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

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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

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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,

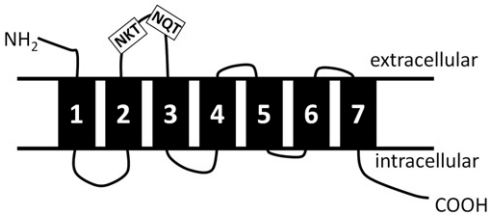

Fig. 1. Predicted topology of HHV-6A gM. The topology of HHV-6A gM was predicted using the TMHMM transmembrane topology prediction server (http://www.cbs.dtu.dk/services/TMHMM/). The approximate location of two N-linked glycosylation sites (NKT, asparagine-lysine-threonine; NQT, asparagine-glutamine-threonine)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

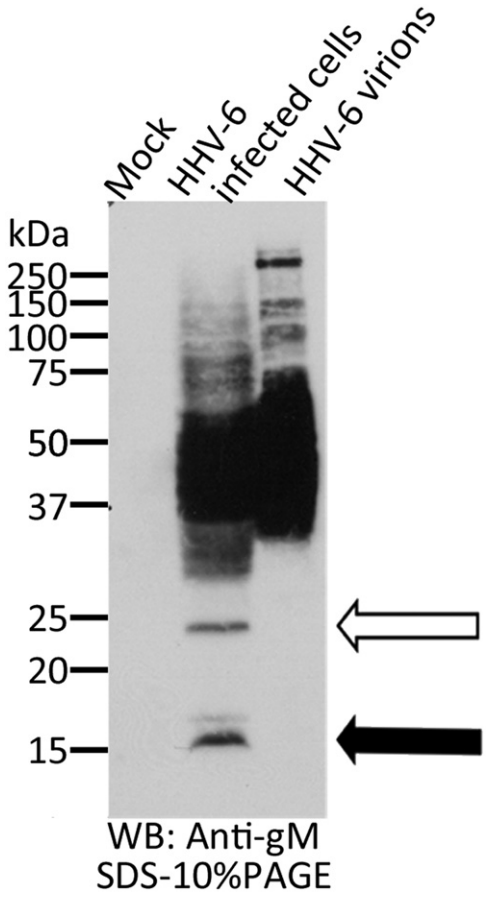

Fig. 2. Expression of gM in HHV-6A-infected cells and virions. HHV-6A-infected or mock-infected HSB-2 cells were harvested at 96 h postinfection. The lysates of HHV-6A-infected or mock-infected HSB-2 cells, or of purified virions were analyzed by western blotting. The blots were reacted with an anti-gM 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