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LOCALIZATION OF VARICELLA-ZOSTER VIRAL GLYCOPROTEINS M AND N

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Cynthia Leigh Zerboni

December 2003

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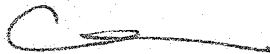

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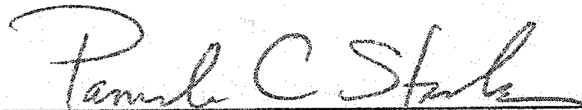

ABSTRACT

LOCALIZATION OF VARICELLA-ZOSTER VIRAL
GLYCOPROTEINS M AND N

by Cynthia Leigh Zerboni

Herpes viral glycoproteins (g) M and N are highly conserved. In some herpes viruses, gM and gN form a complex of unknown function. The localization and interaction of varicella-zoster virus (VZV) gM and gN has not been studied. This thesis examines the localization of VZV gM in infected cells and the localization of gM and gN in transiently transfected cells. VZV gM localizes to infected cell plasma membranes. Epitope tagged VZV gM is expressed in the cytoplasm as well as a perinuclear compartment in transiently transfected MeWo cells. Epitope tagged VZV gN localizes to the same perinuclear compartment but is not expressed in the cytoplasm. Anti-gM antibody immunoprecipitates several large species including the 49 kD gM glycoprotein, as well as a smaller ~25 kDa protein. In conclusion, VZV gM is a membrane protein whereas transiently expressed gM and gN localize to a perinuclear compartment.

ACKNOWLEDGMENTS

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INTRODUCTION

Varicella-zoster virus (VZV) is the human herpesvirus that causes varicella, commonly called chickenpox, as the primary infection in susceptible individuals and may reactivate as herpes zoster, also known as shingles (2). VZV is included in the family *Herpesviridae* based on common genetic organization and virion morphology (47). Herpesviruses have existed for at least 200 million years and infect virtually all animal species. Herpesviruses are divided into three subfamilies (alpha, beta and gamma) based on biological properties. Eight herpesviruses use humans as their natural host. Human herpesviruses include herpes simplex virus-1 (HSV-1), herpes simplex virus-2 (HSV-2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), human herpesvirus-6, -7, and -8 (HHV-6, HHV-7, and HHV-8) (47). HSV-1, HSV-2, and VZV are members of the alphaherpesvirus subfamily.

Alphaherpesviridae

The subfamily *Alphaherpesviridae* includes HSV-1, HSV-2, VZV, and simian varicella virus (SVV), as well as nonprimate herpesviruses pseudorabies virus (PRV), equine herpesvirus I (EHV-1), and bovine herpesvirus (BHV-1). Marek's disease virus type I (MDV) is an avian herpesvirus that shares biological properties of both alpha and gamma subfamilies (7). Inclusion criteria in *Alphaherpesviridae* are variable host range, high multiplicity of infection, and establishment of latency in neuronal ganglia (47). VZV, PRV, BHV-1, and simian varicella virus (SVV) comprise the genus *Varicellovirus*. Inclusion criteria in the *Varicellovirus* genus are the ability

to produce infection of skin, mucous membranes, and the nervous system (47). VZV is the only *Varicellovirus* known to cause disease in humans (47).

VZV tissue tropism

While most alphaherpesviruses infect a broad spectrum of cell types in culture and a range of species and tissue types *in vivo*, VZV infection is largely host restricted. In tissue culture, VZV may be propagated in human fibroblast and melanoma cell lines, guinea pig embryo cells, monkey kidney cells, and less efficiently in EBV-transformed B cells, and neuronal cell lines (3, 14, 18, 30). VZV can also infect and replicate in T-lymphocytes, a characteristic shared with HHV-6 and HHV-7 (41). VZV is distinguished from other alphaherpesviruses in its inability to release infectious particles into the extracellular media of cultured cells (2). The inability to release infectious virus and limited host cell range suggest that VZV may employ means of viral entry or egress not universally shared among other alphaherpesviruses.

VZV structure

Structural features of herpesviral particles are highly conserved. The prototypical infectious particle is approximately 150 nm in diameter and contains a linear double-stranded DNA core within an icosadeltahedral capsid (8). The capsid is surrounded by an amorphous material designated the tegument. The VZV tegument is composed of at least six viral proteins. The tegument and nucleocapsid are surrounded by a viral envelope coated with glycoproteins (8). Varicelloviruses share a genomic arrangement in which two isomeric forms predominate by inversion of shorter unique DNA segments and flanking repeat elements (8). The VZV genome is

125 kilobases which contain open reading frames (ORFs) encoding for approximately 70 proteins, including seven well-characterized viral glycoproteins (8). The virus also contains open reading frames for two uncharacterized glycoproteins (g), gM and gN (11). The glycoproteins of alphaherpesviruses are highly conserved and participate in viral attachment, fusion, and entry into permissive cells.

VZV replication

Entry of VZV into permissive cells is a coordinated process that involves binding of the viral envelope to host cell surface components followed by fusion of the viral membrane with the host cell plasma membrane (8). There is some evidence that alphaherpesviruses can enter cells by direct endocytosis, but that this results in an abortive infection (47). Productive infection following virion entry can only occur if the virus enters by fusion and the host cell contains required elements for viral uncoating, transport to the nucleus, viral gene expression, replication, assembly, and egress (Fig. 1).

In most alphaherpesviruses, including VZV, envelope glycoproteins gB and gC facilitate entry by binding to heparan sulphate moieties on cell surface proteoglycans. Heparan sulphate proteoglycans (HSPGs) are found on the surface of most vertebrate cell types (22, 25). This initial binding step may stabilize the virion on the cell membrane allowing a second co-receptor to bind (25). Cellular proteins which function as herpes viral entry mediators (HVEMs) have been identified for all alphaherpesviruses except VZV. In HSV-1 and HSV-2, viral entry is mediated by the interaction between an HVEM molecule and viral glycoprotein D. VZV does not have a HSV-1 gD homologue (11). The cellular co-receptor and viral ligand which mediate

VZV entry into permissive cells are unknown. The inability to produce cell-free infectious VZV has complicated the search for cell surface components that participate in viral entry.

Following virion entry, the nucleocapsid and associated tegument proteins are transported to the nuclear pore, and the viral genome is deposited inside the nucleus (8).

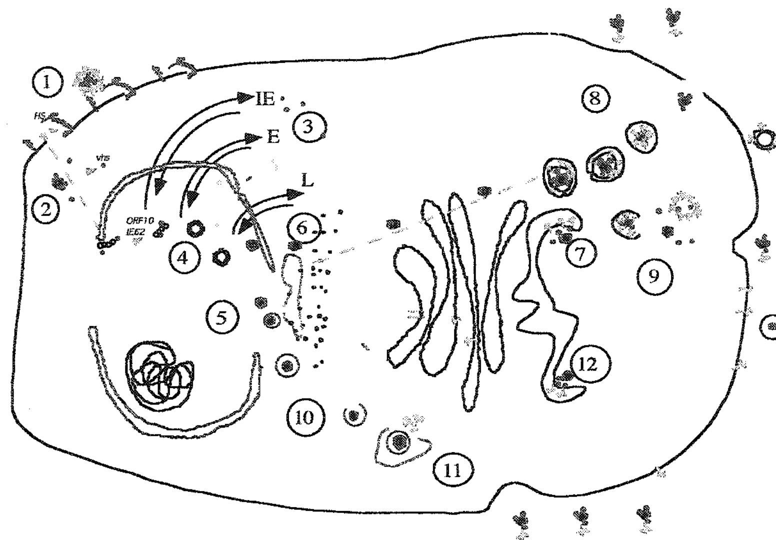

FIG. 1. Replication of VZV. (1) Viral particles attach to cell surface heparan sulphate proteoglycans (HSPGs) and the virion enters by fusion. (2) The nucleocapsid traffics to the nuclear pore and releases the viral genome. (3) Nucleocapsid-associated tegument proteins transactivate a cascade of viral immediate early (IE), early (E) and late (L) transcripts and protein synthesis. (4) Viral replication follows a rolling-circle model. (5) Nucleocapsids are assembled and packaged with the viral genome. (6) Nucleocapsids are enveloped as they exit the nucleus and then de-enveloped at the rough endoplasmic reticulum (RER). Nucleocapsids traffic to *trans* golgi networks where they complex with viral tegument proteins and are taken up into cytoplasmic vesicles containing glycoproteins (7). Vesicles fuse with plasma membranes releasing infectious progeny (8). Some vesicles fuse with pre-lysosomes resulting in particle degradation (9).

Virally-encoded gene transactivators present in the tegument temporally regulate VZV transcription and protein synthesis. Genome replication follows a rolling-circle mechanism (47). Capsid proteins, which are translated in the cytoplasm, are translocated to the nucleus where they are assembled into capsids and packaged with the viral genome.

VZV egress from the infected cell is poorly understood. The re-envelopment model postulates that viral capsids bud through the inner nuclear membrane, acquiring a temporary envelope (8). The inner nuclear membrane is contiguous with the lumen of the rough endoplasmic reticulum (RER). The enveloped capsid then buds through the RER, losing its initial envelope. Naked capsids reacquire the viral envelope at the *trans* golgi network (TGN). At the TGN, tegument proteins associate with the cytosolic stems of viral glycoproteins which have been processed through the *cis* to the *trans* golgi. Naked capsids complex with tegument proteins, and the capsid-tegument complex is packaged into a transport vesicle. The inner folds of the transport vesicle fuses, forming a double enveloped particle. During viral particle egress, the outer layer of the transport vesicle fuses with the plasma membrane, releasing a virion with a single, glycoprotein-studded envelope. Some secretory vesicles fuse with pre-lysosomal compartments in the cell and the viral contents are digested. This may account for the abundant defective particles observed by electron microscopy (47).

VZV pathogenesis

As VZV is a human-restricted pathogen, there is no small animal model to mimic VZV pathogenesis in the natural host. There are three stages of VZV

pathogenesis and disease in the susceptible human host (Fig. 2). The first stage is a 10-21 day incubation period which is initiated by inoculation of respiratory mucosa. During this stage the virus replicates in regional lymph nodes. Replication in T-cells induces a primary viremia which allows for transport of the virus through the circulation. Secondary organs such as liver and spleen may also be sites for viral replication. The second stage is acute disease and is characterized by the appearance of a vesicular rash throughout the epidermis. Following resolution of primary infection, the virus maintains a lifelong latency in the sensory dorsal root ganglia. Dorsal root ganglia become infected through transfer of the virus from T-cells or at nerve endings in skin tissues. The virus may periodically reactivate from this third stage.

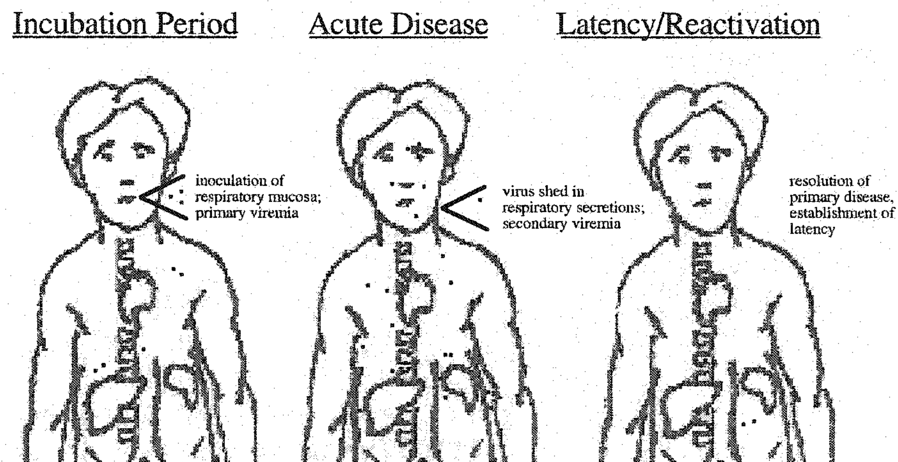

FIG. 2. VZV Pathogenesis. VZV pathogenesis is divided into three stages of disease: the incubation period, acute disease, and the state of latency/reactivation.

VZV glycoproteins

Viral glycoproteins serve several functions including roles in attachment and fusion of the viral envelope with the plasma membrane (Table 1). The role of viral glycoproteins in cell-cell fusion is especially of interest in VZV because of its highly cell-associated nature. Herpes family members encode for up to 13 viral glycoproteins, several of which are highly conserved (gB, gC, gE, gI, gH, gL, and gK) (47). Most alphaherpesviruses encode two glycoproteins, gD and gG, which have no VZV homologue (9).

TABLE 1. VZV glycoproteins with their functions

ORF	gp designation	function
31	gB	fusogen, binds HSPGs
14	gC	entry mediator, binds HSPGs
68	gE	essential, Fc receptor homolog
37	gH	fusogen
67	gI	gE chaperone protein
5	gK	essential virion protein
60	gL	gH chaperone protein
50	gM	unknown
9a	gN	unknown

HSPGs = heparan sulphate proteoglycans

VZV gB and gC function in viral entry by mediating attachment of the virus to heparan sulphate proteoglycans (HSPGs) (22). VZV gB also contributes to fusion of virus and cell plasma membranes during entry (21).

Glycoprotein E is the most abundant glycoprotein on VZV-infected cells (2). VZV gE is required for viral replication (38). Interestingly, gE is dispensable for replication in HSV-1 (47). In both VZV and HSV-1, gE complexes non-covalently with gI and acts as an Ig Fc receptor on infected cells (43). The function of the gE-gI complex is unknown. VZV gI deletion mutants are able to replicate in culture but display a small plaque phenotype (24). Small plaque size and impaired syncytia formation may reflect a role for gI in cell-cell spread. Expression of gI may be required for processing and proper surface expression of gE (24).

VZV gH is highly conserved and the primary viral fusogen (20). Like gE and gI, gH forms a complex with another glycoprotein, gL. Expression of gL appears to be required for correct processing and cell surface expression of gH and thus it has been dubbed a "chaperone protein" (20). VZV gK is a component of the virion and is essential for virus replication (40).

VZV gM and gN

In addition to the seven glycoproteins described (gB, gC, gE, gI, gH, gL, and gK) VZV is predicted to encode two additional glycoproteins (11). VZV ORF50 is predicted to encode a type III transmembrane glycoprotein, designated gM. Glycoprotein M is highly conserved throughout the herpesvirus subfamilies. Multiple sequence alignment of gM proteins in VZV, SVV, PRV, BHV-1, and a gallid herpesvirus demonstrate a high degree of conservation (Fig. 3). Of note, a cysteine

residue at the N-terminal portion of the protein is present in all species examined. ORF9a is predicted to encode a type 1 membrane protein, designated gN. VZV gN is also highly conserved (Fig. 4). Like gM species, all gN species examined share a cysteine residue at the N-terminus. VZV gM and gN are uncharacterized although a stop codon mutant of ORF9a exhibited small plaque size (48).

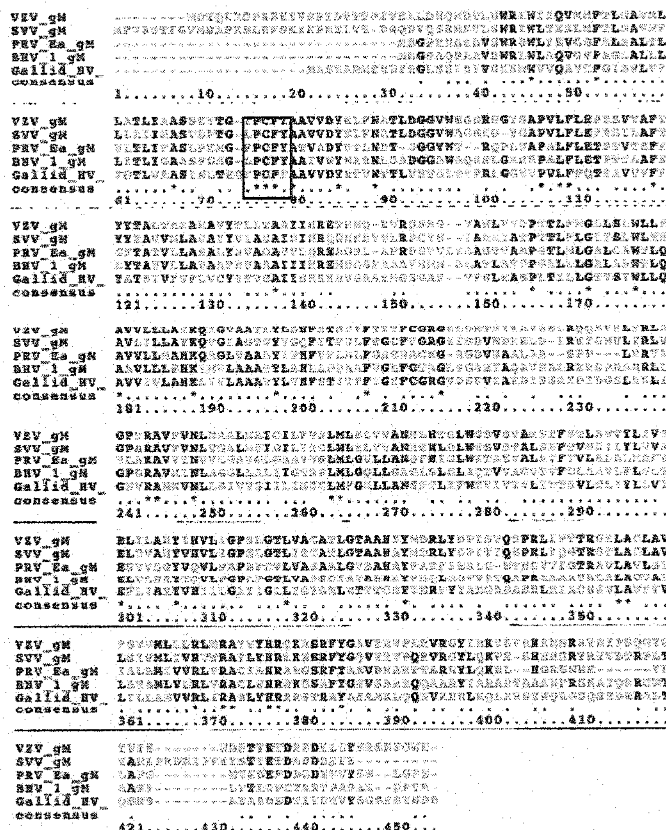

FIG. 3. Comparison of herpesviral gM homologues. Multiple sequence alignment was performed using the ClustalW/BoxShade algorithm. Dark letters represent perfect matches. A consensus sequence is given on the bottom line. Cysteine residues for potential disulfide linkage are indicated by a box.

FIG. 4. Comparison of herpesviral gN homologues. Cysteine residues for disulfide linkage are indicated by boxes.

Herpesviral gM homologues

Despite the highly conserved nature of gM, it has proven to play a non-essential role in alphaherpesviruses HSV-1, PRV, and EHV-1 (13, 36, 44). Among the betaherpesviruses, gM has been shown to be essential for virus replication of HCMV in fibroblasts *in vitro* (23). Across all herpesviruses, gM proteins share four structural motifs: an extracellular hydrophilic N-terminus, a N-linked glycosylation site, six to eight membrane spanning domains, and an intracellular hydrophilic carboxy-terminus (1, 13, 36).

The phenotype of gM-deleted alphaherpesviruses includes small plaque size, reduced syncytia formation, and reduced viral titers *in vitro* (13, 36, 44). Electron microscopy studies of gM-deleted viruses show an accumulation of naked capsids at the *trans* golgi and an absence of secondary envelopment (5). This suggests that gM may play a role in virus maturation and envelopment at the *trans* golgi network. Few studies have characterized gM-deleted herpesviruses *in vivo*. Dijkstra (1997) noted a 100-fold decrease in infectious virus in nasal secretions from piglets inoculated with PRV deleted for gM (12). Vaccination with the gM deleted virus protected the piglets

from challenge with a wild type PRV. In contrast, Masse (1999) found no attenuation using a murine model to examine PRV gM and gN in neurovirulence (39).

Herpesviral gN homologues

Glycoprotein M has been demonstrated to form a complex with gN in BHV-1, PRV, HCMV, EBV, and HHV-8 (27, 31, 33, 35, 54). The conserved cysteine residue in gM and gN indicate a potential for linkage via di-sulphide bridging. Little is known about the function of the gM-gN complex or gM alone in herpesviruses. The amino acid sequence of glycoprotein N predicts a small membrane spanning protein. PRV, HCMV, and EBV gN is an O-linked protein; whereas HSV-1 and BHV-1 gN show no evidence of O-glycosylation (10, 26, 33, 37, 54). In EHV-1, gN (the UL49.5 gene product) is required for appropriate processing and expression of gM (49). This suggests that in EHV-1, gN (like gL) may act as a chaperone protein. Deletion of gN in PRV, however, had no effect on gM processing (10). In EBV, a betaherpesvirus, gM is required for appropriate processing and expression of gN; whereas gN is not required for appropriate processing of gM (32, 33).

Specific aims for this project

To analyze whether gM is essential for viral replication *in vitro*, a deletion mutant of the gM coding region was constructed in a recombinant varicella strain, named rVOka. Immunofluorescence was used to examine the localization of gM in infected cells, and gM ± gN in transiently transfected cells. Western blot and radioimmunoprecipitation were employed to determine the size of VZV gM and any complexed proteins, such as gN.

MATERIALS AND METHODS

Bioinformatic analysis of VZV gM and gN

Software and programs to predict protein composition and function were provided to members of the Computational Molecular and Genetic Medicine (CMGM) program at Stanford University.

Construction of rVOka ΔgM and rV-Oka gM-repair

Use of a cosmid-derived genome for construction of recombinant virus has overcome the difficulty in making viral recombinants of a cell-associated virus. This technique employs five overlapping cosmid clones spanning the entire 125,000 base pair VZV genome (Fig. 5). Individual cosmid clones, containing 20-40,000 bases, may be manipulated using standard recombinant DNA techniques. Digestion with *Avr*II or *Avr*II/*Asc*I releases the genomic segment from the *cos* vector. When co-transfected into susceptible cells, homologous recombination of overlapping sections on genomic segments results in the generation of mature infectious virus containing the mutation of interest (Fig. 5B) (9, 38).

A complete deletion of VZV ORF50 was constructed using mutagenic polymerase chain reaction (PCR) to replace the gM coding region with a *Kpn*I restriction enzyme site in a plasmid subclone of the pvAvr10 (rVOka) cosmid (Fig. 5C). The VZV-genome containing region (minus ORF50) was then reintroduced into the pvAvr10 cosmid clone. The gM deletion was confirmed by sequencing of the pvAvr10 cosmid over the ORF50 coding region. The gM deleted cosmid was named AvrΔgM.

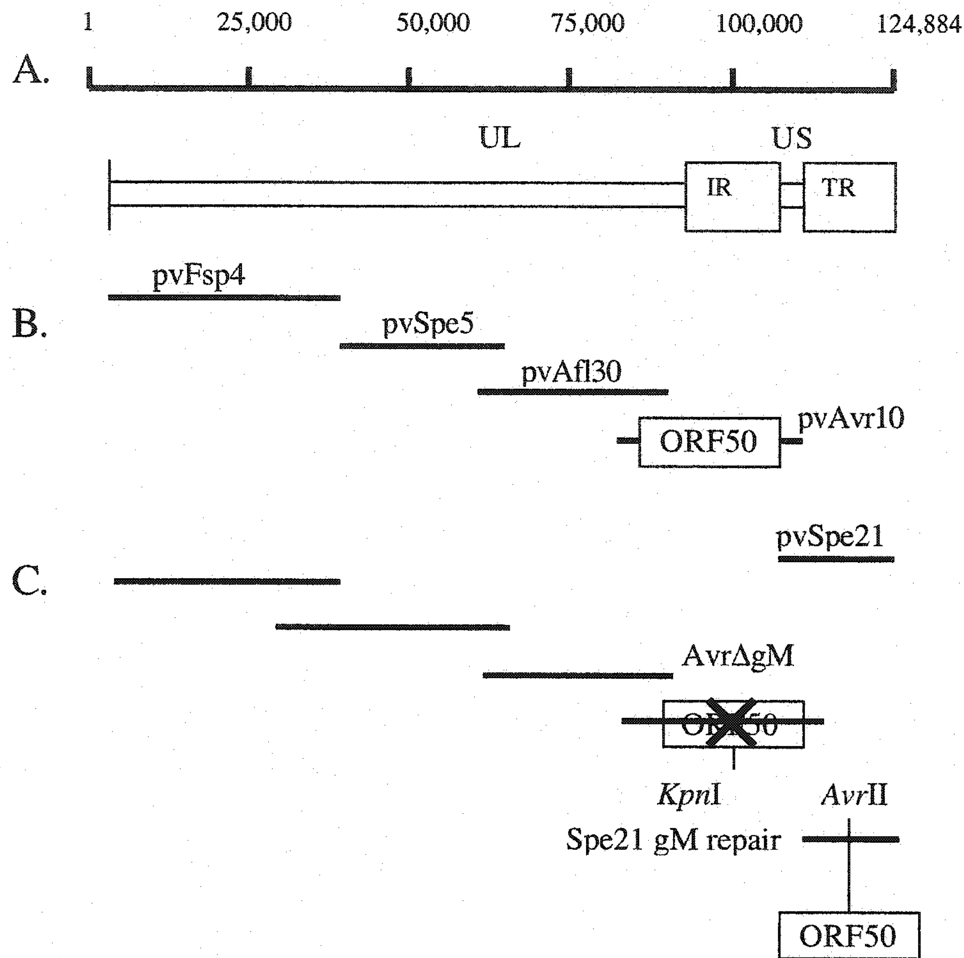

FIG. 5. Recombinant deletion mutant and repair of ORF50. (A) The VZV genome is organized into unique long (UL), unique short (US), inverted repeat (IR) and terminal repeat (TR) regions. (B) The five overlapping cosmid clones that span the VZV genome are pvFsp4, pvSpe5, pvAfl30, pvAvr10 and pvSpe21. ORF50 is located on pAvr10. Co-transfection of all five intact cosmids results in generation of rVOka virus. (C) rVOka deleted for ORF50 (rVOka ΔgM) was generated by substitution of the entire ORF50 coding region for a *Kpn*I restriction site. The ORF50 repair virus was generated by insertion of the ORF50 coding region into a unique *Avr*II site on the pvSpe21 cosmid.

As a control to check for adventitious events during cloning, a repair or complement of the ORF50 coding region was constructed by insertion of ORF50 into a unique non-coding *Avr*II restriction site on the pvSpe21 cosmid. Mutagenic primers were designed to introduce an *Avr*II site 100 nucleotides upstream and downstream of the ORF50 coding sequence to include the promoter region and polyadenylation signal. The repaired cosmid was named Spe21-gMrepair. For co-transfection into a melanoma cell line (MeWo), cosmids were digested with *Asc*I alone or *Asc*I/*Avr*II (pAvr cosmids) to release the VZV genomic segment from the *cos* vector, and transfected using the calcium phosphate method (38). Cytopathic effect is usually evident within 7-21 days post transfection.

Culture of cells and virus

MeWo and 293 cells were maintained in tissue culture media {minimum essential media (MEM) (Mediatech, Washington, D.C.) supplemented with 2mM L-glutamine (Gibco, Gaithersburg, MD), 50 I.U. penicillin, 50 μg streptomycin (Pen/Strep, ICN Biomedicals, Inc. Costa Mesa, CA), 0.5 μg fungizone (Flow Laboratories, McLean, VA), with 10% fetal calf serum (FCS, Tissue Culture Biologicals, Tulare, CA)}. Frozen stocks of cell lines and virus were prepared and stored at -70°C in tissue culture media with 10% dimethyl sulfoxide (DMSO). Unless otherwise stated, all chemicals used were obtained from Sigma-Aldrich Chemical Co., St. Louis, MO.

Immunofluorescence of rVOka infected MeWo cells

Recombinant VOka obtained by co-transfection of intact VZV genome segments was used to infect MeWo cells seeded in 1 mm chamber slides. 24 hours post infection, infected and uninfected cell monolayers were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde and 0.01% Triton X-100 in PBS for 20 minutes at room temperature. Fixed chamber slides were air-dried and blocked overnight with 10% fetal calf serum in PBS at 4°C. Expression and localization of VZV gM was assessed by immunofluorescence using a rabbit polyclonal antibody generated to the C-terminal portion of VZV gM.

The rabbit polyclonal antibody (anti-gM) (a generous gift from Candace Winstead) was generated by insertion of the C-terminal 88 amino acids of VZV gM into the inducible expression vector pGEX-2T (Pharmacia Biotech, Milwakee, WI) (Candace Winstead, personal communication). Bacterial transfection and induction of the pGEX-2T/gM vector results in expression of the C-terminus of gM fused to the glutathione S-transferase (GST) protein. Protein induction, purification, and use as an immunogen for antibody production was performed using the methods of Frangioni and Neel (16). Preimmune rabbit serum was used as a negative control in immunofluorescence studies.

Expression of other VZV proteins was assessed using a human polyclonal antibody (anti-VZV) that primarily recognizes VZV gE. Fluorescein (FITC) conjugated anti-rabbit or Texas Red (TR)-conjugated anti-human secondary antibodies were used for detection (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole,

dihydrochloride). Slides were examined using a Zeiss Axiovert 200 fluorescent microscope.

Mammalian expression vectors

The cellular localization of VZV gM and gN was assessed by transient expression using plasmids designed to epitope tag and overproduce recombinant proteins driven by the T7 promoter. The open reading frames of ORF9a and ORF50 were subcloned into pcDNA4/myc-His and pcDNA4/V5-His (Invitrogen Life Technologies, Carlsbad, CA) (Fig. 6).

To amplify the ORF9a and ORF50 sequences the following PCR primers were used: ORF9a sense primer (5'-AAGATGGGATCAATTACCGCTTC-3') ORF9a antisense primer (5'-CCACGTGCTGCGTAATACAGAA-3'), ORF50 sense primer (5'-CTCCAACCCACTGTTTGATCGC-3'), ORF50 antisense primer (5'-GACATGGGAACTCAAAAGAAGG-3'). The primers were designed to include the Kozak translation initiation sequence. The Kozak consensus sequence for ORF9a is AAGATGG (the start codon is underlined). The Kozak consensus sequence for ORF50 is GACATGG.

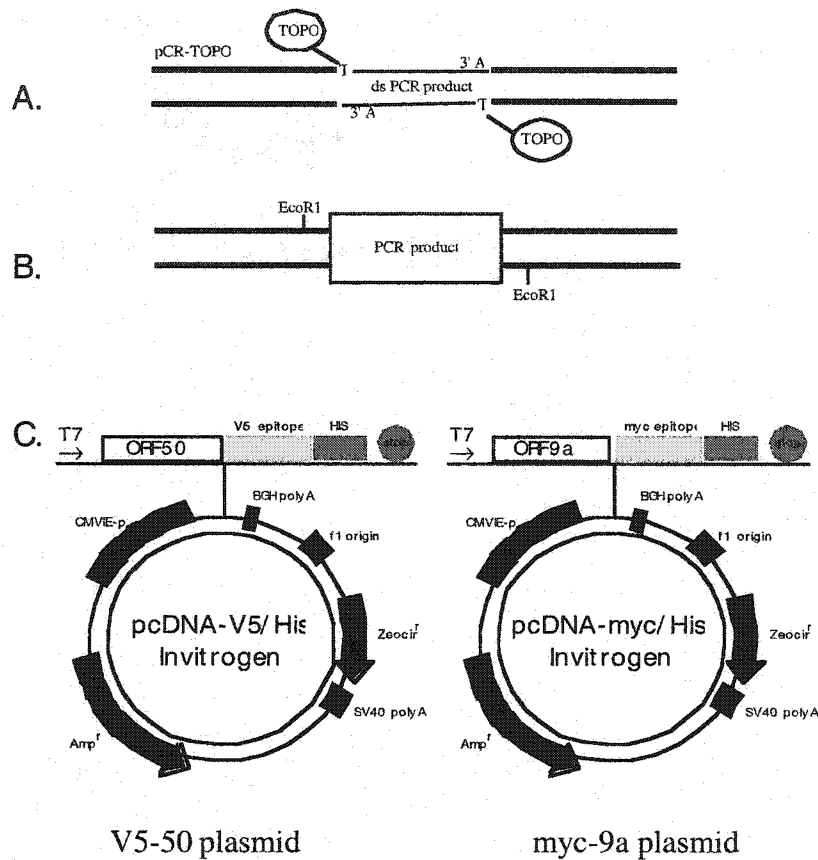

FIG. 6. Diagram of mammalian expression plasmids. ORF50 and ORF9a coding regions (minus the stop codon, plus the Kozak translation initiation sequence) were amplified (A) and TA-cloned into pCR-TOPO using topoisomerase (TOPO) mediated joining. The product was excised from the pCR-TOPO vector by *EcoR*1 restriction (B) and cloned into the Invitrogen pcDNA-V5/His or pcDNA-myc/His plasmids for expression in mammalian cells (C).

For PCR amplification, an Eppendorf mastercycler gradient thermocycler was used with the following program: denaturation 94°C for 30 s, annealing 65°C for 30 s, extension 68°C for 60s per kb of target for 35 cycles. The predicted product size for ORF9a was 283 bp and 1308 bp for ORF50. An enzyme mix consisting of *Taq* and *Pyrococcus* species thermostable DNA polymerases was used (Invitrogen Life Technologies, Carlsbad, CA). Amplification using *Taq* DNA polymerase yields targets with a single deoxyadenosine at the 3' end. The A-overhang permits rapid cloning of the target into a TA cloning vector by topoisomerase-mediated joining. The amplified ORF9a and ORF50 products were first purified using a Qiagen PCR purification kit (Qiagen, Valencia, CA) and then ligated to the pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen Life Technologies, Carlsbad, CA). This step flanks the PCR product with *EcoR*I restriction sites for in-frame cloning into the pcDNA expression vectors.

The TA reaction was electroporated into Top 10F' *Escherichia coli* using a Biorad electroporator and plated on agar plates with the appropriate antibiotics. Colonies were screened for the PCR insert by restriction with *EcoR*I. Two positive colonies were selected for ORF9a and ORF50. The colonies were grown in Luria Bertani (LB) broth with kanamycin and amplicillin, and the DNA was purified using a Qiagen Maxi Prep Kit. The plasmid DNA was digested with *EcoR*I and subjected to agarose gel electrophoresis. The appropriate sized fragments containing the ORF9a and ORF50 coding regions flanked with *EcoR*I sites were excised from the gel and purified using a Qiagen Gel Purification Kit. The ORF9a coding region was ligated into the pcDNA4myc-HIS vector and the ORF50 coding region was ligated into the pcDNA4/V5-His vector at a unique *EcoR*I site. The resulting plasmids, named myc-

9A (myc-tagged ORF9a) and V5-50 (V5-tagged ORF50), were screened for appropriate orientation of the PCR insert and sequenced to verify that the ORF50 and ORF9a open reading frames were in-frame with the epitope tag.

Transient transfection experiments

The V5-50 and myc-9a plasmids were transiently expressed individually and together in MeWo and 293 cells using liposome-mediated transfection. Subconfluent monolayers of MeWo and 293 cells in chamber slides were treated with plasmids plus Lipofectamine reagent (Invitrogen Life Technologies, Carlsbad, CA) for 3 hours. The plasmid pcDNA4/V5-His/lacZ, which expresses β-galactosidase, was transfected in a separate chamber slide to monitor transfection efficiency. 48 hours post transfection, cells were fixed and permeabilized with 4% paraformaldehyde plus 0.01% Triton X-100.

For immunofluorescence, cells in chamber slides were incubated with monoclonal antibodies to myc or V5 epitopes followed by a FITC-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Cell nuclei were stained with DAPI.

Western blot

Cell lysates of rVOka infected and uninfected MeWo cells, as well as cells transiently transfected with the V5-50 and myc-9a plasmids, were prepared to evaluate protein expression by western blot. At 24 hours post infection or 48 hours post transfection, cells were removed from a T75 cm^2 tissue culture flask in ice-cold phosphate-buffered saline. Cells were centrifuged at 800 g x 5 minutes at 4°C to pellet

the cells. Cell pellets were resuspended in 1 ml extract buffer {10 mM TRIS (pH 7.4), 0.15M NaCl, 0.5% Triton X-100, 1% NP-40, 0.01M sodium azide plus protease inhibitor}, vortexed and sonicated. A reducing sample buffer was added to 100ul of cleared cell lysate and the boiled samples were electrophoresed on a 7.5% SDS-PAGE gel. Immobilized proteins were transferred to an immobilon-P (Millipore, Billerica, MA) membrane and stained with amido black solution to verify equal loading of protein samples. After destaining, the membrane was blocked overnight in 5% non-fat milk in PBS at 4°C.

For immunoblot detection of myc-9a and V5-50, a mouse monoclonal antibody to the His epitope was used at a 1:100 dilution as recommended by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA). Anti-gM antibody was used for immunoblot detection of gM in virally infected cells. Chemiluminescent detection was accomplished using an HRP-tagged secondary antibody followed by detection with ECL-Plus reagents (Amersham Pharmacia Biotechnology, San Francisco, CA).

Ammonium sulfate precipitation of anti-gM antibody was performed using 3M ammonium sulfate. Equal volumes of serum and ammonium sulfate were incubated on ice for 60 minutes prior to centrifugation at 10,000 rpm for 15 minutes. Following three rounds of precipitation, the salt pellet was dialyzed against PBS for 3 days with buffer exchanges once a day.

Radioimmunoprecipitation of complexed proteins

The V5, myc, and anti-gM rabbit polyclonal antibodies were bound to protein G sepharose beads and cross-linked with disuccinimidyl suberate (DSS). Transiently

transfected and virally infected cells were radiolabelled with ^{35}S-containing media for 12 hours post infection. After 24 hours, cell lysates were prepared in gentle lysis buffer {10 mM sodium phosphate (pH 7.6), 5 mM EDTA, 0.5% Triton X-100, 0.25% sodium deoxycholate plus protease inhibitor} to preserve weak interactions of protein complexes. The antibody-protein G complex was gently agitated with the cell lysates overnight at 4°C. Protein bead complexes were washed with PBS to eliminate non-specifically bound proteins, and the complexed proteins were eluted from the beads using a low pH elution buffer (Pierce Chemicals, Rockford, IL). Following precipitation, samples were boiled and electrophoresed on a 12% SDS-PAGE gel. A higher percentage gel was used than in western blot experiments to optimally visualize low molecular mass proteins. The gel was fixed, dried, and exposed to film for five days.

RESULTS

VZV gM and gN bioinformatic analysis

VZV ORF50 is predicted to encode a type III transmembrane glycoprotein. Bioinformatic analysis of the gM amino acid sequence predicts a highly processed protein, with six potential membrane spanning domains, and a molecular mass of 48.68 kDa (Fig. 7). The predicted protein sequence contains two potential sites for N-linked glycosylation and several potential phosphorylation sites.

The ORF9a amino acid sequence is predicted to encode a small (87 aa) type 1 membrane protein with a molecular mass of 9.76 kDa (Fig. 8). The ORF9a gene product has an N-terminal signal sequence and one potential membrane spanning domain. The amino acid sequence of VZV gN does not show any consensus motifs (N-X-S/T) for N-linked glycosylation. There are several potential (S/T) sites for O-glycosidic linkage.

```
       on++  +o -+ o o-ooo- - -  -+n  -n o +     n    n o
H3N+-  MGTQKKGPRS EKVSPYDTTT PEVEALDHQM DTLNWRIWII QVMMFTLGAV
          o    o o-oo      o       -o-  n  o -     o  n+  oo
51     MLLATLIAAS SEYTGIPCFY AAVVDYELFN ATLDGGVWSG NRGGYSAPVL
        - +o      oooo  o          oo  o     ++-o+nn+  +noo
101    FLEPHSVVAF TYYTALTAMA MAVYTLITAA IIHRETKNQR VRQSSGVAWL
        - oo          o    n         o+n      o o  +   oo   ooo
151    VVDPTTLFWG LLSLWLLNAV VLLLAYKQIG VAATLYLGHF ATSVIFTTYF
        + + --on  +    n +nn o   o+      o+    n
201    CGRGKLDETN IKAVANLRQQ SVFLYRLAGP TRAVFVNLMA ALMAICILFV
       o   -     n + +o    oo  o   oo oo  o  o    o-       +o +
251    SLMLELVVAN HLHTGLWSSV SVAMSTFSTL SVVYLIVSEL ILAHYIHVLI
        o  o          o  o  +oo  -+ o-  o  no +    oo+  o      o
301    GPSLGTLVAC ATLGTAAHSY MDRLYDPISV QSPRLIPTTR GTLACLAVFS
           +   +  o o++n++o + o    ++    -+ + o ++  +  ++no++o
351    VVMLLLRLMR AYVYHRQKRS RFYGAVRRVP ERVRGYIRKV KPAHRNSRRT
       no on o o  o-n-ooo o- +---   o-+o no   -
401    NYPSQGYGYV YENDSTYETD REDELLYERS NSGWE -COO-
```

FIG. 7. Predicted protein sequence and structure of VZV gM. Chemical reactivity of side groups of each residue have been indicated as follows: acidic (D,E), + = basic K,R,H), o = hydroxyl (S,T,Y/uncharged polar), n = amide (N,Q/uncharged polar), no annotation = nonpolar (G,A,V,L,I,P,F,M,W,C). Predicted functional groups: N-glycosylation sites (2) 80-83 NATL, 413-416 NDST; PKC sites (6) 3-5 TQK, 10-12 SEK, 332-334 SPR, 338-340 TTR, 397-399 SRR, 419-421 TDR; CKII sites (6) 14-17 SPYD, 19-22 TTPE, 319-322 SYMD, 415-418, STYE, 419-422 TDRE, 432-435 SGWE; TKP sites (1) 421-427 REDELLY. Grey-shaded portions indicate potential membrane spanning regions.

```
       o o o     o n      - -oo - n  - +n  + o o  +
 1     MGSITASFIL ITMQILFFCE DSSGEPNFAE RNFWHASCSA
       o - o o        o   o o             o   o+    +  o+ o  +oo
51     RGVYIDGSMI TTLFFYASLL GVCVALISLA YHACFRLFTR SVLRSTW
```

FIG. 8. Predicted protein sequence and structure of VZV gN. Chemical reactivity as described above. Functional groups: PKC site 39-41 SAR, CKII site 22-25 SSGE. Note: there are no consensus sequences (NDT) for addition of N-glycans. Grey-shaded portions indicate potential membrane spanning regions.

Transfection with rVOka ΔgM and rVOka gM-repaired cosmids fail to produce infectious virus

The Arvin laboratory at Stanford University has recombinant cosmid clones spanning the genome of both the vaccine strain of the virus (rVOka) and the wildtype clinical strain from which the vaccine strain was derived (rPOka). The vaccine Oka strain is a tissue culture attenuated version of the parent Oka virus, however the nature of the attenuation is unknown (42).It is unlikely that the attenuation is in the gM or gN coding regions as sequence comparison of rVOka and rPOka gM and gN show no differences (data not shown). Recombinant VOka cosmids were selected for these experiments because rVOka deletion mutants have been more extensively tested *in vitro* and *in vivo* and published previously.

Three independent transfection experiments were performed using intact rVOka cosmids and rVOka cosmids with AvrΔgM ± Spe21gMrepair. Table 2 shows cosmid sets used in each transfection for the target virus. In all three experiments, transfection with the set of five intact rVOka cosmids yielded plaques at days 7-13. However, no virus was recovered from transfection experiments using cosmid sets aimed at recovery of gM deleted or gM repaired virus. Failure to generate mutant virus may indicate that VZV gM is an essential gene. However, the inability to rescue the virus by complementation at the exogenous *Avr*II site suggests that adventitious mutations may be present in the cosmid sets. Alternate theories are presented in the Discussion section.

TABLE 2. Transfection of rVOka cosmids

Target virus	Cosmid set tranfected
rVOka	pvFsp4, pvSpe5, pvAfl30, pvAvr10, pvSpe21
rVOkaΔgM	pvFsp4, pvSpe5, pvAfl30, pvAvrΔgM, pvSpe21
rVOka gM repair	pvFsp4, pvSpe5, pvAfl30, pvAvrΔgM, pvSpe21 gM repair

The rVOka virus recovered from the first transfection experiment was used in all subsequent experiments.

Recombinant VOka gM localizes to the plasma membrane of infected MeWo cells

Recombinant VOka virus obtained by co-transfection of intact VZV genome segments was used to infect MeWo cells seeded in chamber slides. Cells were fixed and permeabilized for antibody staining. Permeabilization of the infected cells is required because the anti-gM antibody recognizes the cytoplasmic C-terminal portion of the glycoprotein. Figure 9 shows VZV gM expression in rVOka infected MeWo cells. As predicted for a multiple membrane spanning protein, VZV gM was readily detected on the plasma membrane of VZV infected cells. VZV infected MeWo cells

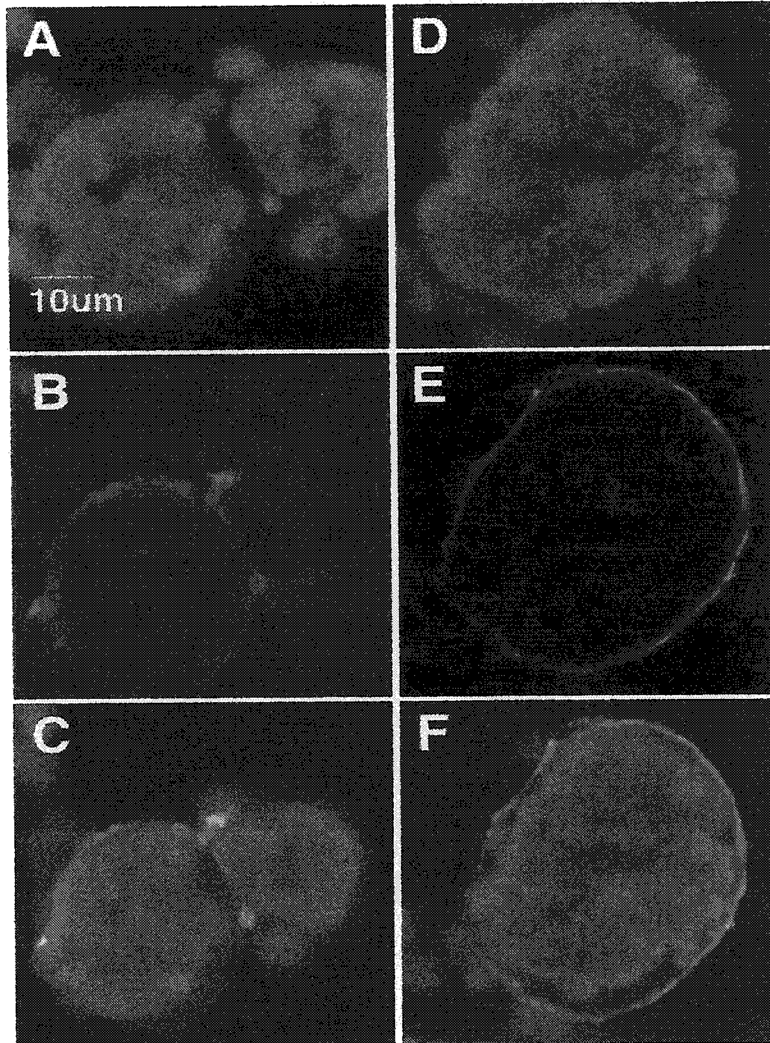

FIG. 9. VZV gM expression in rVOka infected MeWo cells. (A) DAPI stain of uninfected MeWo cells; (B) uninfected cells stained with rabbit pre-immune serum; (C) merged image of A and B. (D) DAPI stain of infected MeWo cells; (E) infected cells stained with rabbit anti-gM antibody; (F) merged image of D and E. Total magnification 200x.

form large, multinucleated syncytia. VZV gM was detected not only on the membrane of individually infected MeWo cells (data not shown) but along the entire syncytial membrane (Fig. 9E, 9F). No cytoplasmic staining was observed. Preimmune serum was used as a negative control and showed minimal background staining (Fig. 9B, 9C).

VZV gM localizes to the plasma membrane with VZV gE

VZV gE is the predominantly expressed protein in VZV infected cells. Anti-VZV, a human polyclonal antibody which primarily recognizes envelope glycoprotein E, was used to demonstrate co-localization of VZV gM with membrane glycoproteins (Fig. 10). VZV gE is expressed along the plasma membrane of infected cell syncytia (Fig. 10C). VZV gM is expressed, although to a lesser extent than gE, along the plasma membrane as well (Fig. 10B). The merged image (Fig. 10D) demonstrates co-localization of VZV gM and gE to the plasma membrane of virus-induced syncytia.

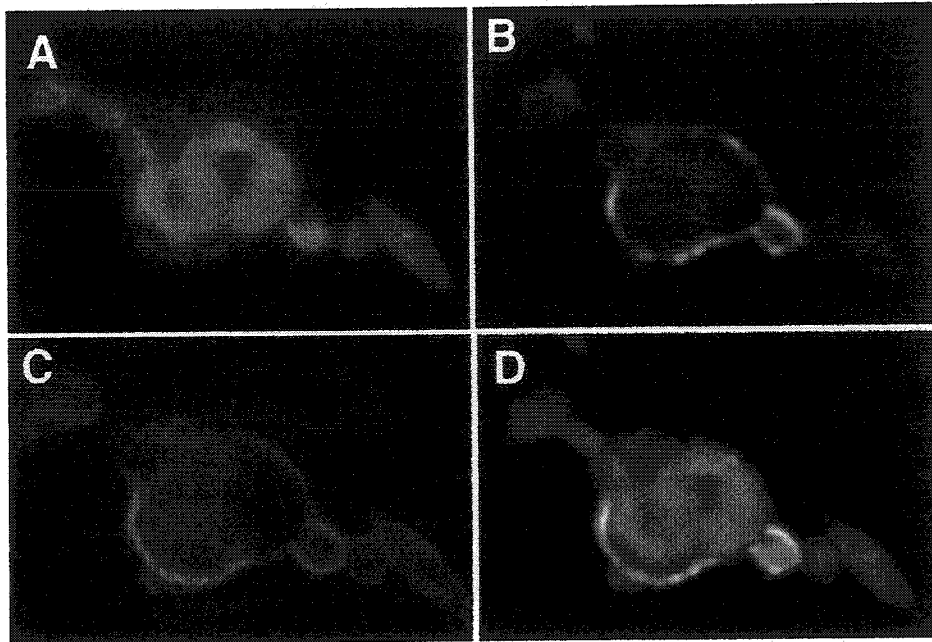

FIG. 10. Co-localization of VZV glycoproteins in infected MeWo cells. (A) DAPI stain infected cells; (B) rabbit anti gM antibody, FITC-conjugated secondary antibody; (C) VZV glycoproteins, predominately gE, stained with anti-human antibody, Texas Red conjugated secondary antibody; (D) merged image. Total magnification 200x.

Transiently expressed VZV gM and gN demonstrate distinct staining patterns; co-expression does not alter localization of either protein

VZV gM and gN were transiently expressed in cells to determine the intracellular localization of the glycoproteins and to see if the presence of one had an effect on the location of the other (Fig. 11). Transient transfection efficiency was 60-80% based on β-galactosidase activity in the pcDNA4/V5-His/lacZ control transfected MeWo and 293 cells. At 48 hours post transfection in MeWo cells, expression of myc-9a alone appeared limited to a perinuclear compartment (Fig. 11A). Expression of V5-50 alone was less restricted, with staining in a perinuclear compartment as well as some cytoplasmic staining (Fig. 11C). Co-transfection of myc-9a with V5-50 did not alter the restricted localization of myc-9a expression (Fig. 11B). Similarly, co-transfection of V5-50 with myc-9a did not alter V5-50 expression (Fig. 11D). Double staining for myc-9a and V5-50 was not possible because both commercial antibodies are mouse monoclonal antibodies. No differences were observed in localization using 293 cells instead of MeWo cells (data not shown).

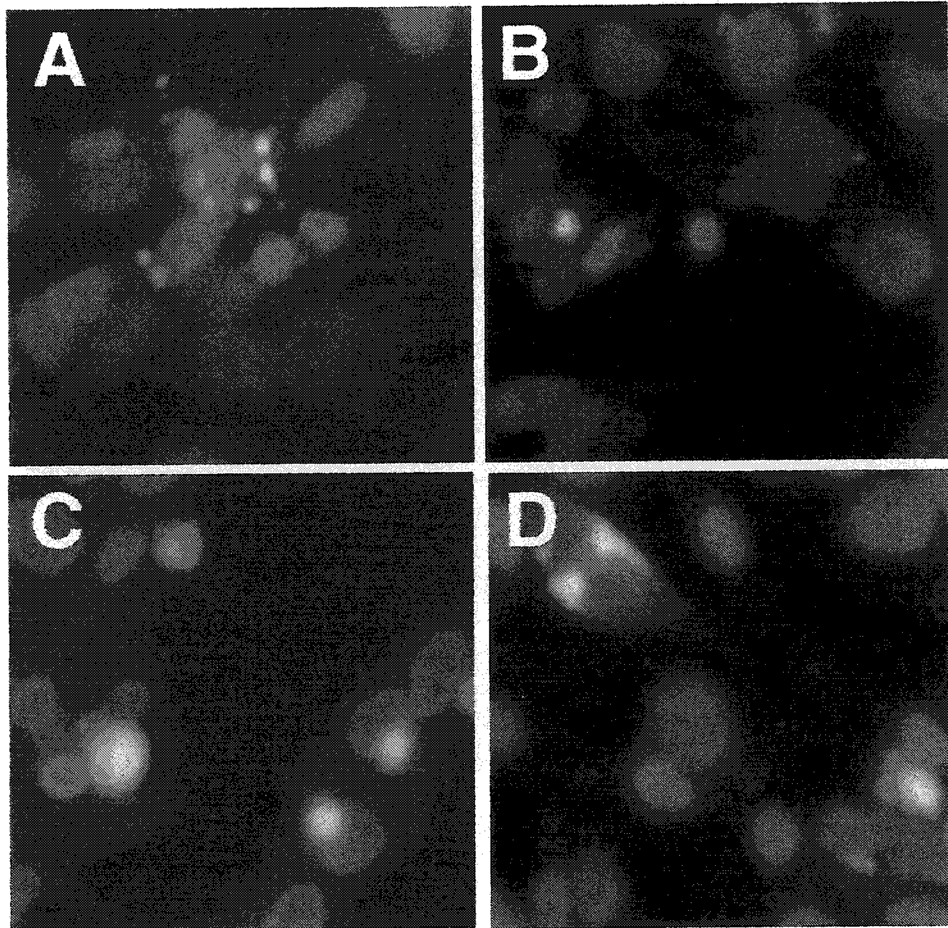

FIG. 11. Transient transfection of epitope tagged gM and gN. (A) myc-9a transfected alone, stained with DAPI and anti-myc; (B) myc-9a and V5-50 co-transfected, stained with DAPI and anti-V5; (C) V5-50 transfected alone, stained with DAPI and anti V5-50; (D) V5-50 and myc-9a co-transfected, stained with DAPI and anti-V5. All antibodies are mouse monoclonal antibodies. FITC-conjugated anti-mouse secondary antibody. Total magnification 200x.

A rabbit polyclonal antibody to VZV gM recognizes several protein species in rVOka infected cells

Western blot analysis was performed to determine the size of VZV gM and gN (Fig. 12). Western blot analysis followed SDS-PAGE separation of cell lysates from rVOka infected and uninfected MeWo cells as well as cells transiently transfected with the V5-50 and myc-9a plasmids.

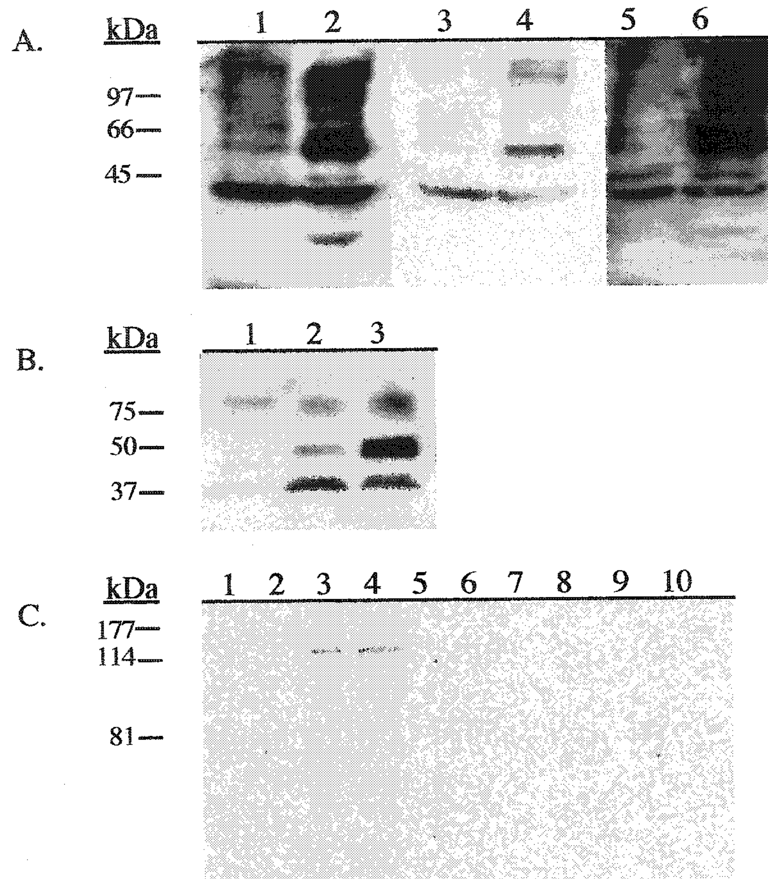

FIG. 12. Western blot detection of VZV gM in infected MeWo cells. Blot A. Lane 1, 3 & 5 uninfected cell lysate. Lane 2, 4 & 6 rVOka infected cell lysate. Lane 1&2, anti-gM bleed 1, 1:500; lane 3&4 anti-gM bleed 2, 1:100; lane 5&6 anti-gM bleed 2, 1:500. Blot B. Lane 1, uninfected cell lysate, lane 2&3 rVOKa infected cell lysate. Blot C. Lane 1&2, tranfection negative control (no dna); lane 3&4, tranfection positive control (his-tagged beta-galactosidase); lane 5&6, V5-50 tranfection, lane 7&8 V5-50/myc-9a co-transfection, lane 9&10, myc transfection. Anti-His antibody was used for immunoblot.

The anti-gM antibody recognized several protein species in infected MeWo cells (Fig. 12A). A wide band at ~45-60 kDa is consistent with the predicted size of VZV gM as a 48 kDa protein.

A smaller protein ~37 kDa protein was evident in the infected cell lysates, but also in the uninfected cell control. In addition to the 45-60 kDa band, several very large molecular mass species were detected in VZV-infected cells but not in uninfected cells. Two bleeds and two dilutions of the rabbit anti-gM antibody were tested, with equivalent results.

To minimize non-specific staining, the anti-gM antibody was subjected to ammonium sulfate precipitation and dialysis prior to retesting. Subsequent western blot analysis (Fig. 12B) did not eliminate the small or very large proteins but did demonstrate that the gM specific band at ~50 kDa appears to be a doublet.

Antibody to the His-tag on gM and gN constructs was reported by the manufacturer as able to recognize a linear epitope. However, western blot analysis of cells lysates from transiently transfected cells did not detect gM or gN. The anti-His antibody did recognize a large protein (>110 kDa) in the LacZ control transfected cells. This is consistent with the predicted size of a His-tagged β-galactosidase of ~120 kDa (Fig. 12C, lanes 3 and 4).

A small ~25,000 Da protein co-immunoprecipitates with ^{35}S-labelled VZV gM

Co-immunoprecipitation experiments were performed to determine if VZV gM complexes with any other viral or cellular proteins (Fig. 13). A radiolabelled approach was used because direct co-immunoprecipitation experiments using the anti-gM antibody lacked the required sensitivity.

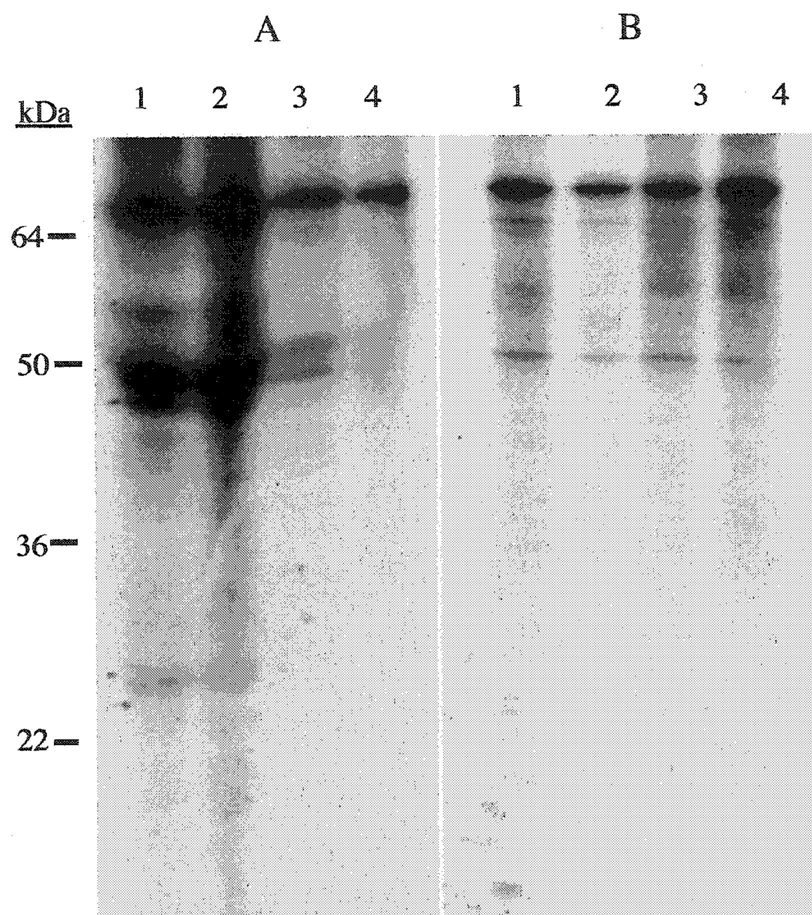

FIG. 13. Co-immunoprecipitation of ^{35}S-labelled gM and complexed proteins. (A) Lanes 1&2, immunoprecipitation of proteins from rVOka infected MeWo cells using rabbit anti-gM antibody; lanes 3&4 immunoprecipitation using pre-immune serum as a negative control. (B) immunoprecipitation from cells transiently tranfected with V5-50 (lane 1), myc-9a (lane 2), V5-50 and myc-9a co-transfected (lane 3&4). Protein was immunoprecipitated using anti-His antibody.

Anti-gM antibody was used to immunoprecipitate ^{35}S-labelled gM and any complex-bound proteins. Preimmune rabbit serum was used as a negative control. Radioimmunoprecipitation resulted in several protein species: a large non-specific ~70 kDa band (also present using the preimmune serum), a faint ~55kDa band, a ~48-50 kDa doublet, and a ~25 kDa band (Fig. 13A).

Two faint bands also appear in the preimmune serum lanes but they are not the same size as the bands in the anti-gM lanes. The large molecular mass species detected by western blot were not present. The ~25 kDa protein is much larger than expected for gN. The predicted size of VZV gN is only 9 kDa. Anti-His antibody was also used to immunoprecipitate ^{35}S-labelled gM and any complex-bound proteins in cells transiently transfected cells with the V5-50 and myc-9a expression vectors. The only evident band is non-specific as it appears in all four lanes (Fig. 13B, lane 2, 4 and 5).

DISCUSSION

This work provides the first report on VZV glycoprotein M. The results indicate that, like other alphaherpesvirus gM proteins, VZV gM is a highly processed membrane protein. While the interaction of VZV gM with gN is still unclear, these data suggest that membrane localization of gM is independent of gN expression. This work also addresses several important questions with regard to gM and gN, which are discussed below.

What is the structure and function of gM and gN? VZV gN shares structural and sequence homology with other gN species in that all are small proteins with an N-terminal signal sequence and a single membrane spanning domain. VZV gN has several potential sites for O-glycosidic linkage. HSV-1 and BHV-1 gN are not O-linked; whereas PRV gN is an O-linked glycoprotein (26, 37, 54). Interestingly, in HSV-1, BHV-1, and shown here for VZV, presence of gN has no effect on processing and localization of gM (1, 34). Lack of O-linked modification may result in a loss of function for gN and its role in gM maturation. Preliminary experiments with O-glycosidase treatment of VZV gN indicates that the protein is not O-linked (Hitoshi Sato, personal communication). Further investigation of VZV gN glycoprotein linkages may provide additional evidence for this theory.

In addition to the high identity of gM homologues (35-48%), all share common structural motifs. Conservation of these structural motifs indicate that gM homologues may serve analogous functions in viral pathogenesis. Although these functions are undefined, gene conservation across herpesviral subfamilies seems at a paradox to the dispensability of the protein.

Several groups have investigated the function of gM and gN in herpesviral pathogenesis. Kari and Gerhrz (1992) reported that HCMV gM has heparan binding potential and speculated a role for the glycoprotein in virion attachment (28). Dijkstra (1996) observed that gM deleted HSV-1 and PRV are slightly impaired for viral penetration and suggested a role for gM in viral entry (13). Osterrieder (1997) predicted that EHV-1 gM may have ion channel activity, however voltage-clamp analysis failed to support this hypothesis (45). More recently, a role as a fusion inhibitory complex has been explored. Using a cocktail of herpesvirus glycoproteins to induce fusion, Klupp (2000) observed a marked inhibitory effect on fusion in the presence of PRV gM alone, as well as the EHV gM-gN complex and the ILTV (avian infectious laryngotracheitis virus) gM-gN complex (29). The inhibitory effect was not limited to herpesvirus-induced fusion, as the same effect was observed with fusion mediated by the F protein of bovine respiratory syncytial virus. Klupp hypothesized that the highly hydrophobic regions on gM may alter membrane "fluidity" making it more difficult for membranes to fuse (29).

In a similar set of experiments, Koyano (2003) induced fusion using herpesvirus glycoproteins as well as the Molony murine leukemia virus envelope protein and reported that co-expression of HHV8 gM with gN (and not alone) can inhibit cell fusion (25). Koyano proposed two mechanisms through which the complex may act to inhibit fusion. The gM-gN complex may alter the distribution of viral fusogens on the cell membrane or inhibit the processing of other viral proteins. Similar experiments can be done using the V5-50 and myc-9a plasmids described in this thesis.

Is VZV gM an essential gene? Attempts to generate a gM-deleted virus using recombinant cosmids failed. While it is possible that VZV gM is an essential gene, the inability to produce virus using the gM rescue cosmid does not support this conclusion. An alternate theory may account for these results. The ORF50 gene encoding gM lies on the complementary strand from nucleotides (nt) 86575 to 87882. ORF51, which encodes for the DNA-replication origin binding protein, lies on the non-complementary strand from nt 87881 to 90388. The start codons for ORF50 and ORF51 are overlapping. Although the promoter for ORF51 has not been mapped, it is possible that the ORF50 deletion removes all or part of the ORF51 promoter and/or upstream activating sequences. A VZV ORF51 deletion mutant has not been attempted but, in HSV-1, the ORF51 homologue is essential. Disruption of VZV ORF51, through deletion of the promoter region, would account for the inability to produce rVOkaΔgM and rVOka gM-repair viruses.

Prior to this study, all gM homologues in herpesviruses had proven to be dispensable except HCMV gM (23). More recently, Tischer and colleages (2002) reported deletion of UL10 (gM) and UL49.5 (gN) genes in a bacterial artificial chromosome (BAC) of the Marek's disease type-1 (MDV) viral genome (53). When the ΔgM/ΔgN BAC was transfected into susceptible cells only single infected cells resulted. The virus was completely impaired for virus spread and plaque formation; and, the phenotype could be rescued by complementation. MDV, like VZV, is a predominately cell-associated virus (7). Also of note, MDV gE, like VZV gE, is an essential protein (53). This data suggests that in cell-associated viruses, proteins that function in cell-cell spread may play a more essential role than in viruses that can

readily release extracellular infectious particles. Based on the similar pathogenesis and composition of MDV and VZV, it is probable that VZV gM is an essential gene.

How is gM distributed in VZV-infected cells? In the transient expression experiments reported, VZV gM (± gN) is expressed in a perinuclear compartment, in the cytoplasm, and on the plasma membrane. In VZV infected MeWo cells, gM is expressed on the plasma membrane. Additionally, VZV gM co-localized with VZV gE, the predominant membrane glycoprotein.

In alphaherpesviruses, there is some evidence that gE and gM may serve partially analogous functions. Brack (1999) made a complete deletion of gM (UL10) in the Bartha strain of PRV and noted a significant impairment of plaque formation; whereas the same UL10 deletion in a wildtype PRV strain had minimal effect (5). Because the Bartha strain is known to lack gE and gI, and possess a defective gC, complementation studies were performed to see if the attentuation due to lack of gM was related to defects in other viral glycoproteins. Interestingly, complementing Bartha strain for gE and gI abrogated the defective growth phenotype (5). Similarly, Seyboldt (2000) deleted gM from the EHV-1 Kentucky A strain which lacks gE and gI coding regions and noted an 80% plaque size reduction (52). The original phenotype was restored by complementation of gE-gI. These results suggest that gE-gI and gM-gN may serve separate, yet partially redundant, functions. As demonstrated here by membrane co-localization, VZV gE and gM are found on infected cell membranes in close proximity to each other, however no direct interaction has been investigated.

Why is gM localization in infected cells different than in transiently transfected cells? Most cellular type III membrane proteins insert with an extracellular N-

terminus and a cytoplasmic C-terminus. Orientation and threading through the plasma membrane is sequence driven and can be affected by glycosylation (19). Inappropriate processing, glycosylation and topology of the transiently transfected protein may hamper epitope recognition on the plasma membrane. It is possible that transiently expressed gM does localize to the plasma membrane, but threading of the protein masks the epitope recognized by the V5 antibody. This explanation is unlikely for gN, which one has only one membrane spanning domain. It is also possible that membrane localization of VZV gM requires another viral protein.

Glycoprotein K is the only other type III membrane glycoprotein encoded by alphaherpesviruses (47). HSV-1 gK is expressed in the cytoplasm and cellular membranes of HSV-1 infected cells; however, transiently expressed V5-tagged HSV-1 gK failed to localize to plasma membranes (15).

What accounts for the perinuclear staining seen in transiently expressed gM and gN? Dal Monte (2001) observed that HCMV gN localizes to perinuclear granular formations and to the cell surface of infected cells (10). Perinuclear granular formations have been previously described as "cap-like structures" and "juxtanuclear structures" (10). The results in figure 11 show a similar staining pattern for both gM and gN. The perinuclear localization could indicate inappropriate processing or glycosylation of the proteins due to the addition of the epitope tag. Improper glycosylation may induce instability making the protein more susceptible to cytoplasmic proteasomes.

What accounts for the multiple bands detected by western blot and co-immunoprecipitation? Protein species detected by western blot included several high molecular mass bands. The HCMV gM-gN complex is reported to migrate as a 70-

100 kDa species (35). EBV gM (BBRF3) is detected as three species (48kDa, 84kDa and 113 kDa) (32, 33). Given the composition of the lysis buffer and presence of reducing agents, it is unlikely that these bands represent a VZV gM-gN complex. There are two explanations for the bands in figure 12. Heat denaturation in the presence of SDS can lead to protein aggregate formation in highly hydrophobic proteins like gM (36). Alternatively, the hydrophobic domains of gM could also contribute to the insolubility of a gM-gN complex or gM-gM dimer under these same conditions. Unfortunately, there is no antibody to VZV gN to test these protein species more rigorously.

^{35}S-labelled immunoprecipitation of gM and any complex-bound proteins resulted in several protein species; however, the large molecular mass species detected by western blot were not present. These large protein complexes or aggregates may have been lost due to the altered lysis conditions and several steps required for immunoprecipitation. The ~25 kDa protein is much larger that expected for gN, if it came from a gM-gN complex. Interestingly, HCMV gpUL73 (gN) is an 18kDa protein but when expressed with gM acquires complex modifications with a dramatic shift in molecular mass to 50-60 kDa (35). The predicted size of VZV gN is only 9 kDa. It is possible that the 25 kDa protein is a complex gN species, but specific antibody reactivity cannot be demonstrated without an anti-gN antibody.

Do VZV gM and gN interact? It is not possible to draw any conclusions from the data presented. It is possible that the ~25 kDa species detected by co-immunoprecipitation with gM in VZV-infected cells is gN; however, there is no direct evidence of antibody reactivity. In gM-gN cotransfected cells, Mach detected a 20 and

35 kDa species that was initially presumed to be gN, however showed no reactivity with gN antisera (35).

It is possible that gM interacts with other VZV or cellular proteins. Fuchs (2002) demonstrated using a yeast two-hybrid system that PRV gE and gM both interact with major tegument protein UL49 (the HSV-1 VP22 homologue) (17). HSV-1 VP22 is a multifunctional protein that, in addition to being a tegument protein, acts as an mRNA transporter (51). VP22 shuttles mRNA from infected to uninfected cells for expression prior to viral infection. HSV-1 VP22, when expressed alone, demonstrates a perinuclear localization similar to VZV gM and gN when transiently expressed (51). In the context of viral infection, VP22 can be detected on the plasma membrane as well (6). VZV UL9 (the VP22 homologue) is predicted to encode a 303 amino acid protein with an approximate molecular mass of 33.3 kDa. VP22 homologues in alphaherpesviruses are non-essential except in MDV and VZV (48, 50). Both PRV gE and gM interact between the cytoplasmic domain of UL49 and the cytoplasmic tail of gE and gM (17). Fuchs (2002) proposes that gE and gM function in secondary envelopment and incorporation of VP22 into capsids (17). A similar approach, using a mammalian two-hybrid system, is in progress to determine if VZV gM and gE interact with VZV UL9 and if gM interacts with gN.

Concluding remarks. Future efforts to describe gM, gN and any complex formation will include experiments to examine glycosylation and oligomeric status using a cocktail of exoglycosidases like Endo H, and Endo F/N-glycanase. Generation of antiserum to gN may provide a tool to verify the identity of the low molecular mass protein in the co-immunoprecipitation experiments.

Glycoprotein M and N will continue to be of great interest. The paradox of conservation of non-essential genes across all herpesvirus families makes glycoprotein M and N important proteins to examine.

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