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The Requirement of Varicella Zoster Virus Glycoprotein E (gE) for Viral Replication and Effects of Glycoprotein I on gE in Melanoma Cells

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The glycoprotein E (gE) of varicella zoster virus (VZV), encoded by ORF68, is the most abundant viral glycoprotein. In the current experiments, we demonstrated that ORF68 deletion was incompatible with recovery of infectious virus from VZV cosmids. Replacing ORF68 at a nonnative *Avr*II site in the genome restored infectivity. Further, we found that VZV gE could be expressed under the control of the Tet-On promoter in stably transfected melanoma cell lines (Met-gE cells) without evidence of toxicity. In these Met-gE cells, gE colocalized with γ-adaptin, a trans Golgi network marker, in perinuclear sites, but did not reach plasma membranes. In order to investigate how infection altered gE localization, we made a recombinant virus, vOka-MSPgE, with ORF68 from the VZV MSP strain. VZV MSP encodes a mutant gE protein (D150N) that lacks the mAb epitope, 3B3 (Santos *et al., Virology* 275, 306–317, 2000), whereas Met-gE protein binds mAb 3B3. Within 48 h after Met-gE cells were infected with vOka-MSPgE, the steady-state distribution of Met-gE protein extended beyond the perinuclear areas to other cytoplasmic sites and to plasma membranes. A second recombinant, vOka-MSPgE without gI (vOka-MSPgEΔgI), was constructed to investigate Met-gE protein distribution in the absence of gI. The redistribution of Met-gE protein which was observed by 48 h after vOka-MSPgE infection did not occur until 5 days (140 h) within vOka-MSPgEΔgI infected cells. After vOka-MSPgE infection of Met-gE cells, most Met-gE protein was in the final 94K mature form by 72 h. However, progression to predominance of mature gE was delayed in Met-gE cells infected with vOka-MSPgEΔgI. These observations confirm our hypothesis that VZV gE is essential, based upon the demonstration of restored infectivity after replacing ORF68 in a nonnative site in the genome, and provide further evidence of the role of gI in facilitating the maturation and intracellular distribution of this critical VZV glycoprotein.

Key Words: varicella zoster virus; glycoprotein E; glycoprotein I; protein trafficking and maturation.

INTRODUCTION

Varicella zoster virus (VZV) is a human alphaherpesvirus that causes two diseases, varicella (chickenpox) and herpes zoster (shingles). Herpes zoster is due to VZV reactivation following prolonged latency in sensory ganglia. The herpesviral glycoproteins function at several points in the replication cycle, including viral attachment, entry, envelopment, cell–cell spread, and egress. The VZV glycoproteins, gB, gC, gE, gH, gI, gK, gL, and the putative glycoproteins, gM and gN, have some homology with those of herpes simplex viruses 1 and 2 (HSV-1 and HSV-2), pseudorabies virus (PRV), and other alphaherpes viruses (reviewed by Kinchington and Cohen, 2000). It has been proposed that VZV envelopment is completed in the trans Golgi network (TGN) of infected cells (Gershon *et al.,* 1994; Wang *et al.,* 2001). The VZV tegument proteins appear to assemble with envelope glycoproteins at the TGN during virion formation.

VZV gE, an integral membrane protein, is the most abundant viral glycoprotein. It is found extensively in the cytoplasm, where it is presumed to be present in the membranes of intracellular vesicles or organelles, as well as on plasma membranes during productive infection. In infected cells, the mature form of gE (94K) is an O-linked and N-linked glycoprotein (Montalvo *et al.,* 1985; Santos *et al.,* 1998). The intracellular trafficking and maturation of gE are not fully understood and may differ within different cell types. Most studies of gE transport have been done in HeLa or Cos-7 cells (Olson and Grose, 1997, 1998; Zhu *et al.,* 1996; Wang *et al.,* 1998). As defined in transient expression assays, gE has targeting sequences for the TGN and it is transported from the ER to the TGN in infected and gE-transfected cells (Alconada *et al.,* 1998; Gershon *et al.,* 1994). Current evidence indicates that most gE in the TGN has been retrieved selectively from the plasma membrane and delivered to the TGN via endosomes (Gershon *et al.,* 1994; Wang *et al.,* 2000). Expression of gE using the vaccinia virus-T7 RNA polymerase transfection system indicates that gE undergoes endocytosis from the cell membrane, is returned to the TGN, and then recycles back to the cell membrane (Olson and Grose, 1997, 1998;

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Wang *et al.,* 2000). VZV gI is also transported to the TGN and to plasma membranes in VZV-infected cells and in gI-transfected cells (Alconada *et al.,* 1996, 1998). The mechanisms by which gI reaches the TGN have not been fully defined. VZV gE forms heterodimers with gI. When gE and gI are expressed in vaccinia recombinants, gI facilitates gE endocytosis and regulates the trafficking of the gE/gI complex, which functions as an Fc receptor on the cell surface (Olson and Grose, 1998). VZV gE can also serve as a navigator glycoprotein, forming complexes that direct signal-deficient viral glycoproteins to the TGN (Wang *et al.,* 1998). In some cell types, gE/gI complex formation may be important for the intracellular localization of gI. In other cells, a critical residue in the C-terminus allows gI to reach the TGN when it is expressed in the absence of gE (Wang *et al.,* 2000).

The purpose of these experiments was to further examine the role of gE in VZV replication and the contributions of gI to gE trafficking in VZV-infected cells. In previous experiments using VZV cosmids, we suggested that gE was probably essential because no infectious virus could be recovered when both ORF68 (gE) and ORF67 (gI) were deleted. Infectious virus was made if ORF67 alone was removed (Mallory *et al.,* 1997). We showed that gE was detected throughout the cytoplasm and on plasma membranes of infected cells when intact gI was present and that gE exhibited a punctate distribution in its absence (Mallory *et al.,* 1997). To prove that gE is required for VZV replication, we made ORF68 deletion mutants and recombinants with ORF68 replaced in the nonnative *Avr*II site. In contrast to other alphaherpesviruses, gE was demonstrated to be essential. When expressed alone, as an inducible protein (Met-gE protein) in melanoma cells (Met-gE cells), gE remained localized at perinuclear sites where γ-adaptin, a TGN protein, was also detected. Localization of Met-gE to the TGN under conditions of steady-state expression in Met-gE cells indicated that the usual gE cycling between the TGN and plasma membranes requires other viral proteins.

As a strategy to examine this question further, we made VZV recombinants containing ORF68 from the VZV MSP strain. VZV MSP is the first gE mutant identified as the cause of a naturally occurring human infection (Santos *et al.,* 1998). This new genotype exhibits a phenotype of accelerated cell to cell spread in cell culture and in SCID-hu skin implants (Santos *et al.,* 2000). The gE encoded by VZV MSP does not bind the monoclonal antibody, 3B3 (Santos *et al.,* 2000), whereas Met-gE protein has the 3B3 epitope. Infection of Met-gE cells with vOka-MSPgE was associated with extensive cytoplasmic redistribution of Met-gE protein and its detection on plasma membranes. These experiments are the first report of a gE-expressing cell line and of the capacity of MSP gE to influence gE maturation and transport. Infection of Met-gE cells with the corresponding gI deletion virus, vOka-MSPgEΔgI, showed that viral proteins other than gI mediated the redistribution of gE to some extent. However, gI enhanced gE expression on plasma membranes. In addition, the maturation of gE to its 94K form was delayed in Met-gE cells infected with vOka-MSPgEΔgI, compared with vOka-MSPgE, indicating that MSP gE trafficking mechanisms were facilitated by its partner, gI. These experiments document that gE is essential for VZV replication and provide further evidence that interactions between gE and gI enhance the synthesis of mature gE and produce the characteristic pattern of gE localization within infected cells.

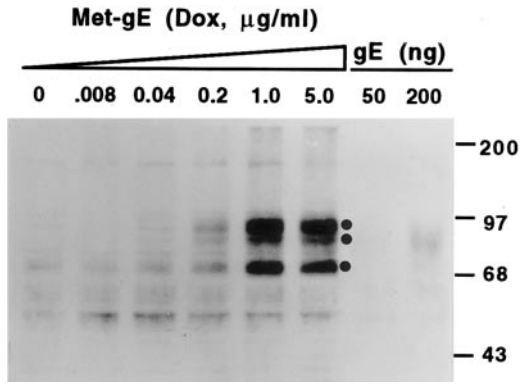

FIG. 1. Dose-dependent effect of doxycycline on gE expression. Met gE cells were grown in 6-well plates for 24 h in medium containing Dox (concentrations from 0, 0.008, 0.04, 0.2, 1.0, and 5.0 μg/ml). Cell lysates were separated by 8.5% SDS–PAGE and transferred to a nitrocellulose membrane. Mouse anti-gE monoclonal antibody (3B3) was used as the probe and detected with anti-mouse antibody conjugated with HRP.

RESULTS

Analysis of gE expression in Met-gE cells

Stable, constitutive expression of gE in melanoma cells was not achieved in our previous experiments (unpublished data) or by Litwin *et al.* (1992). In the current experiments, melanoma cells tolerated inducible expression of gE without evidence of toxicity. Five positive clones of Met-gE cell lines were tested for gE expression after induction with doxycycline (Dox). Two clones, Met-gE1 and Met-gE3, showed relatively low background without Dox and high gE expression when Dox was added at 1 μg/ml. To establish the dose-dependent effect of Dox on gE expression, Met-gE1 cells were grown in 6-well plates for 24 h in medium containing Dox at concentrations of 0, 0.008, 0.04, 0.2, 1.0, and 5.0 μg/ml. Western blots done with anti-gE mAb demonstrated gE expression when cells were induced with Dox concentrations as low as 0.04 μg/ml (Fig. 1). The gE expression was maximal at 1 μg/ml Dox and did not increase with 5.0 μg/ml (Fig. 1). Three forms of gE protein, which were approximately 75K, 90K, and 96K MW, were detected (Fig. 1).

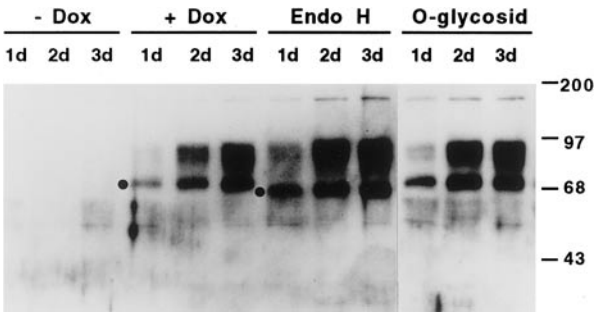

FIG. 2. Enzymatic digestion analysis of gE from gE expressing melanoma cells. Met-gE cells were grown in 6-well plates for 1, 2, and 3 days in medium with (+) or without (−) 1.0 μg/ml Dox. Cells induced with Dox were used for glycosidase digestion analysis. A portion of the product was treated with endo H or O-glycosidase as indicated. Cell lysates were separated by 8.5% SDS–PAGE and transferred to a nitrocellulose membrane. Mouse anti-gE monoclonal antibody (3B3) was used as the probe and detected with anti-mouse antibody conjugated with HRP.

To assess Met-gE glycosylation, Met-gE cells that had been grown for different time periods were analyzed after treatment with endo H or O-glycosidase (Fig. 2). Of the three forms of VZV gE that were detected by Western blot, the 75K protein was endo-H sensitive, showing a decrease in size to 65K (Fig. 2). The 90K and 96K Met-gE proteins showed no alteration with O-glycosidase digestion (Fig. 2), which is in contrast to the decrease in the size of gE recovered from infected cells under these conditions (review: Grose, 1990). These patterns were consistent with retention of some Met-gE in the ER and cis Golgi.

To further examine the distribution of gE in Met-gE cells, Dox-induced Met-gE cells were examined by confocal microscopy using polyclonal antibody to gE and monoclonal antibody to γ-adaptin. γ-Adaptin is a marker for the AP-1 complex, which is restricted to clathrin-coated membranes of the TGN (Seaman *et al.,* 1996; Alconada *et al.,* 1996). Most Met-gE protein was found in perinuclear areas (Fig. 3, center). Colocalization with γ-adaptin was observed, indicating that Met-gE reached the TGN (Fig. 3, right).

Since VZV gE also contains a TGN-targeting motif as well as tyrosine kinase and casein kinase II modification sequences (Olson *et al.,* 1997; Zhu *et al.,* 1995), ORF68 in the Met-gE cells was sequenced to exclude the possibility that mutations in these sequences might have caused the restricted steady-state distribution of gE in Met-gE cells; no nucleotide changes were found. The observations indicate that trafficking of gE expressed as a single viral protein in melanoma cells was disrupted and suggest that gE requires other viral protein(s) as chaperones to achieve the cytoplasmic and plasma membrane distribution observed during VZV infection of melanoma cells.

Effect of gE deletion and repair on the replication of VZV in melanoma cells and in Met-gE cells

As we reported previously, infectious VZV could not be derived from cosmids that had a dual deletion of ORF68 (gE) and ORF67 (gI) (Mallory *et al.,* 1997). Since gI was dispensable, we speculated that gE was probably essential for viral replication. To document the requirement of gE for VZV growth, ORF68 was removed from vOka cosmid DNA. Removal of VZV nucleotides 115808 to 117676 from cosmid pvSpe21 resulted in a complete deletion of the gE coding region (Fig. 4, line 5). Cotransfection of cosmid clones containing full deletions in gE (pvSpe21ΔgE) with pvSpe5, pvPme19, and pvFsp4 yielded no detectable viral plaques. Transfections done with two different Spe21ΔgE cosmids were repeated three times with the same negative result. DNA recovered from transfected cells 3 weeks after cosmid transfection showed no detectable VZV DNA by PCR analysis. As a positive control, intact cosmids pvFsp4, pvSpe5, pvPme19, and pvSpe21 were cotransfected in parallel experiments. The intact cosmids yielded infectious virus consistently, with plaques visible by 6–8 days after transfection.

Met-gE cells incubated in the presence of Dox were transfected with pvSpe21ΔgE and the three intact cosmids to determine whether Met-gE protein could complement the deletion of the gE coding sequence, ORF68, from the cosmid. The formation of small syncytia was observed in Met-gE-transfected cells but no infectious virus was recovered (data not shown). The level of expression of Met-gE may have been insufficient relative to the usual abundant synthesis of gE in VZV-infected cells (Fig. 6, lanes 1–3 vs lanes 7–9).

Repair of gE deletion with VZV MSP gE

The cosmid, Spe21ΔgE-MSPgE@Avr, contains ORF68 from VZV-MSP with 0.45 kb of surrounding noncoding sequence inserted into the *Avr*II site of pvSpe21ΔgE at VZV nt 112853 in the positive orientation (Fig. 4, line 5). In contrast to transfection with the Spe21ΔgE cosmid, restoring ORF68 to the nonnative *Avr*II site and cotransfection with pvFsp4, pvSpe5, pvPme19, and Spe21ΔgE-MSPgE@Avr yielded infectious virus, which was designated vOka-MSPgE. PCR analysis showed the expected 1.9-kb deletion at the endogenous gE site in pvSpe21 (PCR fragment size decreased from 3.2 to 1.3 kb) and the expected 2.3-kb insertion at the *Avr*II site in pvSpe21ΔgE (PCR fragment size increased from 1.1 to 3.4 kb) (data not shown). These experiments confirm that failure to generate infectious virus using the pvSpe21ΔgE cosmid was due specifically to the absence of ORF68 and not to other undetected mutations in the cosmids.

The cosmid Spe21ΔgE/ΔgI-MSPgE@Avr contains ORF68 from VZV MSP inserted into the *Avr*II site as described above and also has ORF67 deleted (Fig. 4, line

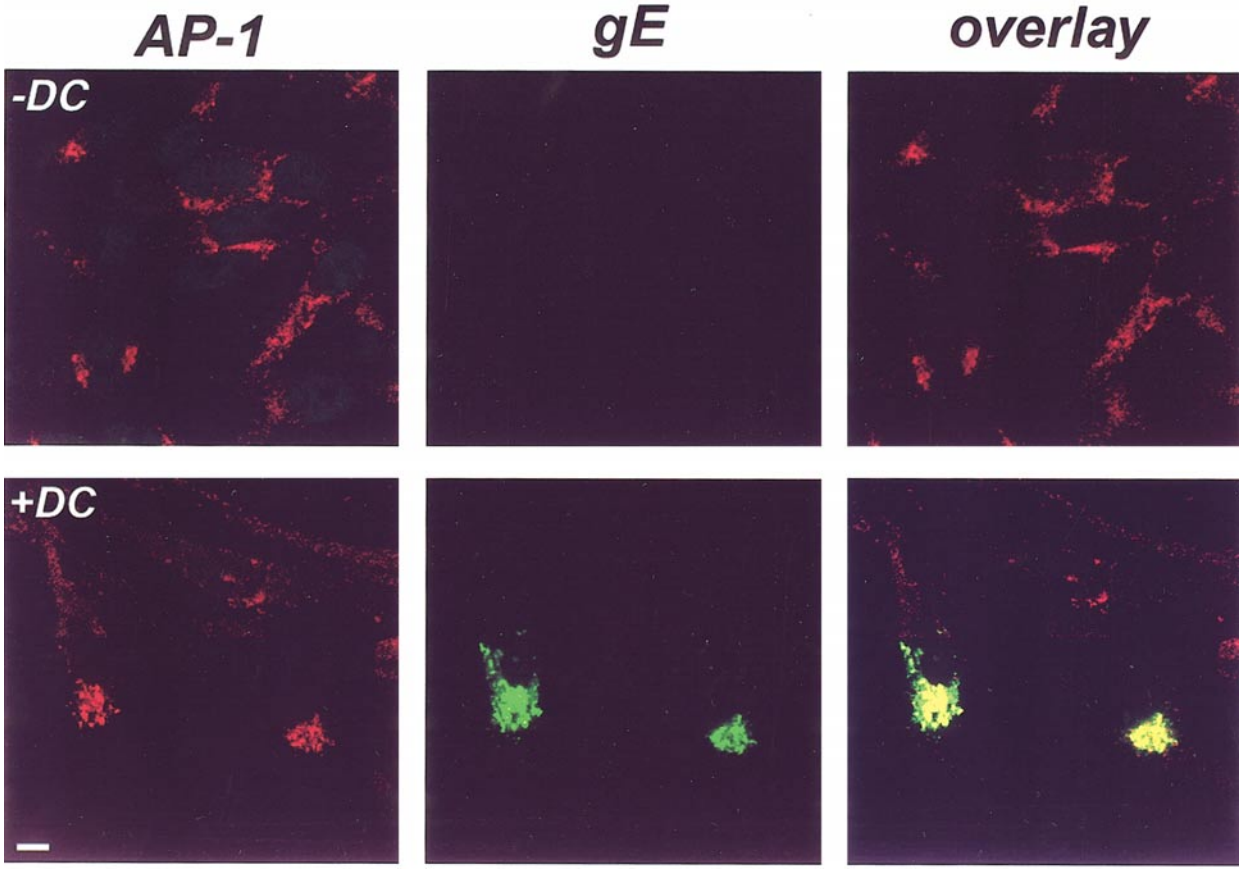

FIG. 3. Localization of gE in Met-gE melanoma cells. VZV gE-expressing cells were grown on glass coverslips and incubated in the absence or presence of 1.0 μg/ml Dox for 3 days. Met-gE cells were fixed and permeablized. The cells were incubated with mouse anti-γ-adaptin monoclonal antibody (AP-1) and human anti-VZV polyclonal antibody (GK). Cells were subsequently washed with PBS–BSA before incubation with goat anti-mouse IgG, conjugated with Texas Red and goat anti-human IgG, and conjugated with FITC. Cells were examined by confocal microscope. AP-1 expression; gE staining; and the overlay of AP-1 and gE staining are shown. Bar, 10 μm.

6). Infectious virus was recovered after cotransfection of Spe21ΔgE/ΔgI-MSPgE@Avr with pvFsp4, pvSpe5, and pvPme19. Initial plaque formation took 10 days longer than the control transfection done with intact pvSpe21 (data not shown). The pattern of initial plaque formation and the limited cell–cell spread of vOka-MSPgEΔgI was similar to that of vOkaΔgI in melanoma cells (Mallory *et al.,* 1997). PCR analysis showed the expected 2.3-kb insertion at the *Avr*II site in pvSpe21ΔgE (PCR fragment size increased from 1.1 to 3.4 kb) (data not shown). These results confirmed that gI was dispensable for VZV replication in melanoma cells (Mallory *et al.,* 1997).

Effect of gI deletion on gE distribution in superinfected Met-gE cells

To further assess the contribution of gI to the intracellular distribution of gE, Met-gE cells were infected with vOka-MSPgEΔgI in the presence of Dox. As in the vOka-MSPgE superinfection experiments, Met-gE cells were infected with vOka-MSPgEΔgI and stained with rabbit anti-IE62 antibody to identify infected cells and with anti-gE mAb, 3B3, in order to assess Met-gE protein localization. The gE visualized in these experiments cannot be MSP gE from the infecting virus because this gE lacks the 3B3 epitope. Uninfected Met-gE cells within the monolayer continued to show gE expression confined to perinuclear regions (Fig. 5A, arrowheads). By 48 h, Met-gE cells that were infected with vOka-MSPgE showed gE detectable throughout the cytoplasm whereas gE remained perinuclear at 48 h after vOka-MSPgEΔgI infection (Fig. 5A, arrows). Since this difference might have been due to the longer replication cycle that is characteristic of gI deletion mutants in melanoma cells, Met-gE cells were examined for 6 days following infection with vOka-MSPgEΔgI. At 5–6 days after superinfection, Met-gE protein was present at sites in the cytoplasm distant from the TGN and at plasma membranes (Fig. 5B, arrows). However, even at these later time points, Met-gE protein was localized in a punctate pattern in the absence of gI (Fig. 5B, 140 h). Dox-induced uninfected cells continued to show perinuclear Met-gE protein at the 140-h late time point (Fig. 5B, top). The data suggest that gI facilitates the normal cytoplasmic distribution of gE within infected cells, although expression of

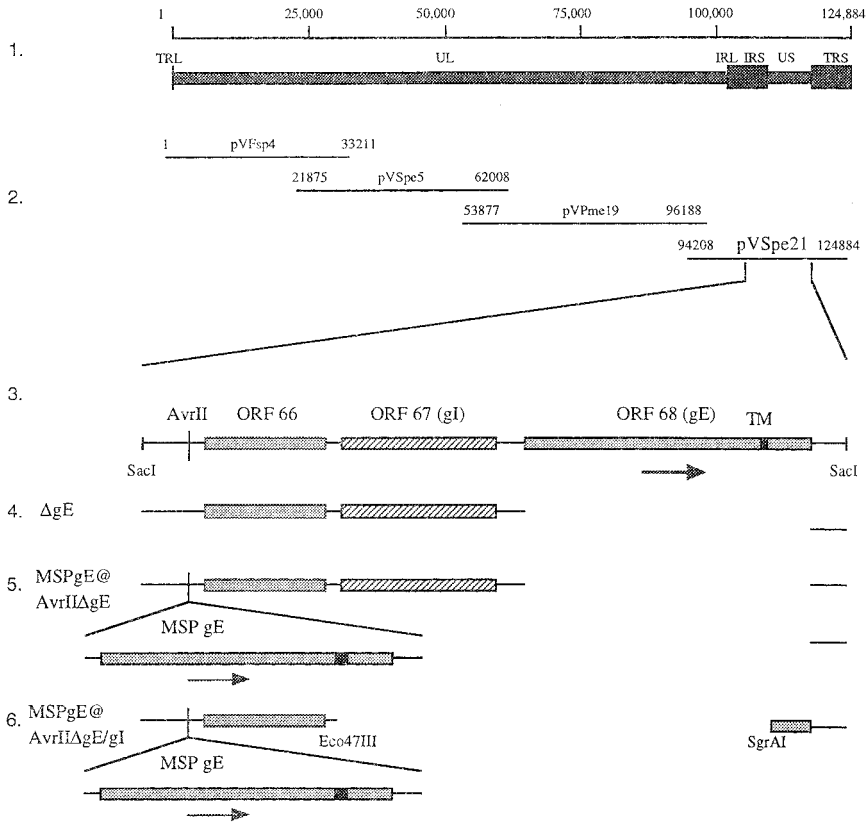

FIG. 4. Construction of VZV pvSpe21 cosmids with deletion of VZV ORF68 or ORF67-68 and insertion of MSP ORF68. gE deletion mutants were constructed with VZV DNA cosmids derived from the Oka strain. Line 1, schematic diagram of the VZV genome with the location of gE; line 2, overlapping segments of the VZV genome used to construct the VZV cosmids; line 3, the subcloned *Sac*I fragment from pvSpe21 containing gE; line 4, deletion of gE; line 5, insertion of MSPgE (ORF68) as well as 454 bp of noncoding sequence (271 bp upstream and 183 bp downstream) into a unique *Avr*II site of pvSpe21ΔgE to generate MSPgE@AvrIIΔgE; line 6, insertion of MSPgE ORF as well as 454 bp of noncoding sequence into a unique *Avr*II site of pvSpe21ΔgI to generate MSPgE@AvrIIΔgE/gI.

VZV proteins other than gI is associated with some intracellular transport of gE to sites beyond the TGN.

Analysis of the effect of VZV superinfection of Met-gE cells on the synthesis of Met-gE protein

The formation of mature gE protein in cells infected with VZV has been studied extensively (reviewed by Grose, 1990). Under conditions described by Montalvo *et al.* (1985), the gE precursor polypeptide is 75K, the high-mannose intermediate form is 81K, the intermediate O-linked glycoprotein is 83K, and the final mature O-linked and N-linked glycoprotein is 94K. In the current experiments, Met-gE cells were inoculated with vOka-MSPgE or vOka-MSPgEΔgI and harvested at different time intervals. Mock-infected Met-gE cells grown in the presence of Dox were used as a control. In these cells, an 80K form of gE was detected at Day 1, and three forms of 80K, 90K, and 96K MW were detected at Days 2 and 3 (Figs. 6A and 6B, lanes 1–3). When Met-gE cells were infected with vOka-MSPgE in the presence of Dox, three bands of 80K, 90K, and 96K were detected at Day 1. This result resembled observations with uninfected Met-gE cells after Dox induction. The lower 80K band is probably the high-mannose form of gE which is endo-H sensitive. There was no change in the 80K form of Met-gE at Days 2 and 3 in induced Met-gE cells (Fig. 6, lanes 2 and 3). However, this protein band was diminished by Days 2 and 3 after infection of Met-gE cells with vOka-MSPgE (Fig. 6A, lanes 8 and 9). By Day 3, most Met-gE was processed to the final 94K form (Fig. 6A, lane 9). These data suggest that viral proteins, including gE itself, may accelerate the maturation of gE during the complex stages of gE processing. When Met-gE cells were infected with vOka-MSPgE in the absence of Dox, some gE was detected (Fig. 6A, lanes 4–6). These observations suggest that VZV transactivator(s) may bind the Tet-responsive PhCMV-1 promoter in the Met-gE cells and turn on low-level gE expression without Dox induction.

Infection with vOka-MSPgEΔgI was used to determine whether gI contributed to gE maturation. When Met-gE cells were infected with vOka-MSPgEΔgI in the presence of Dox, the 80K band was detected at Day 1 (Fig. 6B, lane 7). This band is the same size as the endo-H-sensitive form of gE (Fig. 6B, lane 1). As was observed

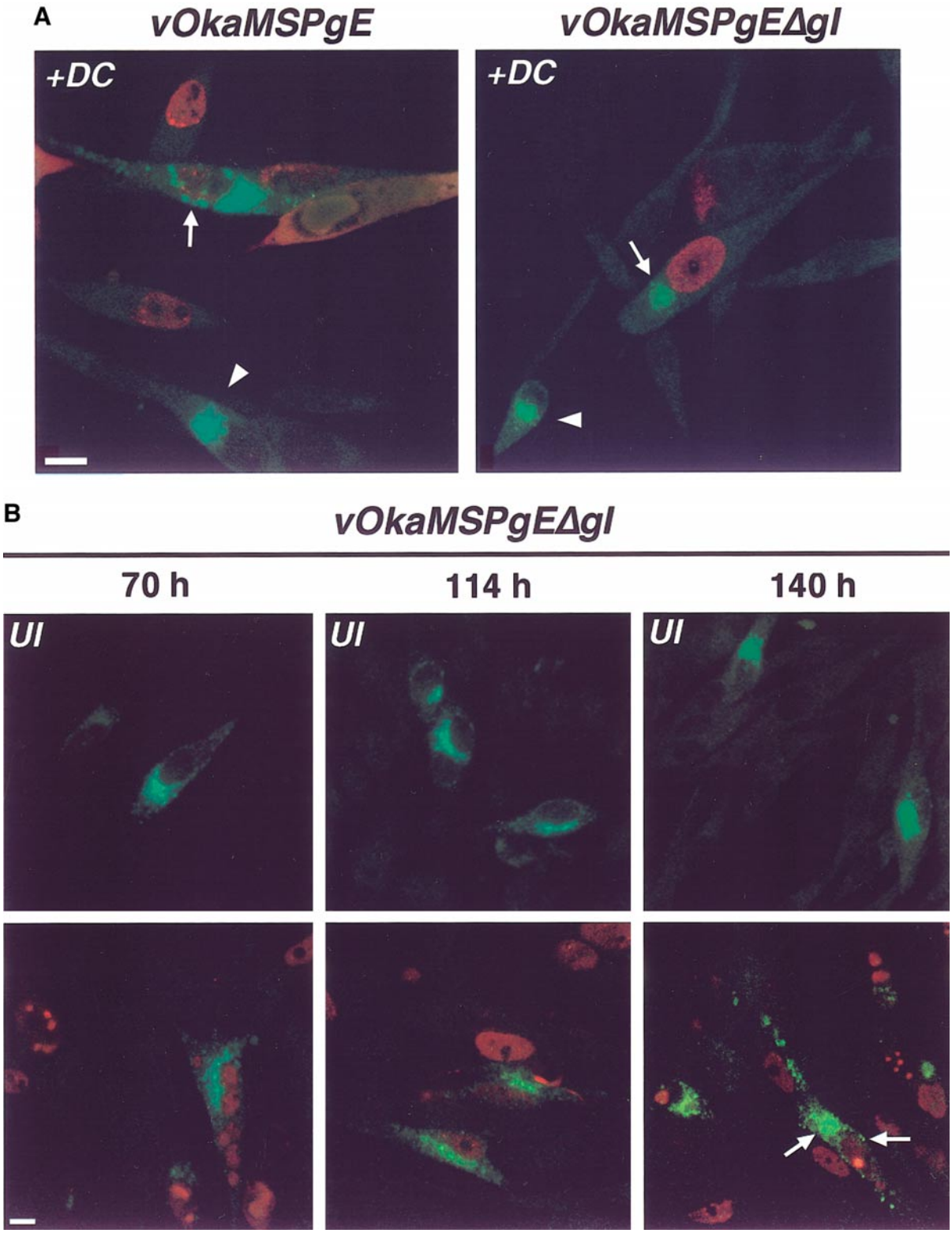

FIG. 5. gE requires VZV infection to reach the cell surface in melanoma cells. Met-gE cells were either mock infected or infected with vOka-MSPgE or vOka-MSPgEΔgI and selected with G418. A. After 48-h incubation in the presence of Dox, vOka-MSPgE- or vOka-MSPgEΔgI-infected cells were fixed and permeablized. B. After 70, 114, and 140 h of incubation in the presence of Dox, vOka-MSPgEΔgI-infected cells and uninfected (UI) cells were fixed and permeablized. The cells were incubated with mouse anti-gE monoclonal antibody (3B3) and rabbit anti-VZV IE62 polyclonal antibody. Cells were subsequently washed with PBS–BSA before incubation with goat anti-mouse IgG, conjugated with FITC and goat anti-rabbit IgG, and conjugated with Texas Red. Bar, 10 μm.

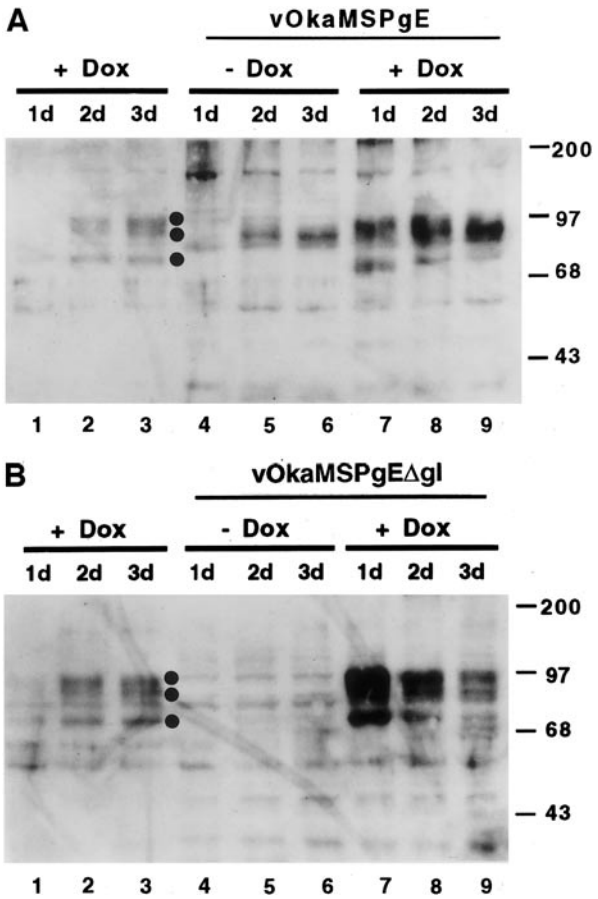

FIG. 6. VZV gE modification after infection of Met-gE cells with vOka-MSPgE or vOka-MSPgEΔgI. Met-gE cells were infected with vOka-MSPgE or vOka-MSPgEΔgI. After 1 week of growth in the presence of G418, the infected cells were transferred to 6-well plates for 1, 2, and 3 days with (+) or without (−) Dox. Cells were harvested and the cell lysates were separated by 10% SDS–PAGE and transferred to a nitrocellulose membrane. Mouse anti-gE monoclonal antibody (3B3) was used as the probe and detected with anti-mouse antibody conjugated with HRP.

when Met-gE cells were infected with vOka-MSPgE, this band was decreased at Days 2 and 3. Two gE forms, of approximately 90K and 96K, could be seen at Days 2 and 3 (Fig. 6B, lanes 8 and 9). However, in contrast to vOka-MSPgE superinfection, the mature, smaller 94K gE did not emerge as the predominant form (Fig. 6A, lanes 8 and 9). This result is consistent with observation of Yao *et al.* (1993), who found that the molecular weight of gE from infected cells resembled that from gE/gI cotransfected cells, while gE from singly transfected cells was not processed to the 94K form. Met-gE was not expressed when Met-gE cells were infected with vOka-MSPgEΔgI in the absence of Dox (Fig. 6B, lanes 4–6). This difference from noninduced MetgE cells infected with vOka-MSPgE probably reflects the fact that gI deletion mutants replicate much less efficiently than intact VZV (Mallory *et al.,* 1997). The failure of gE to shift to the predominant 94K mature form in the absence of gI is consistent with our previous analyses of vOkaΔgI and the gI partial deletion mutant, vOkaΔgI-N (Mallory *et al.,* 1997).

DISCUSSION

VZV, like other alphaherpesviruses, has genes that encode gE and gI proteins within the short unique region of the genome, and gE and gI form heterodimers in infected cells (Litwin *et al.,* 1990). The gE/gI heterodimer complex mediates cell–cell spread and has been shown to be important for infection of neuronal and epithelial cells by HSV and PRV (Card and Enquist, 1995; Dingwell *et al.,* 1994, 1995; Dingwell and Johnson, 1998; Tirabassi and Enquist, 2000). VZV differs from the other alphaherpesviruses, in that gE and gI are the only two glycoproteins encoded in the VZV Us region. VZV gE is a multifunctional protein that has been considered likely to be required for production of infectious virions. This concept is supported by the evidence for its role in VZV envelopment in the TGN, where gE has been shown to interact with other viral glycoproteins and tegument proteins (Gershon *et al.,* 1994). VZV gE also enhances cell–cell contact in polarized epithelial cells, suggesting a role in facilitating cell–cell spread (Mo *et al.,* 2000).

The deletion of the gE homologues of other alphaherpesviruses has adverse effects on replication but does not result in a complete inhibition of virion synthesis (Balan *et al.,* 1994; Jacobs, 1994). In contrast, we speculated that gE might be essential for VZV replication because of the known functions of gE and because we recovered no infectious virus from melanoma cells transfected with cosmids when both ORF68 (gE) and ORF67 (gI) had been deleted (Mallory *et al.,* 1997). VZV gI was identified as nonessential in melanoma cells although its deletion blocked viral replication in Vero cells (Cohen and Nguyen, 1997; Mallory *et al.,* 1997).

The current experiments document that gE is required for VZV replication. No infectious virus was made when melanoma cells were transfected with cosmids from which ORF68 alone was removed, and infectivity was restored when the ORF68 sequence was inserted into a nonnative *Avr*II site in the Us region of the VZV genome. These repair experiments indicate that failure to generate infectious virus when ORF68 was deleted is not likely to have resulted from random mutations elsewhere in the VZV genome. This conclusion is supported further by the reproducibility of the results obtained when ORF68 deletion and repair experiments were repeated with separately derived cosmids. In addition, these experiments showed that infectivity for melanoma cells was restored if gE was reinserted into recombinants that still had deletions of gI. The growth characteristics and plaque morphologies of recombinants obtained after replacing ORF68 were typical of intact VZV, and of gI deletion mutants, respectively. These observations about gE par-

allel our results with gK, encoded by ORF5, which was also proved to be essential for VZV replication by deletion and repair at a nonnative site in the VZV genome (Mo *et al.,* 1999).

Attempts to derive melanoma cells that express gE have not yielded stable cell lines, suggesting that constitutive gE production was incompatible with survival of melanoma cells (unpublished observations; Litwin *et al.,* 1992). However, we found that doxycyline-regulated expression permitted the construction of gE-expressing cell lines (Met-gE cells). After induction, Met-gE cells exhibited relatively limited distribution of gE, with most protein detected in perinuclear areas, where it colocalized with γ-adaptin, a TGN marker. Since VZV gE appears to undergo a continuous cycling between the TGN and plasma membranes, these steady-state experiments are consistent either with gE retention in the TGN or with the failure of gE to remain associated with the plasma membrane long enough to permit detection at this site. In all experiments with induced Met-gE cells, gE was never detected on plasma membranes.

In order to assess the effect of VZV replication on the distribution of Met-gE and its maturation, VZV recombinants were made that expressed the gE from VZV MSP. MSP gE contains a D150N substitution that blocks binding of the monoclonal antibody, 3B3 (Santos *et al.,* 1998). The recombinant virus, vOka-MSPgE, was further modified to delete gI. These recombinant viruses, vOka-MSPgE and vOka-MSPgEΔgI, had growth characteristics in melanoma cells that were indistinguishable from rOka and rOkaΔgI (Mallory *et al.,* 1997). When Met-gE cells were infected with vOka-MSPgE, Met-gE protein exhibited the pattern of intracellular gE localization found in VZV-infected melanoma cells. The cytoplasmic and plasma membrane distribution suggested that Met-gE protein retained the same functional capacity to associate with membranes of intracellular vesicles or organelles as authentic gE that is made during viral replication, but that viral cofactors were needed to permit the characteristic localization of gE within melanoma cells. Based upon our prior observations about VZV gE dimmers (Olson *et al.,* 1997), the simplest explanation is that MSP gE forms a dimmer with Met-gE within the ER and that this dimmer can then be transported through the Golgi to the plasma membrane.

Although gI can be deleted from VZV, our full and partial gI deletion mutants, vOkaΔgI, vOkaΔgI-C, and vOkaΔgI-N, exhibited decreased syncytium formation, punctate gE distribution on cell surfaces, and reduced infectious virus yields in melanoma cells (Mallory *et al.,* 1997). When these mutants were evaluated in human fibroblasts, the TGN cisternae became adherent, viral envelopment was impaired, and virions did not reach post-Golgi structures (Wang *et al.,* 2001). In the current experiments, the distribution of cellular Met-gE was changed in vOka-MSPgEΔgI-infected Met-gE cells. Met-gE protein was detected more diffusely in the cytoplasm of superinfected cells even in the absence of gI. However, the kinetics of gE redistribution was delayed by 3–4 days compared to that of Met-gE cells that were infected with vOka-MSPgE. The punctate pattern of Met-gE localization resembled the aberrant gE localization observed when melanoma cells were infected with full or partial gI deletion mutants (Mallory *et al.,* 1997). Based upon experiments in HeLa cells, gE may not be retrieved efficiently from the plasma membrane in the absence of gI and gI was the dominant protein in modulating the plasma membrane trafficking of the gE:gI Fc receptor complex (Olson and Grose, 1998). In our experiments, some Met-gE appeared beyond perinuclear sites in the cytoplasm of infected cells even in the absence of gI, indicating that other viral proteins can compensate in part for the lack of gI accessory functions. Since recent studies demonstrate that gB interacts with gE to form syncytia, we speculate that gB could contribute to gE trafficking in the absence of gI (Maresova *et al.,* 2001). Alternatively, the effect may be due to gE:gE dimerization alone (Olson *et al.,* 1997).

The typical processing of gE in VZV-infected cells results in the predominance of a mature form of approximately 94K within 72 h. Some differences in gE maturation were observed between Met-gE cells and Met-gE cells infected with vOka-MSPgE. Induced Met-gE protein was detected as 75–80 K, 90K, and 96K forms, whereas Met-gE protein underwent the expected shift to the single 94K mature form when Met-gE cells were infected with vOka-MSPgE. In contrast to superinfection with intact VZV, the emergence of the predominant 94K form was inhibited when Met-gE cells were infected with vOka-MSPgEΔgI. Our previous analysis of melanoma cells infected with vOkaΔgI, vOkaΔgI-C, which expresses the first 209 amino acids of gI, or vOkaΔgI-N, showed that the gI N-terminus was required to achieve the shift to the relative predominance of the mature 94K gE. The current observations provide further evidence that gI facilitates posttranslational modification of gE (reviewed by Grose, 1990; Wang *et al.,* 2001). The N-terminus of gI is known to contain a gE/gI binding domain (Kimura *et al.,* 1998). Further, within a transient transfection system, gE endocytosis is enhanced substantially when gI is cotranslated (Olson and Grose, 1998). Nevertheless, intact gI, including its C-terminal domain, was required to produce the usual titers of infectious VZV, normal plaque morphology (Mallory *et al.,* 1997), and segregation of viral and cellular proteins during virion envelopment in the TGN (Wang *et al.,* 2001).

When Met-gE cells were evaluated for their capacity to complement the deletion of gE, no infectious virus was recovered after transfection of the cosmid, pvSpe21ΔgE, along with the other three intact vOka cosmids, in repeated experiments. The failure to generate infectious virus may be explained by the relatively limited gE syn-

thesis, especially of the mature 94K form, and the kinetics of gE synthesis may not be sufficient to achieve complementation. When induced Met-gE cells were superinfected, they remained fully permissive for VZV replication. Although we had hoped to use the Met-gE cells to determine whether the cell surface expression of gE could interfere with VZV infection in the way that gD expression prevents HSV entry (Campadelli-Fiume *et al.,* 1988; Johnson and Spear, 1989), the limited expression of Met-gE on plasma membranes did not permit us to address this question.

In summary, these observations confirm the hypothesis that gE is essential for VZV replication, based upon the definitive criterion of restored infectivity after reinsertion of ORF68 into the genome. The results using vOka-MSPgE superinfection show that the mutant MSP gE has the capacity to rapidly alter the maturation and distribution of VZV gE. Finally, the experiments provide further evidence that VZV gI, while not required for infectivity, affects the kinetics of gE maturation and facilitates its intracellular distribution for assembly of infectious virus particles.

MATERIALS AND METHODS

Construction of plasmids and cosmids

The complete genome of the VZV vaccine Oka strain (vOka) is contained in four overlapping SuperCos 1 cosmids vectors: pvFsp4 (1–33,211), pvSpe5 (21,875–62,008), pvPme19 (53,877–96,188), pvSpe21 (94,208–124,884) (Fig. 4, line 2). ORF68 extends from nucleotides 115,808 to 117,676, located within the unique short region in the cosmid pvSpe21 (Fig. 4, line 3). A 6-kb DNA fragment from nucleotides 111,911 to 117,989 that contained ORF68 was subcloned into the plasmid vector pBS to generate pSac6A (Mallory *et al.,* 1997). VZV ORF68 was amplified from pSac6A using primers *Sac*IIgEf and *Bam*HIgEb (5′-GCATCCGCGGATGGGGACAGTTAAT-3′ and 5′-GCATGGATCCCGGTGATCACCGGGT C-3′). The PCR product was ligated into the pCR3.1 TA cloning vector, yielding the plasmid, pCR-VZVgE. pCR-VZVgE and pTRE vector (Clontech Laboratories, Inc., Palo Alto, CA) were digested with *Sac*II/*Bam*HI, and the 1.8-kb fragment from pCR-VZVgE was isolated and ligated into pTRE *Sac*II/*Bam*HI to generate pTRE-gE. The expression of gE was driven by the Tet-responsive PhCMV-1 promoter.

The plasmid, pLITMUS ORF66-68, was constructed by inserting the 6.6-kb *Aat*II/*Avr*II fragment from Spe21 into pLITMUS28 vector. For full deletion of VZV gE, primers ΔgEN (5′-TGAATTACAGGCGACCCTCGG-3′) and ΔgEC (5′-TGATCACCGAACCGGGGCAAC-3′) were designed to amplify the sequence adjacent to the gE coding region by using pLITMUS ORF66-68 as the template. The PCR product was gel-purified and ligated to generate pLITMUS ORF66-68ΔgE. After *Aat*II/*Avr*II digestion and gel purification, a 3.8-kb DNA fragment from pLITMUS ORF66-68ΔgE and a 12.5-kb DNA fragment from pLITMUS28/VZV were ligated to yield pLITMUS28/VZVΔgE. Spe21ΔTRs and pLITMUS28/VZVΔgE were digested with *Avr*II/*Srf*I, respectively, and the 16.5-kb fragment from Spe21ΔTRs and 8.9-kb fragment from pLITMUS28/VZVΔgE were ligated to generate Spe21ΔTRs/ΔgE. Spe21ΔTRs/ΔgE was digested with *Avr*II/*Nhe*I; the 13.5-kb fragment was isolated and ligated into the 21.2-kb fragment of Spe21ΔAvrII generated by the *Avr*II/*Nhe*I cleavage to produce Spe21ΔgE (Fig. 4, line 4).

Two cosmids, Spe21ΔgE-MSPgE@Avr and Spe21ΔgE/ΔgI-MSPgE@Avr, were made to restore the deletion of the gE coding region from Spe21ΔgE or Spe21ΔgE/ΔgI (Fig. 4, lines 5 and 6). Plasmid pRS2 was digested with *Avr*II and the DNA fragment containing the MSP ORF68 as well as 454 bp of noncoding sequence (271 bp upstream and 183 bp downstream) was isolated. The 2.3-kb fragment was ligated into *Avr*II linearized cosmids, Spe21ΔgE or Spe21ΔgE/ΔgI, to produce cosmids Spe21ΔgE-MSPgE@Avr and Spe21ΔgE/ΔgI-MSPgE@Avr, respectively.

Cells and gE-expressing melanoma cell lines (Met-gE)

Human melanoma cells were grown in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum, penicillin–streptomycin, and fungizone. The regulator plasmid, pTet-On (Clontech), was transfected into melanoma cells using the Lipofectin method (Gibco BRL, Grand Island, NY). After 4 weeks of selection in medium containing 300 μg/ml of G418, the surviving clones were isolated and expanded for further screening. These stably transfected cells were designated the Tet-On melanoma cells (Met cells).

Met melanoma cell lines were screened using a transient luciferase expression assay. One day before the transient transfections, $1–2 \times 10^5$ Met cells were seeded in 2 ml medium into each well of a 6-well plate. The transient transfections with pTRE-Luc were carried out using Lipofectin. Doxycycline (1 μg/ml) was added to three of the wells. The cells were incubated for 48 h and harvested for the luciferase expression assay (3 Dox+, 3 Dox−). The reaction was placed in a luminometer, and the light produced was measured for a period of 10 s. Over 100 stably transfected clones were analyzed by transient transfection with pTRE-Luc. The clones designated Met19 and Met25 showed relatively low background and high Dox-dependent induction.

The second stable transfection involved the cotransfection of pTRE-gE and pTK-Hyg (Clontech) into the candidate Tet-On cell line (Met19). The pTK-Hyg plasmid allowed for selection with hygromycin B. After 4 weeks of selection in medium containing 300 μg/ml of hygromycin B, the surviving clones were isolated and expanded for further study. These stably transfected cells were designated Met-gE cell lines. Expression of gE was assessed

by Western blot and immunofluorescence. Two clones with a low background of gE in the absence of Dox and high induction of gE in the presence of Dox were used at low passage (<10) for all experiments.

Plasmid DNA with the insert was sequenced using ORF68 sequence primers, gE1837 (5′-CACCGGATAAAAGCGTACTG-3′), gE2311 (5′-GCTTCAACATGCCCGTTAAA-3′), gE2784 (5′-GGGCGTAGGGTTTCTTGTTT-3′), and gE3257 (5′-CAATGAGTCTTTGGCCTTGG-3′). No nucleotide changes were found (data not shown).

Western blot analysis

These samples were incubated at 37°C for 2 h before SDS–PAGE analysis. Aliquots of cell lysates from melanoma cells infected with VZV recombinants were mixed with SDS sample buffer and boiled at 95°C for 5 min. Proteins were separated by 8.5 or 10% SDS–PAGE and transferred to a nitrocellulose membrane. Mouse anti-gE monoclonal antibody, designated 3B3, at a dilution of 1:5000 to 1:10,000 was used as the probe and detected with donkey anti-mouse antibody conjugated with HRP (Amersham, Inc., Buckinghamshire, UK) at a dilution of 1:3000 to 1:5000.

Transfection, DNA isolation, and confirmation of mutants

Cosmid transfection and viral DNA isolation procedures have been described previously (Mo *et al.,* 1999). PCR was used to confirm the expected changes in fragment sizes in cosmids and DNA from recombinant viruses and to sequence the regions spanning the ORF68 deletions and MSPgE insertion into the *Avr*II site. The vOka gE region was amplified by PCR using primers gE1837 and gIf (Mo *et al.,* 2000) to verify the deletion of gE from the native site. The *Avr*II region was amplified by PCR to verify the insertion of the MSPgE cassette at the *Avr*II site 112853. The primers used to determine the sizes of insertion were VZV112580 and VZV113686 (Mallory *et al.,* 1997). The sequence of the inserted ORF68 was verified as identical to VZV MSP ORF68 by the Stanford shared sequencing facility. The recombinant, vOka-MSPgE, that expresses the gE from VZV MSP strain, was generated from vOka cosmids by cotransfection. The second recombinant was vOka-MSPgE without ORF67 (gI), designated vOka-MSPgEΔgI.

Genomic DNA PCR analysis

gE deletion cosmids were cotransfected into gE-expressing cells (Met-gE) in the medium containing 1 μg/ml Dox. Cells were split every 3–4 days at a 1:3 ratio over 9 to 12 days. Cellular and/or viral DNAs were extracted from the transfected cells using DNAzol (Gibco BRL). In cells transfected with intact Spe21 and other three cosmids, the ORF68 sequence would be detected by PCR. In contrast, if Spe21ΔgE recombined with the other cosmids to yield viral genome, PCR would show no ORF68 detected. When primers gEf (Mo *et al.,* 2000) and gE1837 which start at the gE N-terminal and end at 71 bp after gE C-tail are used to amplify diluted DNA template, intact ORF68 in Met gE cells cannot be detected because its DNA annealing site for the second primer is absent.

Immunofluorescence analysis of the distribution of gE

Met-gE cells were infected with vOka-MSPgE or vOka-MSPgEΔgI and harvested when approximately 75% of cells exhibited cytopathic changes. Met-gE cells (3×10^6 cells/T75 tissue culture flask) were incubated for 2 h before the addition of infected cells (3×10^5 cells) to the flask. The infected or mock-infected Met-gE cells were selected in medium containing G418, 300 μg/ml, for 1 week. The infected or mock-infected Met-gE cells were then grown on glass coverslips in medium with Dox, 1 μg/ml, or no drug. After incubation for 24–72 h, cells were fixed and permeablized and incubated with anti-gE mAb, 3B3, and rabbit anti-VZV IE62 polyclonal antibody. The cells were washed with PBS–BSA before incubation with goat anti-mouse IgG, conjugated with FITC and goat anti-rabbit IgG, conjugated with Texas Red. Cells were examined with a Nikon fluorescence microscope using a 40X objective or a Molecular Dynamics MultiProbe 2010 laser scanning confocal microscope. Fluorescence images of stained cells were recorded using Kodak Ektachrome Elite II (ASA 400) films. Developed positive images were then digitized with a slide scanner.

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