Human herpesvirus 6 gM/gN complex interacts with v-SNARE in infected cells

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Human herpesvirus 6 (HHV-6) glycoprotein M (gM) is an envelope glycoprotein that associates with glycoprotein N (gN), forming the gM/gN protein complex, in a similar manner to the other herpesviruses. Liquid chromatography-MS/MS analysis showed that the HHV-6 gM/gN complex interacts with the v-SNARE protein, vesicle-associated membrane protein 3 (VAMP3). VAMP3 colocalized with the gM/gN complex at the trans-Golgi network and other compartments, possibly the late endosome in HHV-6-infected cells, and its expression gradually increased during the late phase of virus infection. Finally, VAMP3 was incorporated into mature virions and may be transported with the gM/gN complex.

INTRODUCTION

Human herpesvirus 6 (HHV-6) belongs to the beta-herpesvirus subfamily (Roizmann et al., 1992). HHV-6 isolates can be classified as HHV-6A and HHV-6B (Ablashi et al., 2013) based on genetic and antigenic differences, cell tropism, and pathogenesis (Ablashi et al., 1991; Aubin et al., 1991; Campadelli-Fiume et al., 1993; Chandran et al., 1992; Mori, 2009; Yamanishi et al., 1988).

Herpesviruses encode several glycoproteins on the envelope of viral particles that work for entry, assembly and egress of the virus. Of these, glycoprotein M (gM) is a remarkable envelope glycoprotein as it is conserved among all herpesvirus subfamilies. Most herpesviruses, including herpes simplex virus type-1 (Baines & Roizman, 1991), pseudorabies virus (Dijkstra et al., 1996) and equine herpesvirus 1 (Osterrieder et al., 1996), do not require gM for replication. Marek’s disease virus (Tischer et al., 2002) and variella-zoster virus (Yamagishi et al., 2008) abolish virus growth in vitro. However, the gM protein of human cytomegalovirus (HCMV), which belongs to the betaherpesvirus subfamily, is essential for the production of infectious virus (Hobom et al., 2000).

HHV-6 gM is a product of the U72 ORF and comprises 343 aa (Kawabata et al., 2012; Lawrence et al., 1995). Post-infection (p.i.), it is translated into a 47–63 kDa protein that is post-translationally glycosylated (Kawabata et al., 2012). It is a type III transmembrane protein with seven membrane-spanning domains and a C-terminal cytoplasmic tail. HHV-6 gM interacts with the product of the U46 ORF, known as gN, to form a complex for transport to the trans-Golgi network (TGN) and endosomal compartments (Kawabata et al., 2012). Finally, the gM/gN complex is incorporated into mature virions. Interestingly, unlike in alpha herpesviruses, HHV-6 gM is essential for virus growth (Kawabata et al., 2012).

To further examine the role of HHV-6A gM during HHV-6 infection, we performed liquid chromatography (LC)-MS/MS analysis to identify the cellular components that interact with the gM/gN complex. The results showed that the gM/gN complex interacts with VAMP3 (vesicle-associated membrane protein 3).

VAMP3 is a v-SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein that resides in recycling endosomes and endosome-derived transport vesicles. v-SNARE interacts with SNARE proteins on target membranes (t-SNAREs) to form trans-SNARE complexes, which draw the two membranes together and drive membrane fusion (Jahn & Scheller, 2006; Jahn et al., 2003; Rothman, 1994; Sollner et al., 1993). SNAREs are cytoplasmic-oriented type I membrane proteins that play a role in intracellular trafficking mechanisms during exocytosis by forming a complex that facilitates the transient fusion of the vesicular and plasma membrane lipid bilayers (Mohrmann & Sørensen, 2012). This membrane fusion is dependent on the formation of a complex between t-SNARE and v-SNARE proteins (Jahn & Scheller, 2006; Jahn et al., 2003).

VAMP3 is localized to recycling endosomes (McMahon et al., 1993) and plays a role in the fusion of recycling endosomes and the plasma membrane by forming a complex with the surface t-SNARE complex, Stx4/SNAP23 (Hu et al., 2007). The VAMP3/Stx4/SNAP23 SNARE complex mediates the long-loop recycling pathway that delivers recycling endosomes and their cargo to the cell.
surface, and plays an important role in regulating the ability of macrophages to effectively adhere and spread on fibronectin (Veale et al., 2011). VAMP3 is also involved in integrin trafficking, cell migration and cell adhesion (Luftman et al., 2009; Tayeb et al., 2005). In addition, it plays a role in the exocytosis of α-granules in platelets (Polgár et al., 2002), as well as in the recycling of endocytosed transferrin receptors to the cell surface (Galli et al., 1994).

Here, we describe the interaction between the gM/gN complex and VAMP3 in HHV-6A-infected cells and discuss the potential for the association between the gM/gN complex and VAMP3 to modify its localization and mediate its incorporation into mature virions.

**RESULTS**

**Identification of cellular proteins interacting with the gM/gN complex**

A recent study showed that the cytoplasmic tail of HCMV gM interacts with the cellular protein FIP4, which is a Rab11-GTPase effector protein important for gM/gN trafficking and for accumulation of the envelope glycoprotein complex in the assembly compartment in HCMV-infected cells (Krzysiañki et al., 2009). To examine the function of HHV-6A gM/gN, we tried to identify the cellular protein(s) that interact with the gM/gN complex. First, gM and gN were cotransfected into 293T cells and gM was immunoprecipitated from the lysates with an anti-gM mAb. Silver staining of gels containing proteins separated from the lysates of the gM/gN-expressing cells revealed several specific bands at approximately 10 kDa; these proteins were not present in the lysates of cells expressing gM alone. One specific band (Fig. 1a, arrowhead) was excised from the gel and subjected to LC-MS/MS analysis. The results of this analysis identified VAMP3 as the interacting protein (Fig. 1b).

To confirm the interaction between VAMP3 and gM/gN, lysates from cells expressing both HA-tagged gM and FLAG-tagged gN were immunoprecipitated with an anti-HA antibody, followed by Western blotting with anti-gM, anti-FLAG or anti-VAMP3 antibodies. As shown in Fig. 1c, VAMP3 was coprecipitated when gM and gN were coexpressed. However, VAMP3 was not coprecipitated when gM or gN was expressed alone. CD63 was not coprecipitated even when gM and gN were coexpressed (Fig. 1c). These results indicate that VAMP3 interacts with the gM/gN complex.

**Interaction between gM and VAMP3 in HHV-6A-infected cells**

To confirm the interaction between the gM/gN complex and VAMP3 in HHV-6A-infected cells, lysates from HHV-6A-infected HSB-2 cells were immunoprecipitated with an anti-gM mAb or an anti-VAMP3 antibody, followed by Western blotting with anti-gM, anti-VAMP3, anti-CD63 or anti-gB antibodies (Fig. 2). Endogenous VAMP3 coprecipitated with gM and vice versa; gB or endogenous CD63 coprecipitated with neither gM nor VAMP3 (Fig. 2b). These results indicate that gM also interacts with VAMP3 in HHV-6A-infected cells. Interestingly, although gM with a molecular mass of 15 kDa was detected in the lysates of HHV-6A-infected cells, it did not coprecipitate with the anti-VAMP3 antibody.

To examine the cellular localization of gM and VAMP3 in HHV-6A-infected cells, an indirect immunofluorescence assay (IFA) was performed using HHV-6A-infected HSB-2 cells at 96 h.p.i. (Fig. 3). Confocal microscopy of HHV-6A-infected HSB-2 cells showed that gM and VAMP3 appear to partially colocalize to the same cellular compartment. In addition, gM and VAMP3 partially colocalized with TGN46, a marker of the TGN (Fig. 3b), and with CD63, a marker of late endosomes and multivesicular bodies (MVB) (Fig. 3a). The expression of endogenous VAMP3 was much lower in uninfected cells than infected cells [Fig. 3a(ii), b(ii)]. These results indicate that VAMP3 may localize with gM to the endosomal compartment in addition to TGN during the late stage of infection. Preimmune serum of guinea pig did not react with either HHV-6A-infected [Fig. 3d(ii)] or uninfected cells [Fig. 3d(ii)], although anti-VAMP3 antibody obtained from the same guinea pig reacted with HHV-6A-infected cells [Fig. 3c(i)] but not uninfected cells [Fig. 3c(ii)].

**VAMP3 is present in purified HHV-6A virions**

Recently, we showed that HHV-6A virions are released through MVBS via the cellular exosomal pathway and that gB and gM are present on exosomes (Mori et al., 2008). To examine whether VAMP3 expressed in HHV-6A-infected cells is present on virions and exosomes, we purified virions from the culture medium of HHV-6A-infected cells. As expected, VAMP3, gM and CD63 were detected by western blotting of the virion fractions (Fig. 4a). Virion fractions were confirmed with the presence of gB (Fig. 4a) and viral DNA (Fig. 4b). In addition, VAMP3 was detected on HHV-6A virions by immunogold labelling electron microscopy analysis [Fig. 4c(i)], but it was rarely detected on virions without primary antibody [Fig. 4c(ii)]. These results indicate that VAMP3 is incorporated into viral particles along with the gM/gN complex.

**Intracellular localization of the gM/gN complex and VAMP3 in cells transiently expressing gM/gN**

We next examined the intracellular localization of gM, gN and VAMP3 (Fig. 5). When plasmids expressing gM and gN were cotransfected into HeLa cells [Fig. 5a(i)], gM/gN colocalized with endogenous VAMP3 in the perinuclear region. However, when gM was expressed alone, it failed to colocalize with VAMP3 [Fig. 5a(ii)]. Because the gM/gN complex localized to the TGN in HHV-6A-infected cells, we hypothesized that the gM/gN complex would interact with...
VAMP3 at the TGN or a TGN-derived compartment. As expected, when gM was coexpressed with gN, it colocalized with VAMP3 and TGN46 (Fig. 5b); however, this colocalization was not observed when gM was expressed alone [Fig. 5a(ii)]. These results suggest that the interaction between the gM/gN complex and VAMP3 occurs at the TGN or a TGN-derived compartment. Glycoprotein M did not colocalize with CD63 even when gM was coexpressed with gN [Fig. 4c(i)]. Non-specific staining of gM was not seen in these cells [Fig. 5a(iii), b(ii), c(ii)].
The kinetics of VAMP3 expression in HHV-6A-infected cells

As shown in Fig. 2, VAMP3 expression in HHV-6A-infected cells was higher than that in mock-infected cells. Therefore, we examined the kinetics of VAMP3 expression in HHV-6A-infected cells. The results in Fig. 6 show that VAMP3 expression increased gradually in the infected cells.

DISCUSSION

Here, we used the transient expression of gM and gN to identify VAMP3 as a cellular molecule that interacts with the HHV-6A gM/gN complex. The interaction between VAMP3 and the gM/gN complex was also confirmed in HHV-6A-infected cells. VAMP3 and gM/gN proteins colocalized at the TGN in cells coexpressing gM and gN, and in HHV-6A-infected cells. This interaction was observed only when gM/gN formed a complex, indicating that the interaction is required for gM/gN complex formation. Previously, we reported that the localization of HHV-6A gM to the TGN was necessary for its interaction with gN (Kawabata et al., 2012). Therefore, the interaction between the gM/gN complex and VAMP3 might also occur at the TGN. It is still not known whether the interaction between VAMP3 and gM requires gM/gN complex formation. Transport of gM to the TGN might be required for this interaction.

VAMP3 also colocalized with CD63, which is a marker of late endosome in HHV-6A-infected cells. In cells transiently expressing gM and gN, however, VAMP3 colocalized with TGN46, but not CD63. This suggests that in infected cells, the localization of VAMP3 may be modified through its interaction with gM/gN, thereby possibly allowing it to localize to the other organelles, such as the late endosome.

Fig. 2. Interaction between gM and VAMP3 in HHV-6A-infected HSB-2 cells. HHV-6A-infected or mock-infected HSB-2 cells were lysed with TNE buffer at 96 h post-infection. The lysates were immunoprecipitated (IP) with anti-gM mAb or anti-VAMP3 Ab (see Methods) and analysed by Western blotting with anti-gM or anti-VAMP3 (BioReagents) Abs (a), anti-gB Ab or anti-CD63 mAb (b). The numbers beside the panels indicate the molecular masses (kDa). WB, Western blotting.
We also found that VAMP3 was incorporated into virions. As the gM/gN complex is expressed on virions and exosomes, complex-associated VAMP3 would be transported along with the gM/gN complex and then released via the exosomal release pathway (Mori et al., 2008).

Although the function of VAMP3 in HHV-6A-infected cells is not known, its interaction with the gM/gN complex may modify the cellular machinery in infected cells. As VAMP3 is incorporated into virions and exosomes, its primary function (to facilitate membrane fusion) may be lost in infected cells. Overexpression of VAMP3 did not affect HHV-6 growth (data not shown). Several v-SNARE proteins with functions similar to those of VAMP3 have been identified (Borisovska et al., 2005). Therefore, the function of VAMP3 may be redundant in HHV-6A-infected cells. It is still not known whether v-SNAREs, including VAMP3, are required for HHV-6 infection. Further studies will be required to address these questions.

**METHODS**

**Cells and viruses.** The HSB-2 T-cell line was cultured in RPMI 1640 medium (Nissui) supplemented with 8% FBS. Human embryonic
kidney cells (293T cells) and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 8% FBS. The HHV-6A strain GS was propagated and titrated in HSB-2 cells. HHV-6A cell-free virus was prepared as previously described (Akkapaiboon et al., 2004). Cord blood mononuclear cells (CBMC) were used for virus propagation (Mori et al., 2004). CBMCs were kindly provided by K. Adachi (Minoh Hospital, Minoh, Japan) and H. Yamada (Kobe University Graduate School of Medicine, Kobe, Japan) and purchased from the RIKEN Cell Bank (BioResource Center). For the usage of CBMCs, the study was approved by the ethics committee of each institution.

Antibodies. Rabbit antibody (Ab) specific for HHV-6A gM or gB (Mori et al., 2008), an AgM-1 mAb against gM (Kawabata et al., 2012), and a U14 mAb against HHV-6 U14 (Takekoto et al., 2005) were used. mAbs against CD63 (clone CLB-gran/12, 435; Sanquin) and α-tubulin (clone B-5-1-2; Sigma), a sheep polyclonal Ab against TGN46 (AbD Serotec), a rabbit polyclonal Ab against VAMP3 (BioReagents) and a goat polyclonal Ab against VAMP3 (Santa Cruz) were used. Alexa Fluor 594-conjugated goat anti-sheep IgG (Molecular Probes), Alexa Fluor 594-conjugated donkey anti-rabbit IgG (Molecular Probes), FITC-conjugated affinity pure F(ab)2 fragment goat anti-guinea pig IgG (Jackson ImmunoResearch Laboratories), and Cy5-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories) were used as secondary antibodies. An anti-VAMP3 monospecific Ab was produced by subjecting guinea pigs to three rounds of immunization with the antigen, which was then expressed in Escherichia coli and purified (Mori et al., 2008).

Immunofluorescence assay. The IFA was performed as described previously (Akkapaiboon et al., 2004; Mori et al., 2004). Specific immunofluorescence was observed under a confocal laser-scanning microscope (FluoView FV1000; Olympus).

Plasmid construction. The HA-tagged gM- and FLAG-tagged gN-expressing plasmids were described previously (Kawabata et al., 2012). The pCAGGS plasmid was kindly provided by Jun-ichi Miyazaki (Osaka University, Japan) (Niwa et al., 1991). To express the recombinant protein, the following primer pair was used to amplify inserts from HSB-2 cells cDNA: for named GST-VAMP3, VAMP3FbamHI (5'-ACCGGATCCTCTACAGGTCCAACTGCTG-CACT-3') and VAMP3rsalI (5'-ACCGTCGACTTACTTGCAATTCT- TCCACCAATATTTC-3'). The PCR products were inserted into the pGEX-4T1 vector (GE Healthcare).

Plasmid transfection. HeLa cells were transfected with the expression plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. The 293T cells were transfected using the calcium phosphate method as described previously (Koshizuka et al., 2010).

Identification of gM/gN-interacting proteins. Plasmids expressing HA-tagged gM and FLAG-tagged gN were cotransfected into 293T cells. Cotransfection of gM and pCAGGS into 293T cells was performed as a control. At 72 h post-transfection, the cells were lysed in TNE buffer (0.01 M Tris/HCl, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1% Nonidet-P-40). After centrifugation at 200 000 g for 1 h, the
supernatants were incubated overnight at 4 °C with an anti-HA antibody conjugated to protein G Sepharose (GE Healthcare). The protein-anti-HA conjugated beads were then washed with lysis buffer and the bound proteins were eluted with 0.1 M glycine/HCl (pH 2.8). After the beads were removed by centrifugation, the supernatants were neutralized by adding 1 M Tris-HCl (pH 9.5). The eluted proteins were then solubilized with sample buffer, separated on a NuPAGE SDS-PAGE system (Invitrogen), and examined by silver staining. Specific bands were analysed by LC-MS/MS to identify the coimmunoprecipitated proteins (Shevchenko et al., 1996; Tang et al., 2013).

**Fig. 5.** Subcellular localization of VAMP3 with the gM/gN complex in HeLa cells transiently expressing gM and gN. HeLa cells were transfected with plasmids expressing gM and gN [a(i), b(i), c(i)], gM and the empty vector [a(ii)], or without vectors [a(iii), b(ii), c(ii)]. The cells were harvested at 48 h post-transfection and fixed. (a) The cells were stained with antibodies against gM, FLAG (for gN) and VAMP3 as well as with Hoechst 33258. (b) Cells were stained with antibodies against gM, TGN46 and VAMP3 as well as Hoechst 33258. (c) Cells were stained with antibodies against gM, CD63 and VAMP3 as well as with Hoechst 33258. Co-stained areas appear white or yellow in the merged panel. Bars, 10 μm.
Fig. 6. Kinetics of VAMP3 protein expression in HHV-6A-infected cells. Whole-cell lysates collected at the indicated time points (h) were analysed by Western blotting. The numbers beside the panels indicate the molecular masses (kDa).

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

Isolation of virion fractions. Virions containing exosomes were collected from the cell culture medium by differential centrifugation and fractionated with a linear sucrose gradient, as described previously (Mori et al., 2008). The fractions were analysed by Western blotting, DNA PCR, and electron microscopy.

Electron microscopy. Immunogold labelling of virions was performed as described previously (Mori et al., 2008). The samples were examined under a Hitachi H-7650 electron microscope.

ACKNOWLEDGEMENTS

We thank E. Moriishi (National Institute of Biomedical Innovation), Mayuko Hayashi and Megumi Ota (Kobe University) for providing reagents, and K. Adachi (Mino City Hospital) and H. Yamada (Kobe University) for the CBMCs. This work was supported by a Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science (JSPS).

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