed that the ORF30-55 loci from the pOka
400 genome were sufficient to maintain the wild-type VZV phenotype in human skin (24).
401 The two chimeric viruses maintaining a pOka phenotype in human skin had either the
402 2096G or both 2096G and 2096A alleles in their genome (referred as G/A polymorphism
403 at position 58793 in (24)), while all the other chimeric viruses having a vOka phenotype
404 in human skin had only the 2096A allele. Importantly no other SNP associated with the
405 pOka- or vOka phenotype in human skin was reported (24). Our current results using the
406 BAC system based on the comparative genomics of pOka and the previous results using
407 vOka and pOka recombinants by the cosmid system (24) show the importance of the
408 loss of the virus population having the ORF31_2096G/gB699R allele from pOka for
409 attenuation of vOka.

410 While pOka was isolated in HEL cells at 37°C, it was subsequently passaged eleven
411 times in HEL cells at 34°C and twelve times in GPEFs at 37°C. The resulting virus was
412 further cultured three times in human diploid, WI-38 cells at 37°C and used as vOka (1).
413 It is thought that passage of the virus in guinea pig cells was the key step resulting in its
414 attenuation. SuduVax, another live attenuated VZV vaccine, originally obtained from a
415 VZV isolate in South Korea was isolated in HEL cells, serially passaged ten times in HEL
416 cells followed by twelve passages in guinea pig embryonic lung fibroblasts and then five

417 passages in HEL cells (22). Adaptation of VZV isolates to guinea pig cells introduced
418 multiple SNPs throughout their genomes, and the allele frequencies at most of these
419 sites vary considerably between vOka and SuduVax (50); however, the six core SNPs are
420 near fixation in both vOka and SuduVax and these SNPs are thought to have a major role
421 for adaptation to growth in guinea pig cells and attenuation in humans. Importantly,
422 SuduVax also has ORF31_2096A as does vOka (50). vOka formed significantly larger
423 infectious foci than pOka in GPEF, and rpOka_gB699Q formed larger foci than
424 rpOka_gB699R in GPEF. However, rpOka_gB699Q showed significantly smaller foci than
425 vOka and foci that were comparable in size to pOka in GPEF (**Supplementary Figure 1**).
426 Since rpOka_gB699Q has a similar attenuation phenotype as vOka in human cells and
427 significantly increased ability for cell-to-cell spread compared to rpOka_gB699R in GPEFs,
428 allele selection of ORF31_2096A/gB699Q might play dual roles in adaptation of vOka to
429 GPEF and its attenuation in human cells. While the data imply that the six core SNPs
430 contribute to vOka adaptation to GPEF, further studies are needed to assess the
431 contribution of the six core SNPs in vOka to attenuation in humans.

432 Among the six core SNPs, the 106227C allele (known as 106262C in
433 Dumas_NC001348.1 and 2872G in ORF62) has been used to discriminate vOka from
434 wild-type VZV as a "vaccine marker" in samples from patients with varicella or HZ,
435 because nearly 100% of the vOka population has this allele. Deep sequencing of pOka
436 genomes identified 106227C in pOka (23.2% in pOka_P9, 21.5% in pOka_R5 and 35.4%
437 in pOka_W) and in the pOkaBAC (**Table 1**). After *in vitro* axonal infection of pOka_P9 or
438 pOka_R5, this "vaccine marker" allele (106227C, ORF62_2872G) in pOka increased along

439 with 58777G (ORF31_2096G). The 106227C allele had no negative impact on
440 rpOka_gB699R in any of the human cells tested. Consistent with this, the six core SNPs,
441 regardless of whether they corresponded to the pOka or vOka SNPs, had no impact on
442 the attenuated phenotype of pOka/vOka chimeric VZVs in a SCID-hu mouse skin
443 xenograft model (24). These results support that the loss of the virus population having
444 the ORF31_2096G/gB699R allele from pOka is a major contributor for attenuation of
445 vOka; further investigation is essential to evaluate the importance of the six core SNPs
446 in vOka attenuation.

447 In herpesviruses, gB functions as a fusogen (class III) for entry of all herpesviruses
448 into cells. Crystal structures of gB post-fusion form from all herpesvirus subfamilies have
449 been resolved (29, 51–54) and all the homologs adopt similar structures (55) including
450 VZV gB (28). In contrast, the structure of the pre-fusion form of gB has been reported
451 only for HSV-1 at an overall resolution of 9 Å using a MPEEV-based approach (40, 56, 57).
452 VZV gB 699R/Q is located in domain V of gB comprising a C-terminal arm that packs
453 against a coiled-coil core formed by domain III in the post-fusion form. This coil-arm
454 complex is reminiscent of the six-helix bundle which may provide the energy to drive
455 membrane fusion in class I fusogens (58–60) as proposed for HSV-1 gB (61). gB 699R/Q
456 in VZV corresponds to gB687E in HSV-1 gB and this glutamic acid is conserved in
457 betaherpesviruses (e.g., human cytomegalovirus [HCMV] strain AD169) and
458 gammaherpesviruses (e.g., Epstein-Barr virus strain B95-8). While the importance of this
459 glutamic acid has not been directly determined in these viruses, mutation of HSV-1 gB
460 at 671I, 681H or 683F within the C terminal arm of domain V (corresponding to 683V,

693R or 695F in VZV, respectively) reduced the ability of HSV-1 gB to execute cell-to-cell fusion (22% to 78% fusion activity compared with wild-type HSV-1) and the combination of all three mutations markedly reduced activity (7% to 9% fusion activity compared to wild-type HSV-1) in a cell-based fusion assay (61). A peptide containing amino acids 678 to 694 of domain V of HCMV gB (corresponding to amino acids 713 to 729 of VZV) inhibited HCMV entry (62) and this effect is postulated to occur by blocking the formation of the post-fusion form of gB (54, 59, 63). Similar to HSV-1 or HCMV, a single amino acid change (699R/Q) in domain V of VZV gB causes a significant difference in its fusion activity in a cell-based fusion assay. In addition, rpOka_gB699R and rpOka_gB699Q differ in the size of infectious foci, a measure of cell-to-cell spread due to fusion, when the viruses are grown in ARPE-19 cells, MeWo cells, or human primary skin fibroblasts. Differences in the ability of these two viruses to infect neurons via axon terminals were detected in the presence of MPEEV expressing the two different variants of gB, gB699R/Q. This might be caused by differences in fusion activity of these two viruses as well as other factors including virus entry at axon terminals or intra-axonal transport of virus. A previous study showed that gB is important for spread of HSV-1 from neurons to epithelial cells (64).

Our results using human primary skin cells suggest that vOka attenuation in human skin is mediated by its reduced replication and spread in skin fibroblasts rather than in keratinocytes. The epidermis is the major site for VZV replication in the skin where lesions laden with VZV virions form, although VZV replicates both in the epidermis and dermis (41). Keratinocytes are the main cell type in the epidermis and several skin

infection models have shown a critical role of epidermal keratinocytes for VZV pathogenesis (42, 65–68). However, it is unclear how VZV is transferred from circulating infected T cells during viremia, or from sensory neuronal axons innervating the epidermis and dermis during virus reactivation, to keratinocytes. While keratinocytes comprise nearly 80% of the cells in the epidermis, fibroblasts comprise about 7% of the dermis and a much lesser percentage of the epidermis (69). Our current data, however, suggest that skin fibroblasts might play an important role in VZV pathogenesis as well as in intrinsic skin immunity to VZV.

We have identified that selection of Oka VZV with gB699Q from a heterogenous population of Oka containing gB699R and gB699Q was important to establish attenuated vOka. One might wonder why gB699Q has been maintained in wild-type circulating VZVs as gB699R should be a virulence factor in human cells and our current data showing that passaging of pOka in human cell culture results in enrichment of gB699R. To our surprise, sequencing data of clinical isolates from patients with varicella or HZ do not show ORF31_2096G/gB699R, although most sequenced VZV belongs to clades other than clade 2 to which pOka (and vOka) belong. Only one sequence from a highly passaged Korean clinical isolate from a patient with HZ (KU926318.1) (Clade 2) has ORF31_2096G, but its frequency data are not available. Analysis of the population diversity of currently circulating VZVs from multiple clades should provide further information on sequences that are important for virulence which are common in multiple clades of VZV as well as certain clade-specific sequences.

Taken together, our comparative genomics analyses based on Illumina deep sequencing of pOka and characterization of SNPs combined with our phenotypic studies of the two variant alleles in gB identify a SNP in ORF31 as a novel factor responsible for attenuation of vOka. A more precise understanding of attenuation of vOka would be important not only to improve the safety of the live-attenuated varicella vaccine, but might also help in development of live-attenuated vaccines against other human herpesviruses for which no vaccines are currently licensed.

Materials and Methods

Cells.

Human embryonic stem cell (hESC; H9)-derived neural stem cells (NSC) (Thermo Fisher Scientific) were cultured and propagated as described previously (21). For differentiation into neurons, NSCs were used after the 3rd to 5th passages. Neurons were differentiated from NSCs on a microfluidic platform as described previously (21, 70) or with slight modifications using Neurobasal Plus Medium with B-27 Plus Supplement (2% [vol/vol]), GlutaMAX-I (2 mM) (Thermo Fisher Scientific) and ascorbic acid (200 µM; Sigma-Aldrich). Human embryonic lung fibroblast MRC-5 cells (JCRB0521, JCRB Cell Bank) were maintained in MEM (minimum essential medium)+GlutaMAX-I (Thermo Fisher Scientific) supplemented with heat-inactivated 8% FBS (fetal bovine serum; Sigma-Aldrich or Biowest). Human retinal pigmented epithelium ARPE-19 cells (CRL-2302, American Type Culture Collection [ATCC]) were maintained in DMEM/F-12 (Dulbecco's modified Eagle medium/nutrient mixture F-12)+GlutaMAX-I (Thermo Fisher

Scientific) supplemented with heat-inactivated 8% FBS. Human melanoma MeWo cells (HTB-65, ATCC) and human embryonic kidney (HEK) 293T (CRL-3216, ATCC) cells were cultured in DMEM+GlutaMAX-I (Thermo Fisher Scientific) supplemented with heat-inactivated 8% FBS. Hs68 (IFO50350, JCRB Cell Bank), neonatal normal diploid skin fibroblasts were cultured and propagated in DMEM+GlutaMAX-I supplemented with heat-inactivated 10% FBS. Human neonatal epidermal keratinocytes, HEKn-APF (Animal Product-Free) (Thermo Fisher Scientific), were cultured and propagated in KBM NHEK-XF2 medium (KOHJIN BIO). MeWo_Cre cells expressing Cre recombinase were previously established (71) and cultured in DMEM+GlutaMAX-I supplemented with heat-inactivated 8% FBS. MeWo_T7pol cells expressing T7 RNA polymerase were established by transfecting a DNA fragment containing a puromycin resistant gene and T7 RNA polymerase expression cassette amplified from pCAG_puro_T7pol plasmid into MeWo cells and selecting in DMEM+GlutaMAX-I supplemented with heat-inactivated 8% FBS and puromycin (0.5 μg/mL) (Sigma-Aldrich). Guinea pig embryo fibroblasts (BioWhittaker) were cultured in MEM+GlutaMAX-I supplemented with heat-inactivated 10% FBS.

Viruses.

The parental strain VZV Oka (pOka), passage 6 was a generous gift from Michiaki Takahashi (Osaka University), previously described (72) and used at passage 9 from its original isolation yielding pOka_P9. pOka in DCV/CID/KU was maintained in MRC-5 cells (pOka_R5) and additionally passaged in MeWo cells (pOka_W). The vaccine strain VZV

548 Oka (vOka; BIKEN) was propagated in MRC-5 cells. Recombinant pOka (rpOka) viruses,
549 rpOka_gB699R and rpOka_gB699Q were reconstituted in MRC-5 cells. The BAC cassette
550 within the reconstituted viruses was excised in MeWo_Cre cells. Cell-free virus was
551 prepared from VZV-infected MRC-5 cells by sonication and centrifugation as described
552 previously (72) using an ultrasonic disruptor (UD-100; TOMY Seiko) at an output level 80
553 for 15 sec.
554
555 ***DNA isolation and Quantitative PCR.***
556 Viral DNA was isolated from VZV-infected cells or cell-free virus using the AllPrep
557 DNA/RNA Mini Kit (Qiagen) or the FavorPrep Blood/Cultured Cell Total RNA Mini Kit
558 (FAVORGEN BIOTECH) in combination with the NucleoSpin RNA/DNA buffer set
559 (Macherey-Nagel).
560 DNA was subjected to quantitative PCR (qPCR) using KOD SYBR qPCR Mix
561 (TOYOBO) in the StepOnePlus Real-time PCR system (Thermo Fisher Scientific) (1 µL of
562 DNA per 10 µL reaction in duplicate). Primer sets used for qPCR are listed
563 (**Supplementary Table 1)**. The qPCR program was 95°C for 2 min (1 cycle), 95°C for 10
564 sec and 60°C 15 sec (40 cycles), and 60 to 95°C for a dissociation curve analysis. When
565 measuring levels of VZV DNA relative to cellular DNA using quantitative PCR, data were
566 expressed as copies of VZV ORF10 DNA compared to copies of cellular CD24 DNA and
567 defined as $2^{-(\text{Ct-value VZV ORF10 - Ct-value CD24})}$. VZV DNA copy numbers in cell-free viruses were
568 calculated by quantitative PCR based on a standard curve using the pOkaBAC genome
569 (1-10^7 copies/reaction) and VZV ORF10 primers.

570

571 ***Whole viral genome Illumina sequencing and data analysis.***

572 Illumina sequencing datasets obtained in our previous analysis of pOka_P9 (passage 9),
573 both as input virus and as virus collected from neuronal infection via axon terminals 14
574 days after infection (21), were reanalysed together with virus obtained at 24 hr post-
575 infection in the same experiments. Additional Illumina sequencing datasets were
576 generated as part of the current study using pOka_R5 (input virus) and virus from
577 neuron infection via axonal terminals at 24 hr and 14 days after infection was used in
578 the current study. Genomic DNA library construction, target enrichment, and
579 sequencing on an Illumina MiSeq were performed as described previously (21). For
580 analysis of the pOka BAC genomes, sequencing libraries were generated using the
581 NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (New England Biolabs) according
582 to manufacturer's instructions and sequenced on an Illumina NovaSeq.

583 For all datasets, sequence reads were trimmed using TrimGalore
584 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) [--paired --length
585 30 --quality 30] and competitively aligned against a genome index comprising both the
586 VZV strain pOka genome (AB097933.1) and HG38 using bowtie2 [--no-discordant --end-
587 to-end --no-mixed] (73). Post-alignment processing was performed using SAMtools (74)
588 and bam-readcount [-w 10 -d 1000000] (https://github.com/genome/bam-readcount)
589 prior to variant calling using variant_caller_v1.0.py
590 (https://github.com/DepledgeLab/vzv-2.0/tree/master/extras).

591

592 ***Plasmids.***

593 ORF31 was amplified by PCR of DNA from VZV pOka-infected MRC-5 cells using primers
594 ORF31up16ecoF and ORF31xhoR (**Supplementary Table 1**). The KOD-Plus-Ver. 2 PCR
595 system (TOYOBO) was used for PCR and the program was 1 cycle of 94°C for 2 min, 30
596 cycles of 98°C for 10 sec, 60°C for 30 sec, and 68°C for 1.5 min. The PCR product was
597 digested with EcoRI and XhoI restriction enzymes and cloned into pCAGGS_MCS_puro
598 plasmid (CAG_empty) via EcoRI and XhoI sites. The resulting ORF31 expression plasmids
599 were named CAG_gB699R and CAG_gB699Q which has guanine (G) and adenine (A) at
600 nucleotide (nt) position 2,096 in ORF31 gene respectively. The ORF31 gene in the
601 plasmids was sequenced by primers CAG1631F and CAG1853R (**Supplementary Table**
602 **1**), which anneal upstream and downstream of the multiple cloning site of the
603 pCAGGS_MCS_puro plasmid. This confirmed that the ORF31 sequences were identical
604 with pOka_AB097933.1 except G at 2096 (2096G) in CAG_gB699R. CAG_gH and CAG_gL
605 plasmids were generated as described for CAG_gB but cloned into pCAGGS_MCS
606 plasmid using primers ORF37up23ecoF and ORF37xhoR or ORF60up21ecoF and
607 ORF60xhoR, respectively (**Supplementary Table 1**). The pCAGGS plasmid was kindly
608 provided by Jun-ichi Miyazaki (Osaka University) (75).

609 A DNA fragment containing pOka ORF31 was amplified by PCR of DNA from VZV
610 pOka-infected MRC-5 cells using primers ORF31up16ecoF and pOkaR60036
611 (**Supplementary Table 1**). The PCR product was cloned into pCR2.1-TOPO TA vector
612 (TOPO TA Cloning Kit) (Thermo Fisher Scientific) and the sequence of ORF31 was
613 confirmed using the primers. The fragment containing ORF31 with 2096G or 2096A was

digested with EcoRI and cloned into the EcoRI site of pBlueScript II SK(-) (Agilent). The plasmids were further digested with SacI and XhoI and cloned into plasmid pST76A-SR using SacI and XhoI sites, resulting pST76A-SR_ORF31_2096G and pST76A-SR_ORF31_2096A. The pST76A-SR shuttle plasmid was a kind gift from by Ulrich H. Koszinowski (Max von Pettenkofer Institut, Ludwig-Maximilians-Universität München) (76).

pT7EMCLuc carrying a firefly luciferase gene under the control of the T7 promoter (77) and pCAGT7 containing the T7 RNA polymerase gene under the control of the CAG promoter (78) were generous gifts from Richard Longnecker (Northwestern University). A T7 RNA polymerase expression cassette was amplified by PCR of pCAGT7 using primers CAG1631F and CAG1878R and fused with a PCR fragment of pCAGGS_MCS_puro (CAG1878F and CAG1631R) using In-Fusion HD Cloning kit according to the manufacturer's instruction (Clontech), resulting in pCAG_puro_T7pol. The KOD-Plus-Ver. 2 PCR system (TOYOBO) was used for PCR and the program was 1 cycle of 94°C for 2 min, 30 cycles of 98°C for 10 sec, 60°C for 30 sec, and 68°C for 3 min.

RecA-mediated BAC mutagenesis in E. coli, reconstitution of recombinant virus and BAC cassette excision.

DH10B *E. coli* containing the pOkaBAC (30) were transformed with plasmid pST76A-SR_ORF31_2096A and recA-mediated allelic exchange was carried out as described previously (71), resulting in pOkaBAC_ORF31_2096A. DH10B cells containing pOkaBAC_ORF31_2096A were transformed with plasmid pST76A-SR_ORF31_2096G

636 and pOkaBAC_ORF31_2096G was generated using the same procedure. pOkaBAC,
637 pOkaBAC_ORF31_2096A, and pOkaBAC_ORF31_2096G were purified (Genopure
638 Plasmid Maxi Kit, Roche Diagnostics), subjected to restriction fragment length
639 polymorphism analysis using BamHI or EcoRI and the region used for allelic exchange
640 was sequenced.

641 The purified BAC genome (1 µg) was mixed with PEImax solution (3 µL) prepared
642 as described (79) and transfected to MRC-5 cells. After cytopathic effects were seen in
643 cells expressing green fluorescent protein within the BAC cassette, cell-free virus was
644 prepared as described above and used to infect MeWo_Cre cells to excise the BAC
645 cassette using the Cre/loxP system, resulting in rpOka_gB699Q (from
646 pOkaBAC_ORF31_2096A) and rpOka_gB699R (from pOkaBAC_ORF31_2096G).

647

648 ***Generation of the membrane protein enriched extracellular vesicles (MPEEVs).***

649 MEPPV_gBs and MPEEV_empty were prepared as described (39) with some
650 modifications. HEK-293T cells (4 x 10^6) cells were plated in 10 cm dishes in 15 mL
651 medium 1 day before transfection. Medium was removed and replaced with 10 mL of
652 fresh medium just prior to transfection. CAG_empty, CAG_gB699R or CAG_gB699Q
653 plasmids (30 µg) were mixed with PEImax solution (60 µL) in KnockoutDMEM/F-12 (250
654 µL per dish) and added to HEK-293T cells. At 16 hr post transfection, medium was
655 replaced with fresh medium and cells were cultured for two days. Supernatants were
656 then filtrated (0.45 µm filters), subjected to purification through Histodenz (Sigma-
657 Aldrich) (20% [wt/vol] in PBS) by ultracentrifugation at 100,000 x g for 1 hr at 4°C in a

P28S rotor (CPWX80; Hitachi Koki), and the pellets were diluted in KnockoutDMEM/F-12 (400 µL) and stored at -80°C until use. Protein quantity in purified MPEEVs was measured using a Qubit 4 Fluorometer and a Qubit Protein Assay Kit (Thermo Fisher Scientific).

Antibodies.

Rabbit anti-gB polyclonal antibody, mouse anti-gB monoclonal antibody (mAb) (clone 8), mouse anti-gE mAb (clone 9), mouse anti-gH mAb (clone VgIII-3) and mouse anti-ORF62 mAb (clone 2-B) were described previously (72, 80–82). Mouse anti-α-tubulin mAb (clone B-5-1-2) and sheep anti-TGN46 antibody were obtained from Sigma-Aldrich and AbD Serotech, respectively. Alexa Fluor 488-conjugated donkey anti-mouse IgG, Alexa Fluor 594-conjugated donkey anti-rabbit IgG, and Alexa Fluor 647-conjugated donkey anti-sheep IgG (Thermo Fisher Scientific) were used for as secondary antibodies for indirect immunofluorescence assays and flow cytometry. Anti-mouse IgG HRP-linked sheep or anti-rabbit IgG HRP-linked donkey antibodies (GE Healthcare Bio-Sciences) were used as secondary antibodies for immunoblotting.

Immunofluorescent staining and confocal microscopy.

Cells on CELLview slides (Greiner Bio-One) were fixed with 4% (vol/vol) paraformaldehyde (PFA)/PBS at room temperature for 20 min, permeabilized with 0.1% Triton X-100/4% PFA/PBS at room temperature for 20 min, and incubated with human Fc receptor blocking solution (5% FBS/PBS containing 10% of Clear Back [MBL Life

680 Science]) at room temperature for 1 hr. Cells were stained with the primary antibodies
681 diluted in 5% FBS overnight at 4°C (1:100 for anti-gB polyclonal antibody, anti-gB mAb
682 and anti-TGN46 antibody), washed with 0.1% Tween 20/PBS (PBS-T) for 5 min 3 times,
683 stained with secondary antibodies (1:300) diluted in 5% FBS/PBS at room temperature
684 for 1 hr, washed with PBS-T for 5 min 3 times, covered with VECTASHIELD Vibrance
685 Antifade Mounting Medium with DAPI (Vector Laboratories), and imaged by an FV1000D
686 confocal microscopy (Olympus).
687
688 ***Immunoblotting.***
689 Cells were incubated in RIPA lysis buffer (0.01 M Tris-HCl [pH 7.4], 0.15 M NaCl, 1%
690 sodium deoxycholate, 1% Nonidet P-40 and 0.1% SDS) on ice for 15 min, sonicated in a
691 water bath for 10 min, and centrifuged at 20,000 x g for 10 min. Supernatant was boiled
692 with LDS Sample Buffer (4X) and Sample Reducing Agent (DTT) (10X) at 100°C for 5 min
693 (Thermo Fisher Scientific). Proteins were separated on 4-12% Bis-Tris Plus Gel in MES
694 SDS Running Buffer (200 V, 25 min) and transferred onto PVDF membranes (0.2 μm)
695 using a Mini Blot Module (20V, 1 hr) in Bolt Transfer Buffer containing 10% methanol
696 and 0.1% Bolt Antioxidant (Thermo Fisher Scientific). The membrane was blocked in a
697 blocking buffer (5% [wt/vol] skim milk/0.1% Tween 20/PBS) at room temperature for 1
698 hr, stained with primary antibodies diluted in the blocking buffer (1:3,000 for anti-gB
699 polyclonal antibody, 1:5,000 for anti-gH mAb) overnight at 4°C, washed with PBS-T for 5
700 min 3 times, stained with the secondary antibodies diluted in the blocking buffer
701 (1:3,000) at room temperature for 1 hr, and washed with PBS-T for 5 min 3 times and

PBS briefly once. Signals were visualized by Chemi-Lumi One Super (Nacalai Tesque, Inc.) and captured using LAS4000mini (GE Healthcare Bio-Sciences). Membranes stained with anti-gB polyclonal antibody were stripped by WB Stripping Solution Strong in accordance with the manufacturer's manual (Nacalai Tesque, Inc.) and reprobed with anti-α-tubulin mAb (1:30,000).

Flow cytometry.

Cells were treated with trypLE (Thermo Fisher Scientific) at 37°C for 5 min, collected in medium (DMEM+GlutaMAX-I supplemented with heat-inactivated 8% FBS), and centrifuged at 200 x g for 4 min. Cell pellets were fixed with 4% PFA/PBS at room temperature for 20 min, washed with 5% FBS/PBS once and incubated with human Fc receptor blocking solution (5% FBS/PBS containing 10% of Clear Back) at room temperature for 1 hr. Cells were incubated with the primary antibody (1:200 dilution in 100 μL of 5% FBS/PBS) on ice for 1 hr, washed with 5% FBS/PBS once and incubated with secondary antibody (Alexa Fluor 488-conjugated donkey anti-mouse IgG; 1:300 dilution in 50 μL of 5% FBS/PBS) on ice for 30 min. Cell surface expression of each viral protein was analyzed using a SA3800 spectrum analyzer (SONY Corporation).

Infectious focus formation and viral growth assays.

Cells (1 x 10^5) were seeded on one well of a 12-well plate 2 days before infection and inoculated with VZV cell-free virus for 1 hr at 37°C. The number of PFU of VZV in the cell-free inoculum was calculated based on titration in MRC-5 cells. After infection, the

724 inoculum was removed, the cells were washed with medium, and cultured. For MeWo
725 cells, the culture medium was supplemented with 3% FBS instead 8% FBS and changed
726 every 3 days.
727 For the infectious focus formation assay, the cells were infected with cell-free virus
728 (100, 10 and 1 µL) and cultured for 7 days. For the viral growth assay, cells infected with
729 50 PFU of cell-free virus (at an MOI of 0.0005) were harvested at 24-hr intervals and
730 then titrated on the same cell type. Infected cells were fixed with 4% PFA/PBS, stained
731 with anti-gE mAb (1 : 10 dilution in PBS) followed by anti-mouse IgG HRP-linked sheep
732 antibody (1 : 6,000 dilution in PBS), and incubated with 3, 3', 5, 5'-tetramethylbenzidine-
733 H peroxidase substrate (Moss, Inc.). For the infectious focus formation assay, images of
734 foci were captured, traced, and areas were measured using ImageJ
735 (http://rsbweb.nih.gov/ij/).
736
737 ***Quantitative cell-to-cell fusion assay.***
738 Effector HEK-293T cells (6 x 10^5 cells/well) were seeded in 12-well plates and transfected
739 in duplicate with plasmids (total 1 µg/well; 0.25 µg for empty, gB, gH, gL and
740 pT7EMCLuc) and 2.5 µL of PEImax solution mixed in 50 µL of knockoutDMEM/F-12.
741 Target ARPE-19 cells (4 x 10^6 cells) were transfected with pCAG_puro_T7pol (8 µg) using
742 Nucleofector II (kit V, program X-005, Amaxa), and the cells were divided into 4 wells of
743 6-well plates and cultured in RPMI1640+GlutaMAX-I supplemented with heat-
744 inactivated 8% FBS (3 mL). At 16 hr post transfection, cells were rinsed with PBS twice,
745 released from wells using trypLE and resuspended in DMEM+GlutaMAX-I supplemented

with heat-inactivated 8% FBS. Target MeWo_T7pol cells were rinsed with PBS once, released from wells using trypLE and resuspended in DMEM+GlutaMAX-I supplemented with heat-inactivated 8% FBS. Effector cells and target cells were then mixed at a 1:1 ratio and co-incubated at 37°C for 24 or 48 hr in 24-well plates. The cells were scraped, centrifuged at 300 x g for 4 min, and incubated in 20 µL of ONE-Glo reagent (Promega) after removal of the supernatant. Luciferase activity was measured by TriStar LB 941 Multimode Microplate Reader (Berthold Technologies).

Data availability.

All sequencing datasets generated as part of this study are available via the European Nucleotide Archive under accession PRJEB53195. Sequencing datasets from our previous work (21) are available via accession PRJEB45678.

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775 **Figure Legends**

776 ***Figure 1. Change in variant frequency of SNPs in pOka before and after axonal infection***

777 ***of human neurons.***

778 The percent of the frequency of variants of all the SNPs in pOka_P9 (**A**) and pOka_R5 (**B**)

779 before axonal infection (input), at 24 hours after infection in **A**, mean of biological

780 triplicates in **B**) and 14 days after infection (mean of biological triplicates in **A** and

781 quadruplicates in **B**) are shown with nucleotide position based on pOka_AB097933.1

782 (see **Table 1**).

783

784 ***Figure 2. Characterization of gB699R and gB699Q expressed by plasmid transfection***

785 ***or by VZV infection.***

786 (**A**) Location of ORF31 in VZV genome (nucleotide numbering based on

787 pOka_AB097933.1), location of the ORF31 SNP 2096G/2096A with corresponding

788 nonsynonymous aa change in ORF31/gB (lower panel). U_L, unique long; U_S, unique short;

789 TR_L, terminal repeat long; IR_L, internal repeat long; IR_S, internal repeat short; TR_S,

790 terminal repeat short; TM, transmembrane region. (**B**) Immunoblotting analysis of

791 gB699R and gB699Q by transfection (left panel) and infection (right panel) in ARPE-19

792 cells using anti-gB polyclonal antibody and anti-α-tubulin mAb as internal control. DTT;

793 dithiothreitol. Molecular mass standards (kDa) are shown at left. (**C** and **D**) Confocal

794 microscopic analysis of gB699R and gB699Q localization by transfection (**C**) and infection

795 (**D**) in ARPE-19 cells using anti-gB mAb and anti-gB polyclonal antibody along with anti-

796 TGN46 polyclonal antibody. Nuclei were stained with DAPI. Images are representative

797 of results from two independent experiments. Transfection efficiency of ARPE-19 cells
798 was about 40% (**B** and **C**). Magnification; x 600 and x 2 digital zoom with 10 μm of white
799 bars.
800
801 ***Figure 3. Comparison of phenotype of VZV with different gB SNPs in different cell types.***
802 Infectious focus size (**A**, **C**, **E**) and virus growth (**B**, **D**, **F**) were compared in MRC-5 (**A** and
803 **B**), ARPE-19 (**C** and **D**) and MeWo (**E** and **F**) cells. Representative data from two
804 independent experiments is shown for each analysis. Infectious focus size is shown in
805 Box and Whisker plots using the Tukey method (n=30-50 foci) measured in each cell
806 type. Red line; mean, gray circle; outliers. P value was calculated by one-way ANOVA
807 with Fisher's LSD correction for multiple comparisons. Each virus titer is shown as a
808 mean (symbol) with standard error of the mean (SEM; bar) of replicates.
809
810 ***Figure 4. Expression and fusion activity of gB/gH/gL in a cell-based quantitative fusion***
811 ***assay.***
812 Total protein expression (**A**), cellular localization (**B**) and cell surface expression (**C**) of gB
813 and gB/gH/gL were compared in HEK-293T cells used as effector cells for the cell-based
814 fusion assay. (**A**) Immunoblotting by anti-gB polyclonal antibody, anti-gH mAb and anti-
815 α-tubulin mAb in the presence of DTT (dithiothreitol). Molecular mass standards (kDa)
816 are shown at left. (**B**) Confocal microscopic analysis using anti-gB polyclonal antibody
817 and anti-gB mAb or anti-gH mAb along with anti-TGN46 polyclonal antibody. Nuclei were
818 stained with DAPI. Magnification is x 600 and x 2 digital zoom; white bars represent 10

μm. (**C**) Flow cytometry using anti-gB mAb or anti-gH mAb. Anti-ORF62 mAb was used as a negative control. Cell surface expression level is shown by MFI (mean fluorescent intensity) obtained from 50,000 events. (**A-C**) Representative data from two independent experiments is shown for each analysis. (**D**) Quantitative luciferase-based cell-to-cell fusion assay. Effector HEK-293T cells expressing VZV glycoprotein(s) with firefly luciferase under the control of the T7 promoter, and target ARPE-19 cells (**left panel**) or target MeWo cells (**right panel**) expressing T7 RNA polymerase were co-incubated for the indicated time and luciferase activity (LUC units) was recorded as a measure of cell-to-cell fusion activity. Representative data from three independent experiments is shown with mean and SEM (standard error of the mean) of four biological replicates. P value was calculated by one-way ANOVA with Fisher's LSD correction. Transfection efficiency of HEK-293T cells by PEImax was more than 80% as shown in panel **B**. Transfection efficiency in ARPE-19 cells by nucleofection was 70-80% by FACS using pCAG_EGFP plasmid.

Figure 5. Reduction of infection by membrane protein enriched extracellular vesicles (MPEEVs) expressing gB.

(**A**) Comparison of gB699R and gB699Q from MPEEVs expressing gB (**left panel**) and rpOka cell-free viruses (middle **panel**) by immunoblotting using anti-gB polyclonal antibody. Other virion components were compared between rpOka_gB699R and rpOka_gB699Q by immunoblotting using anti-gH mAb, anti-pORF63 polyclonal antibody and anti-pORF49 antibody (right panels). DTT; dithiothreitol. Molecular mass standards

(kDa) are shown at left. Image is representative of results from three independent experiments. (**B**) Number of infectious foci generated by each virus after infection in the presence of MPEEVs expressing gB or MPEEV-empty in APRE-19 cells is shown. Biological triplicate data is shown with the mean (red line). P value was calculated by one-way ANOVA with Fisher's LSD correction for multiple comparisons. (**C**) Relative numbers of viral genomes transported to neuronal soma after axonal virus infection in the presence of MPEEV expressing gB or MPEEV-empty are compared between rpOka-gB699R and rpOka-gB699Q. Four biological replicates data are shown with the mean (red line). P values were calculated by one-way ANOVA with Fisher's LSD correction for multiple comparisons.

Figure 6. Comparison of the phenotype of VZV with different gB SNPs in human primary skin cells.

Infectious focus size after infection of HEKn_P3 cells (**A**), Hs68 cells (**B**) and HEKn_P6 cells (**C**) with different VZV isolates. Infectious focus size after infection of HEKn_P3 and HEK_P6 cells with the same VZV isolate (**D**). Data for **D** was derived from **A** and **B**. Representative data from two independent experiments are shown for each analysis. Infectious focus size is shown in Box and Whisker plots using the Tukey method (n=20-50 foci) measured in each cell type. Red line, mean; gray circle, outliers. The P value was calculated by one-way ANOVA with Fisher's LSD correction for multiple comparisons.

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