Virology 429 (2012) 21–28

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Human herpesvirus 6 glycoprotein M is essential for virus growth and requires glycoprotein N for its maturation

Akiko Kawabata [a,b], Chyntia Jasirwan [a], Koichi Yamanishi [c], Yasuko Mori [a,b,*]

[a] Division of Clinical Virology, Kobe University Graduate School of Medicine, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan
[b] Laboratory of Virology and Vaccinology, Department of Fundamental Research, National Institute of Biomedical Innovation, 7-6-8, Saito-Asagi, Ibaraki, Osaka 567-0085, Japan
[c] National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan

ARTICLE INFO

Article history:
Received 7 February 2012
Returned to author for revisions
15 March 2012
Accepted 29 March 2012
Available online 24 April 2012

Keywords:
HHV-6
gM
gN
Glycoprotein

ABSTRACT

Human herpesvirus 6 (HHV-6) is a T-lymphotropic virus belonging to the betaherpesvirus family. Several HHV-6-encoded glycoproteins are required for cell entry and virion maturation. Glycoprotein M (gM) is conserved among all herpesviruses, and therefore thought to have important functions; however, the HHV-6 g has not been characterized. Here, we examined the expression of HHV-6 g, and examined its function in viral replication, using a mutant and revertant gM. HHV-6 g was expressed on virions as a glycoprotein modified with complex N-linked oligosaccharides. As in other herpesviruses, HHV-6 g formed a complex with glycoprotein N (gN), and was transported from the endoplasmic reticulum to the *trans*-Golgi network only when part of this complex. Finally, a gM mutant virus in which the gM start codon was destroyed was not reconstituted, although its revertant was, indicating that HHV-6 g is essential for virus production, unlike the gM of alphaherpesviruses.

Introduction

Human herpesvirus 6 (HHV-6) is a betaherpesvirus related to human herpesvirus 7 (HHV-7) and human cytomegalovirus (HCMV) and is a human pathogen of emerging clinical significance. HHV-6 was first isolated from the peripheral blood lymphocytes of patients with lymphoproliferative disorders and acquired immunodeficiency syndrome (Salahuddin et al., 1986). HHV-6 is categorized as two variants, A (HHV-6A) and B (HHV-6B), on the basis of its in vitro growth properties, DNA restriction site polymorphisms, antigenicity, and host cell tropism (Ablashi et al., 1991; Aubin et al., 1991; Campadelli-Fiume et al., 1993; Chandran et al., 1992). HHV-6B is the causative agent of exanthem subitum (Yamanishi et al., 1988), but the role of HHV-6A in human disease is less clear.

Herpesviruses encode a number of glycoproteins that are present in the envelope of the virion and play important roles in viral infection, including attachment, penetration, cell-to-cell spread, envelopment, and the maturation of nascent viral particles. The genomes of herpesviruses contain a number of genes that are conserved throughout the family *Herpesviridae*, including those encoding glycoprotein (g) B, gH, gL, gM, and gN. Although these genes are conserved, the corresponding proteins appear to have different functional roles in the replication of herpesviruses from different subgroups. For example, gM is reported to be non-essential for the replication of alphaherpesviruses, including herpes simplex virus type-1 (HSV-1) (Baines and Roizman, 1991; MacLean et al., 1993), pseudorabies virus (PRV) (Dijkstra et al., 1996), bovine herpesvirus type-1 (Konig et al., 2002), varicella-zoster virus (VZV) (Yamagishi et al., 2008), infectious laryngotracheitis virus (Fuchs and Mettenleiter, 1999), equine herpesvirus type-1 (EHV-1) (Osterrieder et al., 1997), and EHV-4 (Ziegler et al., 2005). However, the gMs of HCMV, murine gammaherpesvirus 68 and Marek's disease virus are essential for the production of infectious virus (Hobom et al., 2000; May et al., 2005; Tischer et al., 2002).

The gM protein has multiple transmembrane domains and N-linked glycosylation sites, forms a disulfide-linked complex with gN (Jons et al., 1998; Koyano et al., 2003; Mach et al., 2000; Wu et al., 1998), and functions mainly in virion assembly and egress (Brack et al., 1999; Krzyzaniak et al., 2007; Lake and Hutt-Fletcher, 2000; Mettenleiter et al., 2009; Rudolph and Osterrieder, 2002). Consistent with these roles, co-transfected gM and gN colocalize in the *trans*-Golgi network (TGN) and endosomal compartment (Mach et al., 2000; May et al., 2005; Mettenleiter et al., 2009), where herpesvirus secondary envelopment occurs (Mettenleiter et al., 2009). The formation of the gM–gN complex is required for gM's transport from the endoplasmic reticulum (ER) to the TGN and endosomal compartment (Krzyzaniak et al., 2007; Mach et al., 2000). Recently, antibodies against the gM–gN complex were shown to have a neutralizing function in HCMV infection (Shimamura et al., 2006). These

* Corresponding author at: Division of Clinical Virology, Kobe University Graduate School of Medicine, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. Fax: +81 78 382 6879.
E-mail addresses: ymori@med.kobe-u.ac.jp, ymori@nibio.go.jp (Y. Mori).

0042-6822/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2012.03.027

findings suggest that the gM–gN complex is also involved in the virus entry into host cells.

The gM cytoplasmic tail contains several trafficking motifs that are conserved in alpha-, beta-, and gamma-herpesviruses have important functions. These include YXXΦ motifs, which have been linked to AP2 binding (Owen and Evans, 1998), and an acidic cluster, which may be involved in TGN targeting (Voorhees et al., 1995). Removal of a YXXΦ motif in the cytoplasmic tail of the MuHV-4 gM prevents the virus's productive replication (May et al., 2008). The replication of HCMV is also blocked by the deletion or mutation of the gM carboxy-terminal cytoplasmic tail sequence (Krzyzaniak et al., 2007). In addition, the HCMV gM cytoplasmic tail was shown to interact with a cellular component, the Rab11 effector protein FIP4 (family of interacting protein 4) (Krzyzaniak et al., 2009). The HSV-1 gM may play similar roles in viral replication, because both gD and gH/gL are efficiently internalized and targeted to intracellular compartments when co-expressed with gM (Crump et al., 2004), and gM is important for the assembly of infectious HSV-1, due to its ability to control the localization of gH/gL (Ren et al., 2011). However, the detailed functions of HHV-6 g during virus infection are still unknown.

HHV-6 gM is a product of the U72 ORF, and composed of 343 amino acids. It is predicted to be a type III glycoprotein and is composed with seven membrane-spanning domains. The gM of HSV-1 and VZV is reported to colocalize with a marker for the Golgi apparatus and to partially colocalize with a marker for the TGN in infected cells (Baines et al., 2007; Yamagishi et al., 2008). We found that HHV-6A gM is expressed on the TGN, late endosomes, and multivesicular bodies (MVBs) in infected cells (Mori et al., 2008), suggesting that HHV-6 gM may be involved in virus assembly, virion maturation, and egress.

We report here that HHV-6 gM was incorporated into mature virus particles with complex N-linked oligosaccharides and that it associated with a product of the U46 ORF, the herpesvirus gN homolog, and that this interaction was critical for gM's trafficking. Furthermore, we found that gM was essential for virus production, in contrast to the gM of many alphaherpesviruses.

Results

Characterization of HHV-6A gM in infected cells and purified virions

To analyze the expression of HHV-6A gM in infected cells, GS-infected cells were harvested at 96 h postinfection, lysed, and subjected to western blotting with an anti-gM Mab. In infected cells, the gM was detected as a multiple-band smear extending from approximately 36–52 kDa, below which were two well-demarcated single bands, at 15 and 24 kDa (Fig. 2).

Purified virions were also subjected to western blotting with the anti-gM Mab. Interestingly, in the purified virions, gM was detected as broad multiple bands from 34 to 75 kDa, but the smaller bands of 15–24 kDa, found in the infected cells, were not detected. These results showed that HHV-6A gM, like the gM of other herpesviruses, is a component of the virions.

HHV-6A gM was predicted to be a type III membrane protein that possesses seven transmembrane domains and has two potential N-linked glycosylation sites at the putative second extracellular loop (Fig. 1; TMHMM prediction server; http://www.cbs.dtu.dk/services/TMHMM/). Given the broad multiple bands detected in both infected cells and virions, as described above, we examined gM's glycosylation state in HHV-6A-infected cells and in purified virions, by treating the lysates with two kinds of endoglycosidase, endo H and PNGase F. The digested proteins were analyzed by western blotting with an anti-gM Ab. The gM in both infected cells and virions was resistant to endo H treatment,

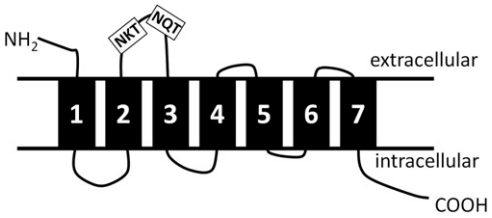

Fig. 1. Predicted topology of HHV-6A gM. The topology of HHV-6A gM was predicted using the TMHMM transmembrane topology prediction server (http://www.cbs.dtu.dk/services/TMHMM/). The approximate location of two N-linked glycosylation sites (NKT, asparagine-lysine-threonine; NQT, asparagine-glutamine-threonine) is indicated.

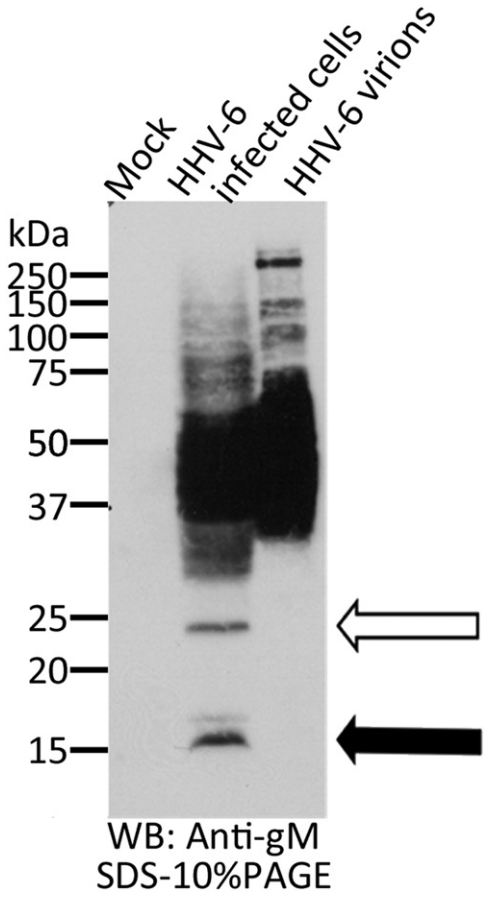

Fig. 2. Expression of gM in HHV-6A-infected cells and virions. HHV-6A-infected or mock-infected HSB-2 cells were harvested at 96 h postinfection. The lysates of HHV-6A-infected or mock-infected HSB-2 cells, or of purified virions were analyzed by western blotting. The blots were reacted with an anti-gM Mab. The cells were harvested at 96 h postinfection and lysed. The numbers beside the panels show molecular masses. White arrow indicates the 24 kDa gM. Black arrow indicates the 15 kDa gM. WB, Western blotting.

and showed no shift in its electrophoretic mobility (Fig. 3(a). After PNGase F digestion, however, the broad bands of gM were shifted to approximately 30 kDa in both infected cells and virions (Fig. 3(b)), but the smaller bands found in the infected cells were not changed. These results indicated that HHV-6A gM was glycosylated with complex N-linked oligosaccharides and incorporated into virions as an envelope glycoprotein.

To confirm that HHV-6A gM behaves as a late protein, the timing of its presence in infected cells was examined by PFA treatment (Fig. 4). GS-infected HSB-2 cells were maintained for 48 h in culture medium with or without PFA. As shown in Fig. 4, neither gM nor the late protein gQ1 was detected in cells treated

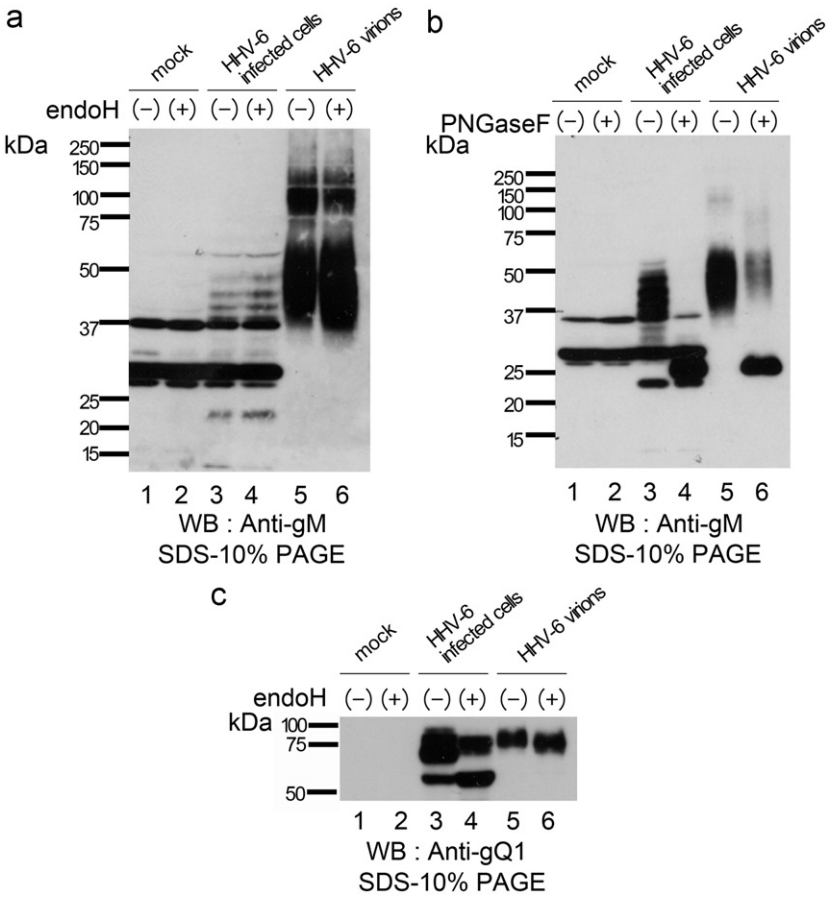

Fig. 3. Glycosidase digestion patterns of HHV-6A-infected HSB-2 cells and purified virions. The lysates were digested with endo H (a) or PNGase F (b) and analyzed by western blotting. The blots were reacted with an anti-gM Ab or anti- gQ1 (AgQ1-119) (c) as positive control of endo H digestion. The cells were lysed with TNE buffer at 96 h postinfection. The numbers beside the panels show molecular masses. WB, Western blotting.

with PFA, while the immediate-early protein IE1 and the early protein U27 were detected in the PFA-treated cells in both infected cells and virions. These results indicate that gM belongs to the late class of HHV-6A proteins.

Complex formation of HHV-6A gM and gN

Generally, gM has been reported to form a complex with gN (Jons et al., 1998; Mach et al., 2000, 2005). Therefore, to examine if HHV-6 g also binds gN, 293T cells were cotransfected with HA-AgM and AgN-FLAG (Fig. 5(A)). The cells were harvested at 72 h post transfection, lysed, and subjected to western blotting with an anti-gM or anti-FLAG Ab. When 293T cells were transfected with a plasmid expressing gM alone, gM was detected as broad multiple bands of 47–63 kDa and 28–35 kDa (lane 2 of upper panel in Fig. 5(B-a)). However, when 293T cells were cotransfected with plasmids expressing gM and gN, the size distribution of the multiple gM bands changed (lane 1 of upper panel in Fig. 5(B-a)). These results indicate that HHV-6A gM was modified in the presence of gN. In contrast, HHV-6A gN was detected as a 13 kDa band regardless of gM expression (lower panel in Fig. 5(B-a)).

The lysates from cells co-expressing HA-AgM and AgN-FLAG were then immunoprecipitated with an anti-HA Ab for gM or anti-FLAG Ab for gN, followed by western blotting with an anti-gM Ab or anti-FLAG Ab (Fig. 5(B-b)). When gM or gN was expressed alone, the anti-HA Ab precipitated only gM (lane 2 in Fig. 5(B-b)) and the anti-FLAG Ab precipitated only gN (lane 4 in Fig. 5(B-b)), as expected. However, in the lysates of gM- and gN-coexpressing cells, the anti-HA Ab precipitated both gM (lane 1 of upper panel in Fig. 5(B-b)) and gN (lane 1 of lower panel in Fig. 5(B-b)), and the anti-FLAG Ab also precipitated both gM (lane 3 of upper panel in Fig. 5(B-b)) and gN (lane 3 of lower panel in Fig. 5(B-b)). These results indicated that the HHV-6A gM and gN formed a complex in the absence of any other viral factors, and that gN was required for the efficient modifications of gM.

Transportation of HHV-6A gM and gN

To confirm that gM formed a complex with gN and to define the intracellular compartment in which this association occurred, we transiently expressed gM and gN or gM alone in 293T cells, followed by an IFA with antibodies directed against the viral protein, epitope tag, or markers of the secretary pathway. 293T cells were cotransfected with HA-AgM and AgN-FLAG or transfected with HA-AgM alone, and the IFA was performed at 72 h post transfection (Figs. 6 and 7). When HHV-6A gM was expressed alone, it partially colocalized with calnexin, a marker of the ER (Fig. 6(B-a)), but not with TGN46 (Fig. 6(B-b)). In contrast, when the expression plasmids for gM and gN were cotransfected into 293T cells (Fig. 7), gM colocalized with gN at the TGN. These results indicated that the formation of a complex of HHV-6A gM and gN was required for the efficient transport of gM to the distal compartments of the secretary pathway.

Generation of gM mutant virus and its revertant

It was previously shown using BAC mutagenesis that gM is essential for the in vitro replication of HCMV (Krzyzaniak et al.,

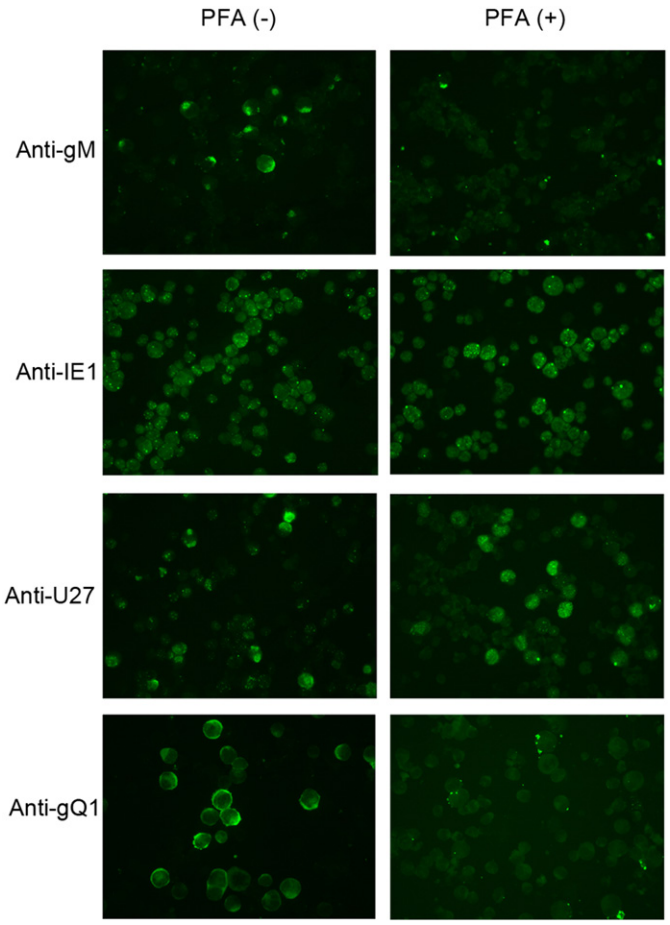

Fig. 4. IFA of HHV-6A-infected HSB-2 cells with PFA treatment. HSB-2 cells were infected with HHV-6A in the presence (+) or absence (−) of PFA. The cells were harvested at 48 h postinfection and stained with anti-gM, -IE1, -U27, and -gQ1 antibodies.

2007). Recently, we constructed the HHV-6 BAC and successfully reconstituted the infectious virus (Tang et al., 2010). Therefore, using the established HHV-6 BAC mutagenesis, we examined whether HHV-6 gM was essential for virus production. We constructed a gM mutant using established methods for BAC mutagenesis, HHV-6ABACgMM1L in which the start codon of gM was mutated, and its revertant, HHV-6ABACgMM1Lrev, and confirmed the mutation by sequencing. We then attempted to reconstitute infectious virus using these BACs. Infectious viruses could be reconstituted from HHV-6ABAC and HHV-6ABACgMM1Lrev, but not from HHV-6ABACgMM1L. We then confirmed the expression of gM and gQ1 by western blotting using anti-gM antisera and an anti-gQ1 Mab. As shown in Fig. 8, gM and gQ1 were detected in the HHV-6ABAC and HHV-6ABACgMM1Lrev reconstituted cell lysates, but not in those of HHV-6ABACgMM1L. These results indicate that the HHV-6 g is essential for virus growth.

Discussion

In this study, we found that HHV-6A ORF U72 and U46 encode the gM and gN homologs, respectively, which bind to each other to form a complex. These results were consistent with previous descriptions of the gM–gN complex in alpha-, beta- and gamma-herpesviruses, indicating that the gM–gN protein complex is conserved throughout the family *Herpesviridae*.

We found that gM was modified with complex N-linked oligosaccharides in HHV-6-infected cells, and that its full glycosylation required its expression with gN. Unfortunately, because we could not produce an antibody to HHV-6 gN, the expression of gN and its interaction with gM were not confirmed in HHV-6-infected cells or virions.

Here gM was found to be a virion component that was detected on western blots as multiple bands in virions and infected cells. Interestingly, 24 kDa and 15 kDa forms of gM were also detected in infected cells, but not in purified virions. These bands probably represent the carboxyl terminus of gM, because the antibodies used here recognize its C-terminal end. Although the functions of these two proteins are unknown, they are unlikely to be important after viral budding, since they are not present in virions. Because these bands have not been detected for the gM of other herpesviruses (Koyano et al., 2003; Mach et al., 2000; Osterrieder et al., 1997; Yamagishi et al., 2008), the 24 kDa and 15 kDa gM proteins may have a specific function for HHV-6. As shown in Fig. 3(b), the multiple bands of gM showed a shift in their electrophoretic mobility to approximately 27 kDa after PNGase F treatment in infected cells and virions. When infected cell lysates were digested with PNGase F, the gM bands were shifted to sharp band, probably representing the gradual addition of glycans to gM and possibly reflecting gM's formation of a multimeric complex.

When gM was transiently expressed alone, it was retained in the ER (Fig. 6), whereas its coexpression with gN caused gM to be transported to more distal sites in the secretory pathway (Fig. 7), as shown for other herpesviruses (Mach et al., 2000). As shown Fig. 5(B), the multiple bands of 37–50 kDa and the 15 kDa band of gM were detected when it was coexpressed with gN, however they were absent when gM was expressed alone, and the expression of the 24 kDa band decreased, indicating that the interaction with gN is important for gM's processing.

It had been unknown whether HHV-6 gM was needed for infection. Therefore, we constructed a gM point mutant virus that does not express gM. Our data showed that without the expression of gM, no infectious virus could be reconstituted from the HHV-6ABAC genome, although the revertant virus could be, indicating that gM was essential for HHV-6 propagation (Fig. 8).

Further analyses of HHV-6 g will be required to elucidate its roles in infected cells and virions.

Materials and methods

Cells and viruses

T-cell lines (HSB-2 and J JHAN cells) were cultured in RPMI-1640 medium supplemented with 8% fetal bovine serum. Human embryonic kidney cells (293T cells) were cultured in Dulbecco's modified Eagle's medium supplemented with 8% fetal bovine serum. Umbilical cord blood mononuclear cells (CBMCs) were prepared as described previously (Mori et al., 2003).

The HHV-6A strain GS was propagated and titrated in HSB-2 cells. HHV-6A cell-free virus was prepared as described elsewhere (Akkapaiboon et al., 2004). Virions were purified as described previously (Mori et al., 2008). Briefly, supernatants from infected cells were used for virion preparation and virions were separated by 5–50% Histdenz (Sigma) gradient. The virions-containing fractions were determined by polymerase chain reaction (PCR) of viral DNA and western blot of virion proteins. Phosphonoformic acid (PFA), which inhibits viral DNA synthesis, was used to determine whether gM is an early or late protein. HSB-2 cells were infected with HHV-6A, cultured in medium with PFA (300 μg/ml), and harvested at 48 h postinfection.

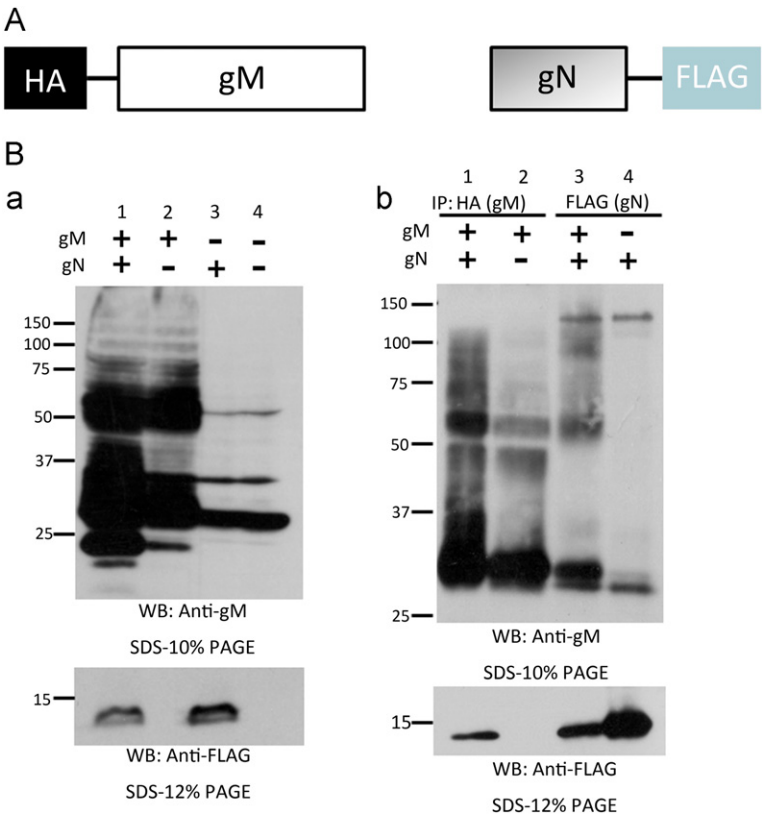

Fig. 5. Detection of the gM–gN complex in transfected 293T cells. (A) Schematic representations of HA-tagged gM and FLAG-tagged gN. (B) (a) 293T cells were transfected with plasmids expressing HA-AgM, AgN-FLAG, or with pCAGGS alone as a negative control. The cell lysates were analyzed by western blotting. The blots were reacted with anti-gM antiserum or an anti-FLAG Ab for gN. The cells were harvested at 72 h post transfection and lysed. (b) The cells were lysed with TNE buffer at 72 h post transfection. The lysates were immunoprecipitated (IP) with an anti-HA Ab for gM or anti-FLAG Ab for gN and analyzed by western blotting. The blots were reacted with the anti-gM antiserum or anti-FLAG Ab. The numbers beside the panels show molecular masses. WB, western blotting.

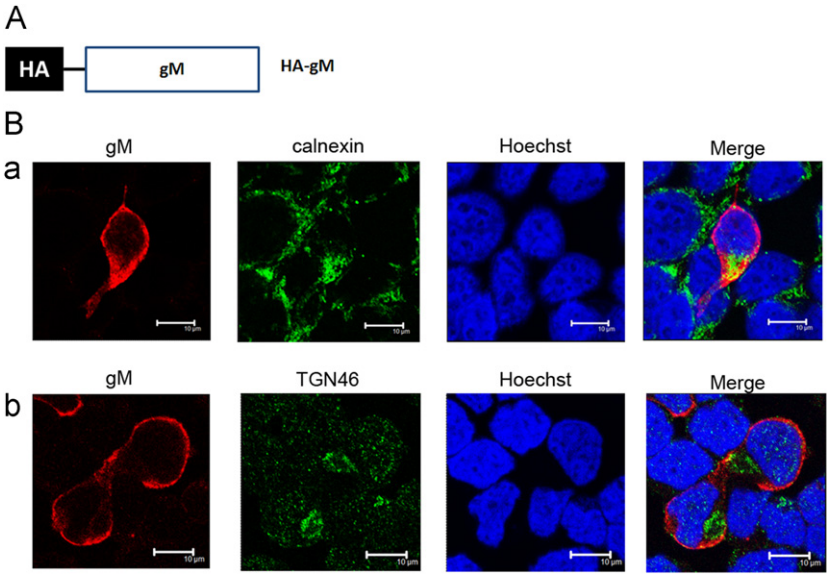

Fig. 6. Subcellular localization of gM in transfected 293T cells. (A) Schematic representation of HA-tagged gM. (B) 293T cells were transfected with HA-AgM. The cells were harvested at 72 h posttransfection, then fixed and stained for IFA with antibodies against gM, calnexin (a), or TGN46 (b). Costained areas appear yellow in the merged panel. Scale bars: 10 μm.

Antibodies

The rabbit monospecific antibodies (Abs) against HHV-6 gM were described previously (Mori et al., 2008), and the anti-HHV-6 gM monoclonal antibody (Mab), named AgM-1, was produced in our laboratory. Hybridoma clones producing Mabs against HHV-6 gM were produced by three immunizations of mouse footpads. The gM–gN complex coexpressed in 293T cells was used as an antigen for immunization, and the detailed purification method is described below. The Mab AIE1 against HHV-6A immediate early-1 protein

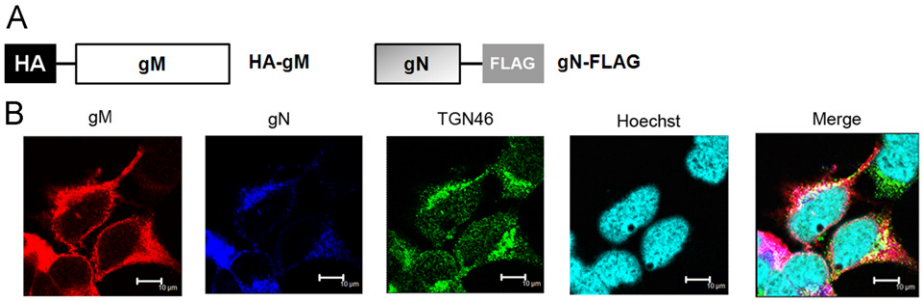

Fig. 7. Subcellular localization of gM with gN in transfected 293T cells. (A) Schematic representations of HA-tagged gM and FLAG-tagged gN and (B) 293T cells were transfected with pCAGGS-HA-AgM and pCAGGS-AgN-FLAG. The cells were harvested at 72 h posttransfection and then fixed and stained for IFA with antibodies against gM, FLAG for gN, and TGN46. Costained areas appear white in the merged panel. Scale bars: 10 μm.

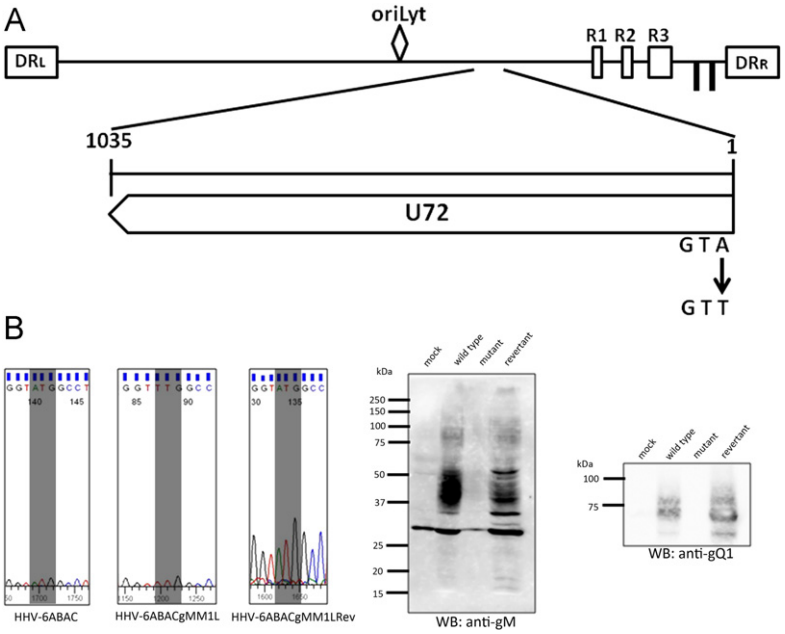

Fig. 8. gM is essential for HHV-6 growth. (A) Schematic representation of the HHV-6A genome and the HHV-6A gM gene region. The HHV-6 genome consists of three major internal repeat elements (R1, R2, and R3), the origin of replication (oriLyt), and the direct repeats (DR_L and DR_R). White box indicates the predicted open reading frame (ORF) encoding gM, numbered from the start (position 1) to the stop (position 1035) codon. The position of the mutated start codon within gM (ATG mutated to TTG) is indicated at right. (B) Mock- or reconstituted virus-infected CBMC lysates from HHV-6A BAC genomes (HHV-6ABAC [wild type], HHV-6ABACgMM1L [mutant], and HHV-6ABACgMM1Lrev [revertant]) were subjected to SDS-PAGE, followed by western blotting (WB), and probed with anti-gQ1 or -gM antibodies. Numbers beside the panels show molecular masses in kilodaltons.

(IE-1) and Mab AgQ1-119 against HHV-6A gQ1 were described previously (Huang et al., 2006; Mori et al., 2003). The Mab U27 A42-1 against the HHV-6 early protein U27 was produced as described previously (Okuno et al., 1992). The Mab against calnexin (clone: AF18; Abcam), and sheep polyclonal Ab, TGN46 (AbD Serotec) were used as cellular markers. Anti-HA Ab (clone: HA-7; Sigma) and anti-FLAG Ab (clone: M2; Sigma) were also used. Alexa Fluor 488-conjugated F(ab')2 fragment of donkey anti-mouse IgG, Alexa Fluor 488-conjugated donkey anti-sheep IgG, Alexa Fluor 594-conjugated donkey anti-rabbit IgG (Molecular Probes), and Cy5-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc) were used as secondary antibodies.

Immunofluorescence assay

The indirect immunofluorescence assay (IFA) was performed as described previously (Akkapaiboon et al., 2004). Specific immunofluorescence was observed with a confocal laser-scanning microscope, Leica DMIRE2 (Leica Microsystems).

Western blotting

Western blotting was performed as described previously (Akkapaiboon et al., 2004).

Glycosidase digestion

For endoglycosidase digestion, endoglycosidase H (endo H) and peptide *N*-glycosidase (PNGase F) were purchased from New England Biolabs. The lysates were resuspended in digestion buffer and digested with endo H or PNGase F as specified by the manufacturer.

Plasmid construction

To generate an expression plasmid for N-terminal HA-tagged HHV-6A gM, primers AgMHAecoRIF and AgMecorIR (shown in Table 1) were used to amplify inserts from HHV-6A (strain GS) cDNA. The finished construct, named HA-AgM, was made by inserting the PCR products into pCAGGS (Niwa et al., 1991) at

Table 1
Primer sequence.

Primer name	Sequence
AgMHAEcoRIF	5′-accgaattccacagagctaccatgtacccatacgatgttccagattacgctgcctccagt-3′
AgMecorIR	5′-accgaattcctaaagtgtcactcgttttcat-3′
AgNFMluI	5′-accacgcgtaaataacgcttaaatgagttg-3′
AgNfragRxhoI	5′-accctcgagttacttgtcgtcatcgtctttgtagtcataacccttcaccgtctctgaag-3′
AgMFKan	5′-gagtatcgttctgaaacctgttctccatcacagagctggtttggcctccagtcgtgtagaagcatgacgacgataagtagggataac-3′
AgMRKan	5′-gatccgtaaattaatggtgtctacacgactggaggccaaaccagctctgtgatggagaacaaccaattaaccaattctga-3′
AgMKanRevF	5′-atcgttctgaaacctgttctccatcacagagctggtatggcctccagtcgtgtagaaggatgacgacgataagtaggg-3′
AgMKanRevR	5′-agatccgtaaattaatggtgtctacacgactggaggccataccagctctgtgatggagaacaaccaattaaccaattctga-3′

the *EcoR*I restriction site. To generate a plasmid expressing C-terminal FLAG-tagged HHV-6A gN, primers AgNfragRxhoI and AgNFMluI (shown in Table 1) were used to amplify inserts from HHV-6A (strain GS) cDNA. The completed construct, named AgN-FLAG, was made by inserting the PCR products into pCAGGS at the *Xho*I and *Mlu*I restriction sites.

Plasmid transfection

293T cells were transfected with expression plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Immunoprecipitation

293T cells cotransfected with plasmids HA-AgM and AgN-FLAG were lysed in TNE buffer (0.01 M Tris-HCl [pH 7.4], 0.15 M NaCl, 1 mM EDTA, 1% Nonidet-P-40) for 30 min on ice. After centrifugation at 200,000 × g for 1 h, the supernatants were incubated with anti-HA Ab- or anti-FLAG Ab-protein G-Sepharose (GE Healthcare Biosciences) complex at 4 °C for 4 h. The immunocomplexes were washed with TNE buffer to remove unbound proteins. The precipitated proteins were solubilized with sample buffer (32 mM Tris-HCl [pH 6.8], 1.5% SDS, 5% glycerol, 2.5% 2-mercapto-ethanol) and separated by SDS-PAGE. The proteins in the precipitates were detected by western blotting as described above.

Construction of the gM mutant and its revertant

To generate the recombinant virus with a point mutation in the start codon for gM, we used a two-step Red-mediated mutagenesis procedure (Adler et al., 2000; Kato et al., 2008; Tischer et al., 2006) in an *E. coli* GS1783 strain that harbors the HHV-6ABAC genome (Tang et al., 2010). The primer pairs AgMFKan and AgMRKan used for the amplification are shown in Table 1. The recombination using two-step Red-mediated mutagenesis was performed as described previously (Tang et al., 2011; Tang et al., 2010). We named the resultant BAC, HHV-6ABACgMM1L.

To generate the gM revertant, in which the start codon of gM was repaired, the same procedure used to generate HHV-6ABACgMM1L was performed, except that GS1783 harboring HHV-6ABACgMM1L, and the primers AgMKanRevF and AgMKanRevR, as shown in Table 1, were used.

The method for generating HHV-6A recombinant viruses was described previously (Tang et al., 2010). We named the resultant BAC, HHV-6ABACgMM1Lrev. The detailed method for reconstituting infectious virus using HHV-6A BACs was described previously (Tang et al., 2011, 2010).

Acknowledgments

We thank Dr. Gregory A. Smith (Department of Microbiology–Immunology, Northwestern University, Chicago, IL) for the *E. coli* GS1783, Dr. Nikolaus Osterrieder (Department of Microbiology and Immunology, College of Veterinary Medicine, Cornell University, Ithaca, NY) for the pEP-KanS plasmid, Dr. Ulrich H. Koszinowski (Max von Pettenkofer-Institute, Ludwig-Maximilians-University, Munich, Germany) for the pHA-2 plasmid, and Dr. J. Miyazaki (Osaka University, Osaka, Japan) for the pCAGGS. We thank Dr. Kazushige Adachi (Minoh City Hospital) and Dr. Hideto Yamada (Department of Obstetrics and Gynecology, Kobe University Graduate School of Medicine) for providing the CBMCs.

We thank Drs. Tetsuo Koshizuka (National Institute of Biomedical Innovation: NIBIO, Japan) and Tomohiko Sadaoka (Kobe University) for their useful advice and comments, and Dr. Huamin Tang (Kobe University) and Ms. Eiko Moriishi (NIBIO) for their technical assistance.

This study was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan and a Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science (JSPS).

References

Ablashi, D.V., Balachandran, N., Josephs, S.F., Hung, C.L., Krueger, G.R., Kramarsky, B., Salahuddin, S.Z., Gallo, R.C., 1991. Genomic polymorphism, growth properties, and immunologic variations in human herpesvirus-6 isolates. Virology 184 (2), 545–552.

Adler, H., Messerle, M., Wagner, M., Koszinowski, U.H., 2000. Cloning and mutagenesis of the murine gammaherpesvirus 68 genome as an infectious bacterial artificial chromosome. J. Virol. 74 (15), 6964–6974.

Akkapaiboon, P., Mori, Y., Sadaoka, T., Yonemoto, S., Yamanishi, K., 2004. Intracellular processing of human herpesvirus 6 glycoproteins Q1 and Q2 into tetrameric complexes expressed on the viral envelope. J. Virol. 78 (15), 7969–7983.

Aubin, J.T., Collandre, H., Candotti, D., Ingrand, D., Rouzioux, C., Burgard, M., Richard, S., Huraux, J.M., Agut, H., 1991. Several groups among human herpesvirus 6 strains can be distinguished by Southern blotting and polymerase chain reaction. J. Clin. Microbiol. 29 (2), 367–372.

Baines, J.D., Roizman, B., 1991. The open reading frames UL3, UL4, UL10, and UL16 are dispensable for the replication of herpes simplex virus 1 in cell culture. J. Virol. 65 (2), 938–944.

Baines, J.D., Wills, E., Jacob, R.J., Pennington, J., Roizman, B., 2007. Glycoprotein M of herpes simplex virus 1 is incorporated into virions during budding at the inner nuclear membrane. J. Virol. 81 (2), 800–812.

Brack, A.R., Dijkstra, J.M., Granzow, H., Klupp, B.G., Mettenleiter, T.C., 1999. Inhibition of virion maturation by simultaneous deletion of glycoproteins E, I, and M of pseudorabies virus. J. Virol. 73 (7), 5364–5372.

Campadelli-Fiume, G., Guerrini, S., Liu, X., Foa-Tomasi, L., 1993. Monoclonal antibodies to glycoprotein B differentiate human herpesvirus 6 into two clusters, variants A and B. J. Gen. Virol. 74 (Pt 10), 2257–2262.

Chandran, B., Tirawatnapong, S., Pfeiffer, B., Ablashi, D.V., 1992. Antigenic relationships among human herpesvirus-6 isolates. J. Med. Virol. 37 (4), 247–254.

Crump, C.M., Bruun, B., Bell, S., Pomeranz, L.E., Minson, T., Browne, H.M., 2004. Alphaherpesvirus glycoprotein M causes the relocalization of plasma membrane proteins. J. Gen. Virol. 85 (Pt 12), 3517–3527.

Dijkstra, J.M., Visser, N., Mettenleiter, T.C., Klupp, B.G., 1996. Identification and characterization of pseudorabies virus glycoprotein gM as a nonessential virion component. J. Virol. 70 (8), 5684–5688.
Fuchs, W., Mettenleiter, T.C., 1999. DNA sequence of the UL6 to UL20 genes of infectious laryngotracheitis virus and characterization of the UL10 gene product as a nonglycosylated and nonessential virion protein. J. Gen. Virol. 80 (Pt 8), 2173–2182.
Hobom, U., Brune, W., Messerle, M., Hahn, G., Koszinowski, U.H., 2000. Fast screening procedures for random transposon libraries of cloned herpesvirus genomes: mutational analysis of human cytomegalovirus envelope glycoprotein genes. J. Virol. 74 (17), 7720–7729.
Huang, H., Li, Y., Sadaoka, T., Tang, H., Yamamoto, T., Yamanishi, K., Mori, Y., 2006. Human herpesvirus 6 envelope cholesterol is required for virus entry. J. Gen. Virol. 87 (Pt 2), 277–285.
Jons, A., Dijkstra, J.M., Mettenleiter, T.C., 1998. Glycoproteins M and N of pseudorabies virus form a disulfide-linked complex. J. Virol. 72 (1), 550–557.
Kato, A., Tanaka, M., Yamamoto, M., Asai, R., Sata, T., Nishiyama, Y., Kawaguchi, Y., 2008. Identification of a physiological phosphorylation site of the herpes simplex virus 1-encoded protein kinase Us3 which regulates its optimal catalytic activity in vitro and influences its function in infected cells. J. Virol. 82 (13), 6172–6189.
Konig, P., Giesow, K., Keil, G.M., 2002. Glycoprotein M of bovine herpesvirus 1 (BHV-1) is nonessential for replication in cell culture and is involved in inhibition of bovine respiratory syncytial virus F protein induced syncytium formation in recombinant BHV-1 infected cells. Vet. Microbiol. 86 (1–2), 37–49.
Koyano, S., Mar, E.C., Stamey, F.R., Inoue, N., 2003. Glycoproteins M and N of human herpesvirus 8 form a complex and inhibit cell fusion. J. Gen. Virol. 84 (Pt 6), 1485–1491.
Krzyzaniak, M., Mach, M., Britt, W.J., 2007. The cytoplasmic tail of glycoprotein M (gpUL100) expresses trafficking signals required for human cytomegalovirus assembly and replication. J. Virol. 81 (19), 10316–10328.
Krzyzaniak, M.A., Mach, M., Britt, W.J., 2009. HCMV-encoded glycoprotein M (UL100) interacts with Rab11 effector protein FIP4. Traffic 10 (10), 1439–1457.
Lake, C.M., Hutt-Fletcher, L.M., 2000. Epstein-Barr virus that lacks glycoprotein gN is impaired in assembly and infection. J. Virol. 74 (23), 11162–11172.
Mach, M., Kropff, B., Dal Monte, P., Britt, W., 2000. Complex formation by human cytomegalovirus glycoproteins M (gpUL100) and N (gpUL73). J. Virol. 74 (24), 11881–11892.
Mach, M., Kropff, B., Kryzaniak, M., Britt, W., 2005. Complex formation by glycoproteins M and N of human cytomegalovirus: structural and functional aspects. J. Virol. 79 (4), 2160–2170.
MacLean, C.A., Robertson, L.M., Jamieson, F.E., 1993. Characterization of the UL10 gene product of herpes simplex virus type 1 and investigation of its role in vivo. J. Gen. Virol. 74 (Pt 6), 975–983.
May, J.S., Colaco, S., Stevenson, P.G., 2005. Glycoprotein M is an essential lytic replication protein of the murine gammaherpesvirus 68. J. Virol. 79 (6), 3459–3467.
May, J.S., Smith, C.M., Gill, M.B., Stevenson, P.G., 2008. An essential role for the proximal but not the distal cytoplasmic tail of glycoprotein M in murid herpesvirus 4 infection. PLoS One 3 (5), e2131.
Mettenleiter, T.C., Klupp, B.G., Granzow, H., 2009. Herpesvirus assembly: an update. Virus Res. 143 (2), 222–234.
Mori, Y., Akkapaiboon, P., Yang, X., Yamanishi, K., 2003. The human herpesvirus 6 U100 gene product is the third component of the gH–gL glycoprotein complex on the viral envelope. J. Virol. **77** (4), 2452–2458.
Mori, Y., Koike, M., Moriishi, E., Kawabata, A., Tang, H., Oyaizu, H., Uchiyama, Y., Yamanishi, K., 2008. Human herpesvirus-6 induces MVB formation, and virus egress occurs by an exosomal release pathway. Traffic 9 (10), 1728–1742.
Niwa, H., Yamamura, K., Miyazaki, J., 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108 (2), 193–199.
Okuno, T., Shao, H., Asada, H., Shiraki, K., Takahashi, M., Yamanishi, K., 1992. Analysis of human herpesvirus 6 glycoproteins recognized by monoclonal antibody OHV1. J. Gen. Virol. 73 (Pt 2), 443–447.
Osterrieder, N., Neubauer, A., Fakler, B., Brandmuller, C., Seyboldt, C., Kaaden, O.R., Baines, J.D., 1997. Synthesis and processing of the equine herpesvirus 1 glycoprotein M. Virology 232 (1), 230–239.
Owen, D.J., Evans, P.R., 1998. A structural explanation for the recognition of tyrosine-based endocytotic signals. Science 282 (5392), 1327–1332.
Ren, Y., Bell, S., Zenner, H.L., Lau, S.Y., Crump, C.M., 2011. Glycoprotein M is Important for the Efficient Incorporation of Glycoprotein H/L into HSV-1 particles. J. Gen. Virol.
Rudolph, J., Osterrieder, N., 2002. Equine herpesvirus type 1 devoid of gM and gp2 is severely impaired in virus egress but not direct cell-to-cell spread. Virology 293 (2), 356–367.
Salahuddin, S.Z., Ablashi, D.V., Markham, P.D., Josephs, S.F., Sturzenegger, S., Kaplan, M., Halligan, G., Biberfeld, P., Wong-Staal, F., Kramarsky, B., et al., 1986. Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. Science 234 (4776), 596–601.
Shimamura, M., Mach, M., Britt, W.J., 2006. Human cytomegalovirus infection elicits a glycoprotein M (gM)/gN-specific virus-neutralizing antibody response. J. Virol. 80 (9), 4591–4600.
Tang, H., Hayashi, M., Maeki, T., Yamanishi, K., Mori, Y., 2011. Human herpesvirus 6 glycoprotein complex formation is required for folding and trafficking of the gH/gL/gQ1/gQ2 complex and its cellular receptor binding. J. Virol. 85 (21), 11121–11130.
Tang, H., Kawabata, A., Yoshida, M., Oyaizu, H., Maeki, T., Yamanishi, K., Mori, Y., 2010. Human herpesvirus 6 encoded glycoprotein Q1 gene is essential for virus growth. Virology 407 (2), 360–367.
Tischer, B.K., Schumacher, D., Messerle, M., Wagner, M., Osterrieder, N., 2002. The products of the UL10 (gM) and the UL49.5 genes of Marek's disease virus serotype 1 are essential for virus growth in cultured cells. J. Gen. Virol. 83, 997–1003.
Tischer, B.K., von Einem, J., Kaufer, B., Osterrieder, N., 2006. Two-step red-mediated recombination for versatile high-efficiency markerless DNA manipulation in Escherichia coli. Biotechniques 40 (2), 191–197.
Voorhees, P., Deignan, E., van Donselaar, E., Humphrey, J., Marks, M.S., Peters, P.J., Bonifacino, J.S., 1995. An acidic sequence within the cytoplasmic domain of furin functions as a determinant of trans-Golgi network localization and internalization from the cell surface. EMBO J. 14 (20), 4961–4975.
Wu, S.X., Zhu, X.P., Letchworth, G.J., 1998. Bovine herpesvirus 1 glycoprotein M forms a disulfide-linked heterodimer with the U(L)49.5 protein. J. Virol. 72 (4), 3029–3036.
Yamagishi, Y., Sadaoka, T., Yoshii, H., Somboonthum, P., Imazawa, T., Nagaike, K., Ozono, K., Yamanishi, K., Mori, Y., 2008. Varicella-zoster virus glycoprotein M homolog is glycosylated, is expressed on the viral envelope, and functions in virus cell-to-cell spread. J. Virol. 82 (2), 795–804.
Yamanishi, K., Okuno, T., Shiraki, K., Takahashi, M., Kondo, T., Asano, Y., Kurata, T., 1988. Identification of human herpesvirus-6 as a causal agent for exanthem subitum. Lancet 1 (8594), 1065–1067.
Ziegler, C., Just, F.T., Lischewski, A., Elbers, K., Neubauer, A., 2005. A glycoprotein M-deleted equid herpesvirus 4 is severely impaired in virus egress and cell-to-cell spread. J. Gen. Virol. 86 (Pt 1), 11–21.