progeny viruses was completely dependent on the interaction with ORF44p at 41DFDE44. These results suggest that ORF44p is fully functional only in the presence of ORF49p and vice versa and has essential functions during infection, which are independent of the interaction with ORF49p or redundantly supported by other viral factors in the absence of ORF49p.

In the absence of the interaction with ORF49p during infection, ORF44p was detected throughout the cytoplasm and rarely colocalized with the TGN (or with a reorganized organ containing TGN-derived membranes known to be induced by viral infection, although it has not been found in VZV infection), the recognized site of viral assembly; however, incorporation of ORF44p into viral particles was comparable to that observed in wild-type virus infection. These results indicate that ORF44p was not directly incorporated into the particles through the TGN via its interaction with ORF49p, at least in the absence of ORF49p. In HSV-1, the amount of pUL16 packaged into the viral particles was severely reduced in the absence of pUL11 (50), but there are some other interaction partners that potentially function in incorporating pUL16 into the viral particles (i.e., pUL21 and glycoprotein E) (51, 52). In VZV, by global screening using the yeast two-hybrid system, some candidates for ORF44p binding partner have been reported (28, 29), but in our observations, none of these viral proteins other than ORF49p could accumulate ORF44p on the TGN; one viral protein could alter the localization of ORF44p into the nucleus; however, whether it functions in the incorporation of ORF44p into the viral particles remained unclear (T. Sadaoka and Y. Mori, unpublished observation). Anyway, additional ORF44p binding partners active during either the wild-type virus or ORF49-defective virus infection remain to be identified so far, and the complexity of the herpesvirus protein-protein network requires a solid approach to elucidate the essential roles of ORF44 during viral infection further through the interactions with other viral proteins.

In summary, in the present study, we established a *trans*-complementation system for ORF49 and identified ORF44p as the binding partner for ORF49p. We showed that (i) ORF49p functions in the efficient production of infectious virus, (ii) no other viral factor is required for binding, (iii) residue 129F of ORF44p is critical not only for binding to ORF49p but also for progeny virus production/reconstitution, (iv) the carboxyl-terminal half of the acidic cluster (41DFDE44) of ORF49p is the binding motif for ORF44p, and (v) the efficient production of infectious progeny virus by ORF49p is dependent on its interaction with ORF44p. Further analyses of the role of ORF44 mediated by its interaction with ORF49 or other as yet unidentified viral proteins may shed light on the conserved infection mechanisms of the *Herpesvirinae* and those unique to VZV.

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ORIGINAL ARTICLE

MHC class I molecules are incorporated into human herpesvirus-6 viral particles and released into the extracellular environment

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ABSTRACT

Human herpesvirus-6 (HHV-6), which belongs to the betaherpesvirus subfamily, mainly replicates in T lymphocytes. Here, we show that MHC class I molecules are incorporated into HHV-6 viral particles and released into the extracellular environment. In addition, HHV-6A/B-infected T cells showed reduced surface and intracellular expression of MHC class I molecules. The cellular machinery responsible for molecular transport appears to be modified upon HHV-6 infection, causing MHC class I molecules to be transported to virion assembly sites.

Key words human herpesvirus-6A/B, MHC class I, viral particles.

Human herpesvirus 6 (HHV-6), which belongs to the betaherpesvirus subfamily (1), was first isolated from peripheral blood lymphocytes obtained from patients with lymphoproliferative disorders (2). HHV-6 isolates are classified as HHV-6A and HHV-6B based on genetic and antigenic differences and their cell tropism (2–5). Primary infection with HHV-6B causes exanthem subitum (6). The diseases caused by HHV-6A are so far unknown. HHV-6B mostly infects infants and remains latent in more than 90% of the population (7).

In general, herpesviruses use several strategies to evade host immune responses. For example, viruses may inhibit MHC class I-associated antigen presentation to escape detection by cytotoxic T lymphocytes. Several proteins expressed by herpesviruses block the transport of antigenic peptides from the cytosol to the endoplasmic reticulum (8–11), whereas others retain (12–14) or destroy class I molecules, or deliver them to lysosomes for degradation (15–18). The result is reduced surface expression of MHC class I molecules, enabling the virus to evade host immune surveillance.

HHV-6A, but not HHV-6B, downregulates expression of MHC class I in dendritic cells (19). HHV-6 U21 binds to and diverts MHC class I molecules to an endolysosomal compartment, effectively removing them from the cell surface and providing a possible means of immune escape (20).

Here, we show that expression of MHC class I molecules by infected cells is downregulated with incorporation into HHV-6 viral particles, suggesting a possible mechanism by which the virus escapes host immune surveillance.

MATERIALS AND METHODS

Cells and viruses

CBMCs were prepared as described previously (21). CBMCs were provided by K. Adachi (Minoh Hospital, Minoh,

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List of Abbreviations: CBMC, umbilical cord blood mononuclear cell; LC-MS/MS, liquid chromatography-tandem mass spectrometry; HHV-6A, human herpesvirus-6A; HHV-6B, human herpesvirus-6B; MVB, multivesicular body; TGN, *trans*-Golgi network.

Japan) and H. Yamada (Kobe University Graduate School of Medicine, Kobe, Japan) and purchased from the Cell Bank of the RIKEN BioResource Center, Tsukuba, Japan. Virus stocks were also prepared as described previously (21, 22). HSB-2 and MT-4 cell lines were used in this study (23). HHV-6A (strain GS) and HHV-6B (strain HST) were prepared as previously described (21).

Antibodies

Monoclonal antibody (Mab) OHV-1 (24) and a polyclonal antibody against gB (23, 25) have been described previously. The following other Mabs were purchased: MHC class I (clone: W6/32; Bio Legend, San Diego, CA, USA), CD63 (clone: CLB-gran/12, 435; Sanquin Blood Supply, Amsterdam, the Netherlands), and α-tubulin (clone: B-5-1-2; Sigma, St Louis, MO, USA). The following secondary antibodies were used: Alexa Fluor 488- or 594-conjugated F(ab′)2 fragment of goat anti-mouse or rabbit immunoglobulin G (IgG) (Invitrogen, Tokyo, Japan) and anti-mouse IgG, horseradish peroxidase-linked whole antibody (from sheep) (GE Healthcare, Piscataway, NJ, USA).

Virion and exosome isolation

Virions and exosomes were purified as previously described (23, 26). The collected fractions were used for western blotting, electron microscopy or liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Liquid chromatography-tandem mass spectrometry

The fractions described above were analyzed by LC-MS/MS. Proteins were diluted tenfold with 9.8 M urea. The solutions were adjusted to pH 8.5, reduced with 13 mM dithiothreitol at 37°C for 1.5 hr and alkylated with 27 mM iodoacetamide in the dark for 1 hr. The protein mixtures were further diluted with 100 mM triethylammonium bicarbonate (pH 8.5) to reduce urea to 1 M, and digested with 4 μL of 1 mg/mL trypsin–tosyl phenylalanyl chloromethyl ketone solution. Samples were digested overnight at 37°C. Following digestion, lysates were acidified by adding 10% trifluoroacetic acid. The samples were desalted using peptide cleanup C18 spin tubes (Agilent Technologies, Santa Clara, CA, USA) and vacuum-dried. NanoLC-MS/MS analyses were performed on an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) as described previously (27), while spray voltage was changed to 1800 V. Peptides and proteins were identified by automated database searches using Proteome Discoverer v.1.1 (Thermo Fisher Scientific) against all entries of the Swiss Prot protein database (version 3.26) with a precursor mass tolerance of 10 p.p.m., a fragment ion mass tolerance of 0.8 Da, and strict trypsin specificity, allowing for up to two missed cleavages. Cysteine carbamidomethylation was set as a fixed modification, and methionine oxidation was allowed as a variable modification.

Western blotting

Western blotting was performed as described previously (28, 29).

Electron microscopy

Electron microscopy was performed as described previously (30).

Briefly, the virion-containing pellets were resuspended in 2% (w/v) paraformaldehyde solution buffered with 0.1 M phosphate (pH 7.2). Next, 5 μL of the resuspended pellet was loaded onto formvar–carbon-coated grids to adsorb the virions. Immunostaining was then performed. The virions were incubated with mouse anti-gB, anti-MHC class I or anti-CD63 antibody for 1 hr at room temperature, followed by goat anti-mouse IgG conjugated to 10 nm colloidal gold particles (GE Healthcare) for a further 1 hr at room temperature. After immunolabeling, the samples were washed in distilled water, stained for 5 min with uranyl oxalate, pH 7.0, washed again, embedded in a mixture of 1.8% methylcellulose and 0.4% uranyl acetate, pH 4.0, at 4°C, air-dried, and observed under a Hitachi H-7100 electron microscope (Hitachi, Tokyo, Japan). For the control experiments, samples were incubated with the secondary antibody alone.

Flow cytometry

MT-4 cells were infected with HHV-6B. At 72 hr post-infection, the cells were fixed with 4% (w/v) paraformaldehyde at room temperature for 15 min and incubated with anti-MHC class I Mab at 37°C for 1 hr. The cells were then stained with an appropriate secondary antibody at 37°C for 30 min. For the control experiments, samples were incubated with the secondary antibody alone. Stained cells were analyzed using a flow cytometer (ec800; Sony, Tokyo, Japan).

Immunofluorescence assay

Immunofluorescence assay was performed as described previously (28). Briefly, MT-4 cells were infected with HHV-6B. At 72 hr post-infection, the cells were fixed with cold acetone–methanol (7:3) and incubated at 37°C

for 1 hr with an anti-HHV-6 gB rabbit antibody or an anti-MHC class I Mab. After washing for 10 min with PBS containing 0.02% Tween-20, the cells were incubated with an appropriate secondary antibody at 37°C for 30 min, followed by Hoechst33342 at 37°C for 40 min. After washing as described above, signals were detected by a confocal laser-scanning microscope (Olympus FluoView FV1000; Olympus, Tokyo, Japan).

RESULTS

Virion and exosome isolation

Extracellular viral particles containing exosomes were purified from the culture supernatant of HHV-6A (strain GS)-infected HSB-2 or HHV-6B (strain HST)-infected MT-4 cells. The particle-containing fractions were confirmed by western blotting with an anti-gB antibody (23, 25). Next, the particle-containing fractions were analyzed by LC-MS/MS (27), which detected many cellular proteins (unpublished data). Of the host proteins detected, our analyses focused on MHC class I molecules.

Virion- or exosome-associated fractions contain MHC class I molecules

To verify expression of MHC class I within viral particles, the proteins in fractions 3–10 were separated by SDS–PAGE and analyzed by western blotting with anti-gB rabbit, anti-MHC class I or anti-CD63 antibodies. As shown in Figure 1, gB protein was detected in fractions 5–6 whereas MHC class I was detected primarily in fractions 6–8. We have previously reported that the MVB marker, CD63, is incorporated into virions and exosomes (23); therefore, expression of CD63 was also examined. As expected, CD63 was detected in fractions 5–10 (Fig. 1c). To confirm expression of MHC class I within both virions and exosomes, negative staining of fractions 6 and 7 were performed, followed by electron microscopy (30). Fraction 6 contained mainly viral particles of diameter approximately 200 nm. Both MHC class I (Fig. 1e) and gB protein (Fig. 1d) were present in these particles. Fraction 7 contained mainly exosomes of diameter approximately 50–100 nm (Fig. 1f). These exosomes contained MHC class I, which confirmed the results of the western blotting experiments. Taken together, these results indicate that MHC class I molecules are present in exosomes and virions released from HHV-6B-infected cells.

Downregulated expression of MHC class I molecules on the surface of HHV-6B-infected cells

Downregulation of MHC class I occurs in many different virus-infected cells (31–37). Because MHC class I molecules were incorporated into virions, HHV-6-infected MT-4 cells might show an apparent downregulation in cell surface expression. To confirm this, HHV-6B- or mock-infected cells harvested 72 hr post-infection were fixed and then stained with an anti-MHC class I antibody. Surface expression of MHC class I was then analyzed by flow cytometry. As expected, HHV-6B-infected cells showed downregulated cell surface expression of MHC class I when compared with mock-infected cells (Fig. 2a). This reduced expression was confirmed by western blot analysis (Fig. 2b), indicating that expression of MHC class I molecules within HHV-6-infected cells (not just expression on the cell surface) was also downregulated. Next, the localization of MHC class I molecules in these cells was assessed after they had been fixed and co-stained with anti-MHC class I and gB antibodies. MHC class I in infected cells was localized mainly within intracellular compartments, and colocalized with the envelope glycoprotein gB during the later stages of infection; however, MHC class I was mainly localized to the plasma membrane in mock-infected cells (Fig. 2c).

DISCUSSION

Here, we used mass spectrometry-based proteomics analysis to show that MHC class I molecules are incorporated into HHV-6 viral particles. Downregulation of MHC class I molecules in virus-infected cells is an important mechanism by which viruses evade immune surveillance (31–37). We showed that downregulation of MHC class I molecules occurs in T cells infected by HHV-6. MHC class I molecules are incorporated into viral particles and exosomes and then released into the extracellular environment, suggesting a possible strategy for escaping host immune responses. In addition, MHC class I molecules incorporated into virions and exosomes may assist viral entry. Further studies are needed to address this question.

We have previously reported that immature HHV-6 particles bud into TGN or TGN-derived vesicles (which are produced in HHV-6B-infected cells), that vesicles containing mature virions become MVBs, and that virions and exosomes are released into the extracellular environment via an exosomal secretary pathway (23). It is possible that MHC class I molecules are transported into the TGN-derived membranes from which the virions bud and then incorporated into virions within infected cells without being recycled (Fig. 3).

Within infected cells, MHC class I molecules colocalized with the gB protein in the cytoplasm indicating that, like viral glycoproteins, they are sorted into vesicles. The reduction in the total (both cell surface and

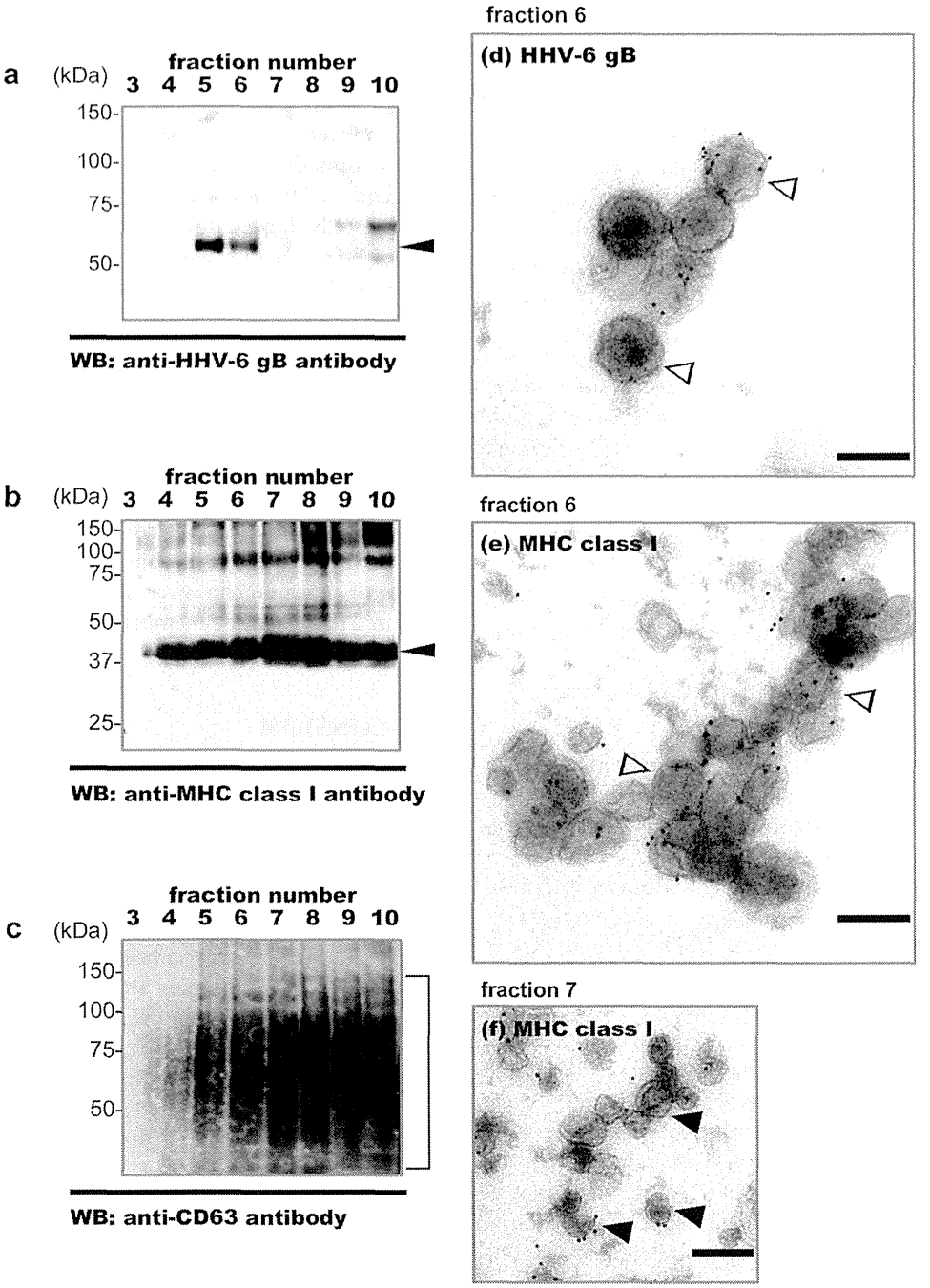

Fig. 1. MHC class I molecules are incorporated into virions and exosomes and released from HHV-6B-infected cells. Virions and exosomes were collected from the culture medium of HHV-6B-infected cells by sucrose density gradient centrifugation and examined by (a–c) western blotting and (d–f) electron microscopy. Western blots with (a) anti-gB rabbit, (b) anti-MHC class I (W6/32) or (c) anti-CD63 (CLB-gran/12, 435) antibodies are shown. The same amount of each protein fraction was added to each well of the gel. Immunogold labeling of (d) gB in fraction 6 and of (e,f) MHC class I in fractions 6 and 7. The fractions were collected from the bottom of tube. Hollow arrowheads, labeled virions; filled arrowheads, exosomes. Scale bars: 200 nm (d–f).

intracellular) expression of MHC class I in HHV-6-infected cells suggests that some of them may be transported to lysosomes and degraded, as this route is the same as that used to transport particles to MVBs.

Although several host proteins are usually expressed on the surfaces of uninfected cells, they are expressed in the same intracellular compartments as those in which viral particles incorporated. Newly formed compartments

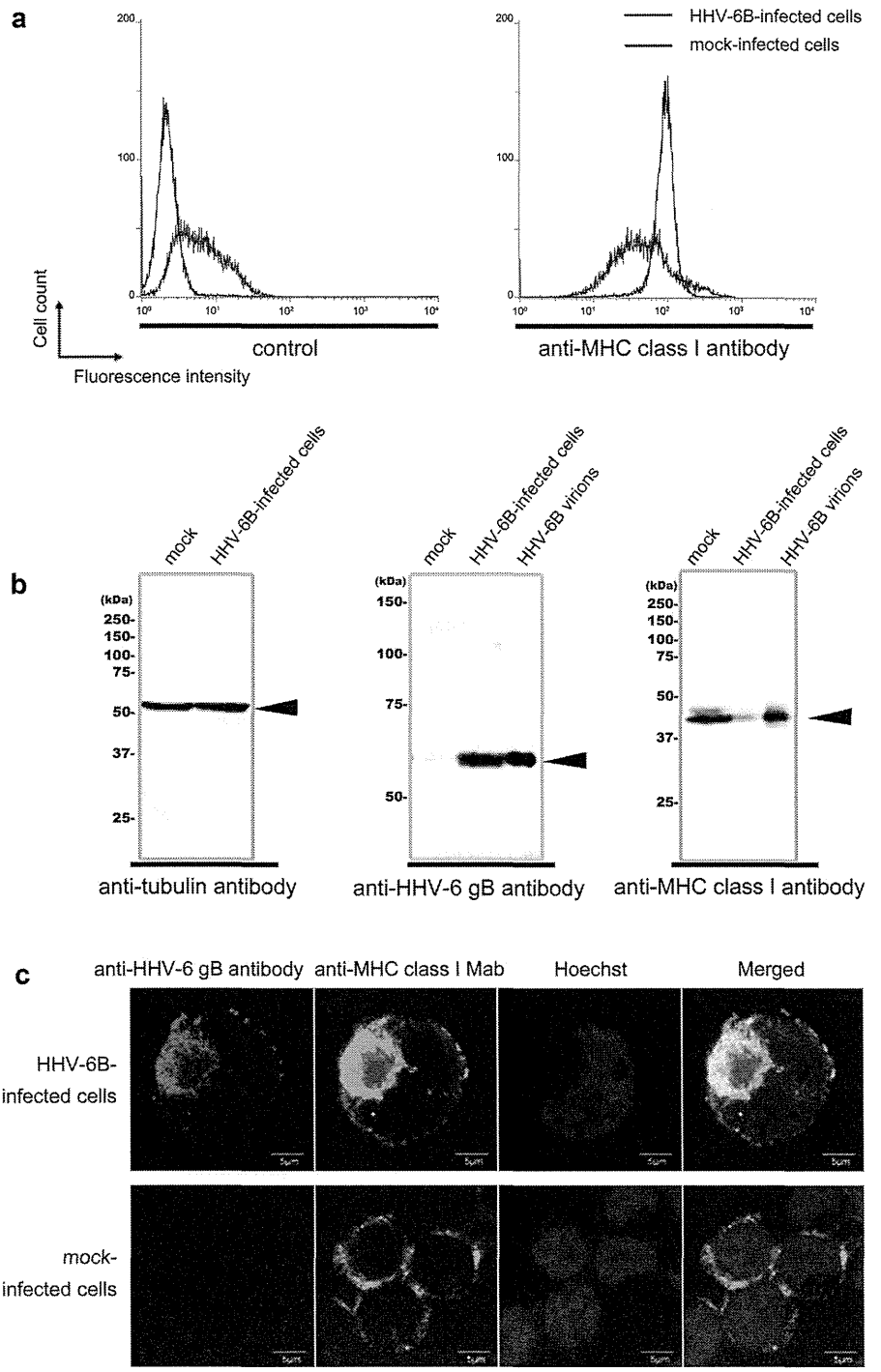

Fig. 2. Expression of MHC class I in HHV-6B-infected cells. (a) Expression of MHC class I on the surface of HHV-6B-infected cells is downregulated. HHV-6B-infected or mock-infected cells were harvested at 72 hr post-infection and fixed with 4% (w/v) paraformaldehyde. Fixed cells were stained with an anti-MHC class I antibody followed by staining with a secondary antibody prior to flow cytometric analysis. Control samples were incubated with the secondary antibody alone. Black histogram, mock-infected cells; blue histogram, HHV-6B- infected cells. (b) The total expression of MHC class I in HHV-6-infected cells was reduced. HHV-6B-infected or mock-infected cells were harvested at 72 hr post-infection and cell lysates prepared for western blotting. Purified HHV-6B virions were also used for western blotting. (c) MHC class I colocalizes with HHV-6B gB in intracellular compartments. HHV-6B-infected or mock-infected cells were harvested at 72 hr post-infection and fixed in cold acetone–methanol. Fixed cells were stained with antibodies against HHV-6 gB or MHC class I and with Hoechst33342. The stained cells were observed under a confocal microscope. The merged panels show the colocalized HHV-6 gB and MHC class I molecules. Single sections are shown. Scale bars: 5 micro meter.

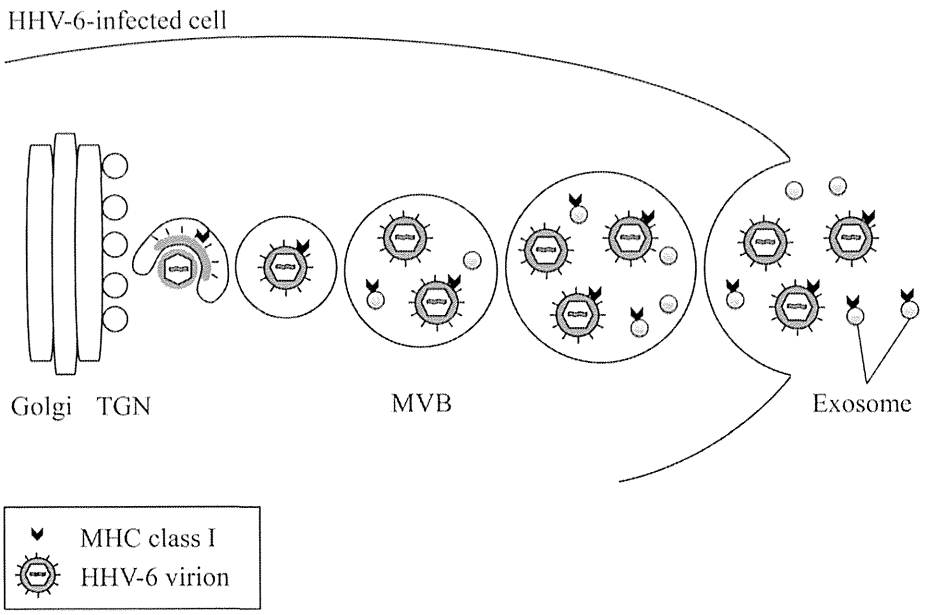

Fig. 3. Scematic representation of the fate of MHC class I molecules in HHV-6-infected cells. MHC class I molecules are transported to TGN- or post-TGN-derived vacuoles in HHV-6-infected cells and then incorporated into virions and intracellular small vesicles, which later become exosomes. Finally, MHC class I molecules are released from HHV-6-infected cells along with virions and exosomes.

within HHV-6-infected cells may show the combined characteristics of early and late endosomes. Recycling to early endosomes in HHV-6-infected cells may be modified or defective; therefore, several cellular proteins that use the same recycling system may be incorporated into virions and exosomes.

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DISCLOSURE

The authors declare that they have no competing interests.

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