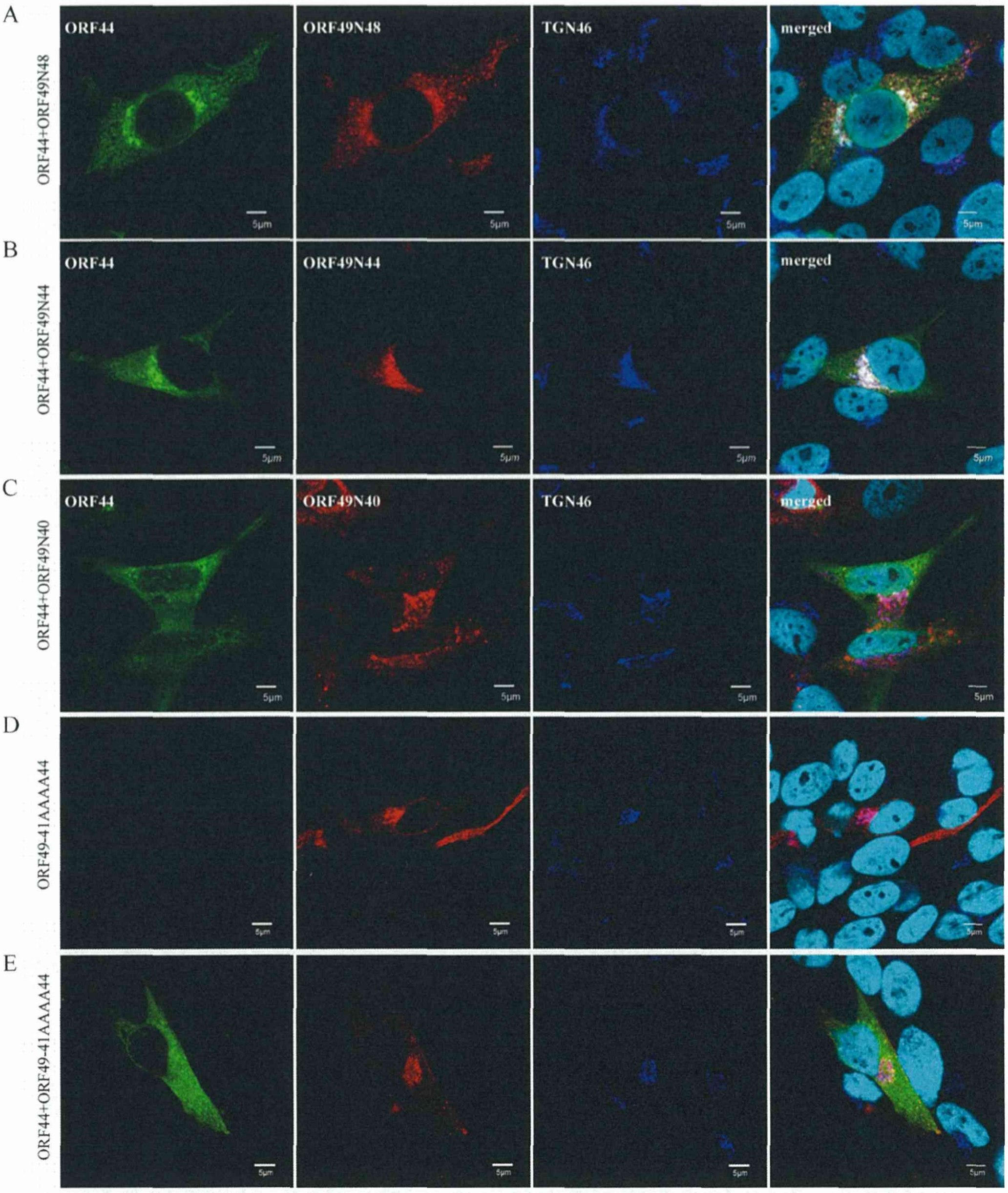

FIG 9 Localization and accumulation of ORF44p in MeWo cells expressing ORF49 mutant proteins. MeWo cells were cotransfected with CAG/ORF44 and CAG/ORF49N48 (A), CAG/ORF49N44 (B), CAG/ORF49N40 (C), or CAG/ORF49-41AAAA44 (E), or transfected with CAG/ORF49-41AAAA44 alone (D). Cells were fixed at 48 h posttransfection and triple labeled for ORF44 (green), ORF49 (red), and TGN46 (blue). Nuclei were stained with Hoechst 33342 (cyan). Scale bars, 5 μm.

Furthermore, ORF49-41AAAA44p did not form a complex with ORF44p or ORF44F129Ap (Fig. 6B, lane 5 or 6) despite the efficient coexpression of all proteins (Fig. 6A, lanes 5 and 6).

The carboxyl-terminal half of the acidic cluster of ORF49p plays a central role in the function of ORF49p during infection. rpOkaORF49-41AAAA44 showed almost the same phenotype as rpOkaORF49M1L, including the loss of the interaction with ORF44p (Fig. 2E, lane 1), the dispersed localization of ORF44p without accumulation on the TGN (Fig. 10A), impaired growth as assessed by plaque size and infectious-center assays (Fig. 11A and B, respectively), and reduced production of infectious progeny virus (to 3 to 10% of the wild-type level [Table 2]), excluding the apparent ORF49-41AAAA44p expression (Fig. 2D, lane 1, and Fig. 10A). These defects were completely rescued by revertant virus infection (Fig. 2D and E, lanes 2, Fig. 10B, and Fig. 11A and B) or by exogenous ORF49p in MeWoORF49 cells (Fig. 11A and B and Table 2). The expression of ORF49-41AAAA44p was detected as a faint and faster-migrating band than that of ORF49p in the revertant virus infection, but an equal amount of ORF44p was detected in both viruses with gH and ORF61p (Fig. 2D, lanes 1 and 2). Furthermore, whereas the interaction between ORF49-41AAAA44p and ORF44p was not detected at all (Fig. 2E, lane 1),

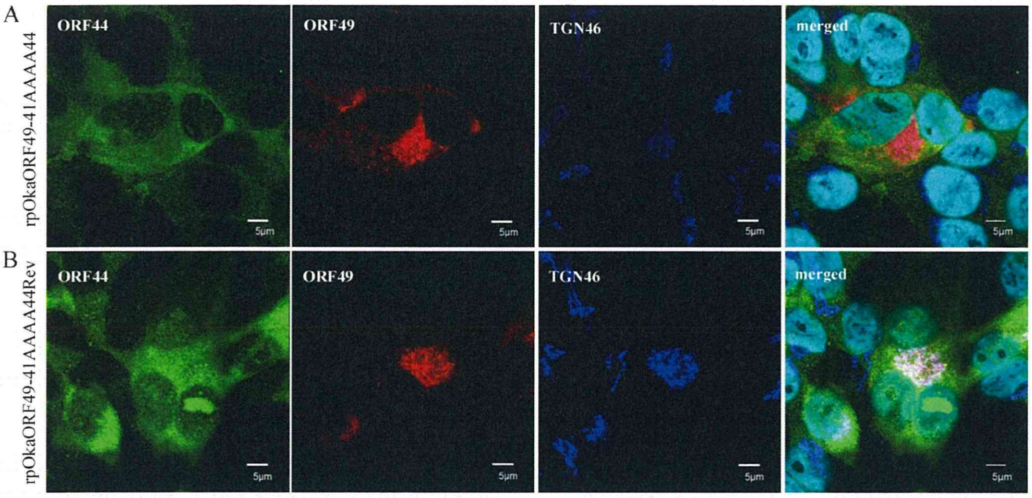

FIG 10 Localization of ORF44p and ORF49p in rpOkaORF49-41AAAA44-infected MeWo cells. rpOkaORF49-41AAAA44-infected MeWo cells (A) and rpOkaORF49-41AAAA44Rev-infected MeWo cells (B) were fixed at 48 hpi and triple labeled for ORF44p (green), ORF49p (red), and TGN46 (blue). Nuclei were stained with Hoechst 33342 (cyan). Scale bars, 5 μm.

ORF44p was incorporated into the rpOkaORF49-41AAAA44 particles in the absence or presence of exogenous ORF49p (Fig. 2F, lane 1 or 2), which was also the case in rpOkaORF49M1L infection (Fig. 2D and F).

Taken together, our results indicated that ORF49p functions in the efficient production of progeny viruses required for VZV infection through its interaction with the essential protein ORF44p.

DISCUSSION

In VZV, ORF49 encodes a nonessential tegument protein that is one of the cell-tropic factors in cell culture (6). In human fetal lung fibroblast MRC-5 cells, the growth of ORF49-defective virus is identical to that of its parental virus, whereas in the human melanoma MeWo cell line, it shows reduced growth; however, the cell tropism of VZV for these two most permissive cell lines has not been studied. In the previous study, we showed that it is a cell-tropic factor, although the step(s) at which ORF49 functions, including the entry, host gene modulation, viral gene expression, viral particle assembly, or egress, remained unclear. Nevertheless, we showed that it may play an important role in the production of a complete virion (6).

VZV is highly cell associated *in vitro*, producing extremely small amounts of infectious virus even when isolated intracellularly (in a particle-to-PFU ratio that varies from approximately 40,000 to 1,000,000) (39, 40), and the particles detected by electron microscopy (EM) analysis appear to be degraded, even in cells infected by the wild-type virus (41–43). The low infectivity and the presence of few or no infectious particles in the cell culture

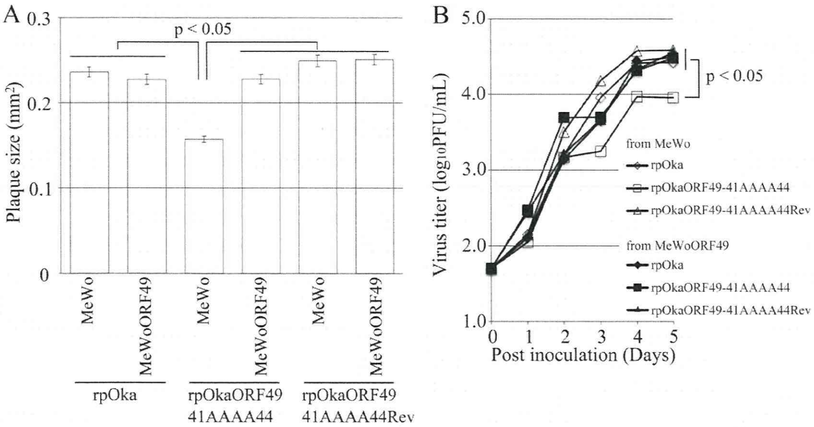

FIG 11 Growth properties of ORF49-41AAAA44 mutant virus in MeWo and MeWoORF49 cells. (A) Comparison of plaque sizes among recombinant viruses. MeWo cells or MeWoORF49 cells were infected with rpOka, rpOkaORF49-41AAAA44, or rpOkaORF49-41AAAA44Rev (50 PFU/well) and cultured for 7 days. Infected cells were stained with an anti-gE Ab, and the plaques were traced and measured by ImageJ software. Plaque size is shown with the standard error of the mean. Statistical significance was determined by Student's *t* test. (B) Growth kinetics of recombinant viruses on MeWo cells and MeWoORF49 cells. MeWo cells or MeWoORF49 cells were infected with rpOka, rpOkaORF49-41AAAA44, or rpOkaORF49-41AAAA44Rev (50 PFU/well), harvested at the indicated times, serially diluted, added to newly prepared MeWo cells, and cultured for 5 days. The plaques were stained with an anti-gE Ab and counted. Each point represents the mean titer for two wells of one experiment. Two experiments were performed independently. Statistical significance was determined by Student's *t* test.

supernatant in VZV have made it difficult to construct a *trans*-complementation system. In such a system, the target viral gene is expressed on permissive cells on which the target gene-deleted virus is only capable of efficient replication similar to the wild-type virus infection on the parental cells. The infection of parental cells by the target gene-deleted virus isolated from cells expressing the target gene enables analysis of its function. This method is useful to confirm that the deletion phenotype is not caused by undesired mutations, which can also be determined by generating the revertant virus to repair the mutated gene within the viral genome. In addition, it can also be used to identify the target gene function, which cannot be determined by simply generating the mutant and revertant viruses and has been used widely in the mutagenic analysis of other herpesviruses, especially in the analysis of structural proteins. However, to the best of our knowledge, this system has been used successfully to analyze gene function in only one report (44) and to confirm that the deletion phenotype is independent of undesired mutation in two reports (45, 46) in VZV research.

In the present study, we established a *trans*-complementation system for ORF49. The ORF49 *trans*-complementation system allowed identification of the precise function of ORF49, which could not be determined by generating its defective virus following EM analysis on MeWo cells. In the EM analysis, no significant differences between the wild-type and ORF49-defective viruses were detected with regard to the intracellular and cell surface viral particle counts or morphology (T. Sadaoka and Y. Mori, unpublished observation), possibly leading to their obvious difference in infectivity, which was reduced by 10-fold or higher in the defective virus. Additionally, in both viruses, the infected cells or viral particles isolated from the same quantity of infected cells contained almost the same amount of viral proteins. The *trans*-complementation system in combination with the results of other analyses described above indicated that ORF49 functions in the production of efficient infectious viruses. The results of EM analysis and immunoblotting suggested that the ORF49 defect did not cause the reduction of viral protein synthesis and viral particle assembly and egress. The cell-free virus titration and plaque formation analyses using the *trans*-complementation system showed that the ORF49p released from the virion into the cells during the entry step was not functional, but *de novo* ORF49p synthesized during lytic replication functioned in the production of efficient infectious virus required for cell-free and cell-to-cell viral transmission modes. However, how the deletion of ORF49 impaired infectivity remained unclear.

To gain further insight into the function of ORF49 during VZV infection, we confirmed ORF44p as its binding partner, as reported in other herpesviruses (12–15), and examined the conserved interaction between these proteins by analyzing their binding properties. We identified 129F in ORF44p as being essential for accumulation on the TGN through the interaction with ORF49p: whether it functions in the binding directly or indirectly is unknown. Simultaneously, 41DFDE44 of the carboxyl-terminal half of the acidic cluster within ORF49p was identified as the binding motif for ORF44p. Among these critical amino acids of ORF44p and ORF49p, each phenylalanine seems to function in the binding. As the phenylalanine is an aromatic and hydrophobic amino acid, it prefers to be buried in protein hydrophobic cores. However, 129F of ORF44p is surrounded by polar amino acid 128T and charged amino acid 130K and 42F of ORF49p by charged amino acids 41D, 43D, and 44E, and there is possibility that these two phenylalanines are exposed at the protein surface. The phenylalanine side chain is fairly nonreactive and is thus rarely directly involved in protein function, although it can play a role in substrate recognition. In particular, hydrophobic amino acids can be involved in binding/recognition of hydrophobic ligands, and the aromatic side chain can also be involved in interactions with other aromatic side chains via stacking interactions (47). In coexpression of ORF44 and ORF49, ORF49F42A mutation alone disrupted the interaction and failed to accumulate ORF44p on the TGN, as seen in ORF49-41AAAA44 mutation, while individual ORF49D41A, -D43A, or -E44A mutation had no effect on them (T. Sadaoka and Y. Mori, unpublished observation). On the other hand, ORF44F129A showed an impaired phenotype in terms of their interaction and its accumulation on the TGN, and neither ORF44T128A nor K130A mutation had any effect on them. However, in the context of infection, ORF49F42A alone could not abrogate the interaction and had no effect on virus growth (T. Sadaoka and Y. Mori, unpublished observation) different from that of ORF49-41AAAA44 mutation (discussed below), while only ORF44F129A was truly lethal for infectious virus production/reconstitution, and again ORF44T128A and ORF44K130A had no effect. These findings may prompt us to conclude that the core machinery of the binding is the noncovalent attractive force between two aromatic rings of phenylalanine and that the additional binding force via charged amino acids around 42F of ORF49p is required in the context of infection; however, there is another possibility—that the ORF44F129A mutation just disrupts the protein structure itself, leading to the loss of interaction. As mentioned above, phenylalanine prefers to be buried in protein hydrophobic cores, and the interaction among the aromatic residues is also important in the protein folding and the structural stabilization of protein (48, 49). Our results about the interaction property revealed that the binding domain within ORF44p is located at the first 136 residues and 129F is essentially involved in the binding, but could not be determined as the precise binding domain, and at the same time, there was no apparent degradation or lower expression of ORF44F129Ap in comparison with ORF44p in both prokaryotic expression and eukaryotic expression systems. To address these issues, further analyses by making an N-terminal truncation for refining the interaction domain and another 129F substitution with tyrosine, which differs only in that it contains a hydroxyl group in place of the ortho hydrogen on the benzene ring, for more preferable substitution to maintain structural stability will be helpful and ongoing.

Assessment of the function of ORF44 in the context of infection did not reveal new findings, with the exception of the F129A mutant, which showed the same phenotype as the deletion mutant. The ORF44 deletion and F129A mutation were lethal for progeny virus production/reconstitution in MeWo and MRC-5 cells; however, an effective *trans*-complementation system for ORF44 as for ORF49 was not successfully established, and at what step(s) in the lytic infection ORF44 essentially functions remained unclear. To find the nonessential but important functions of ORF44 in the context of infection, we turned back to analyzing the ORF49 function by generating ORF49-41AAAA44 virus, in which ORF49p specifically lost the interaction with ORF44p, following comparison of the phenotype between ORF49-defective virus and ORF49-41AAAA44 virus. The rpOkaORF49-41AAAA44 virus showed the same phenotype as the rpOkaORF49M1L virus, indicating that the function of ORF49p in the efficient production of 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

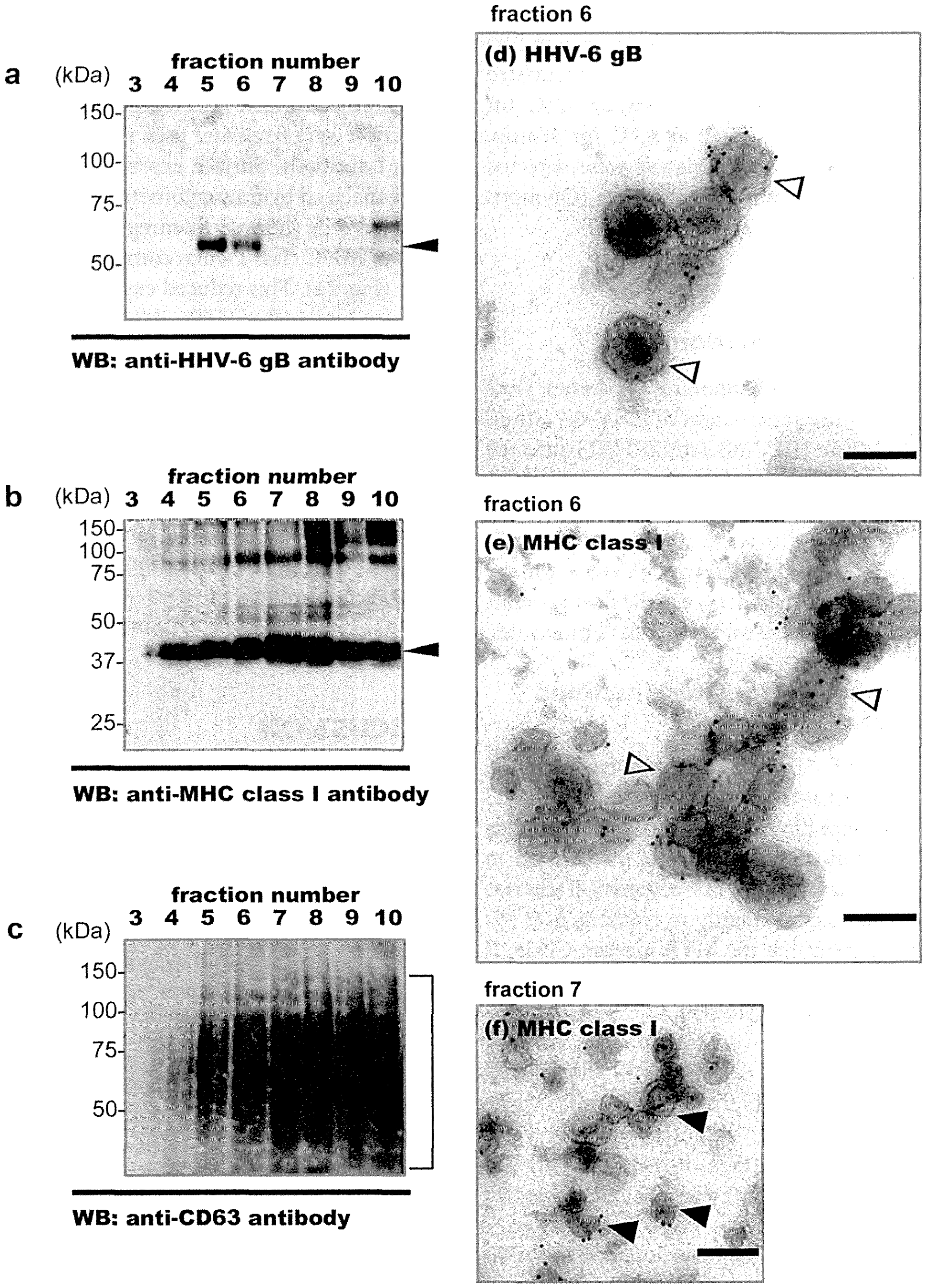

Fig. 1. MHC class I molecules are incorporated into virions and exosomes and released from HHV-6B-infected cells. Virions and exosomes were collected from the culture medium of HHV-6B-infected cells by sucrose density gradient centrifugation and examined by (a–c) western blotting and (d–f) electron microscopy. 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

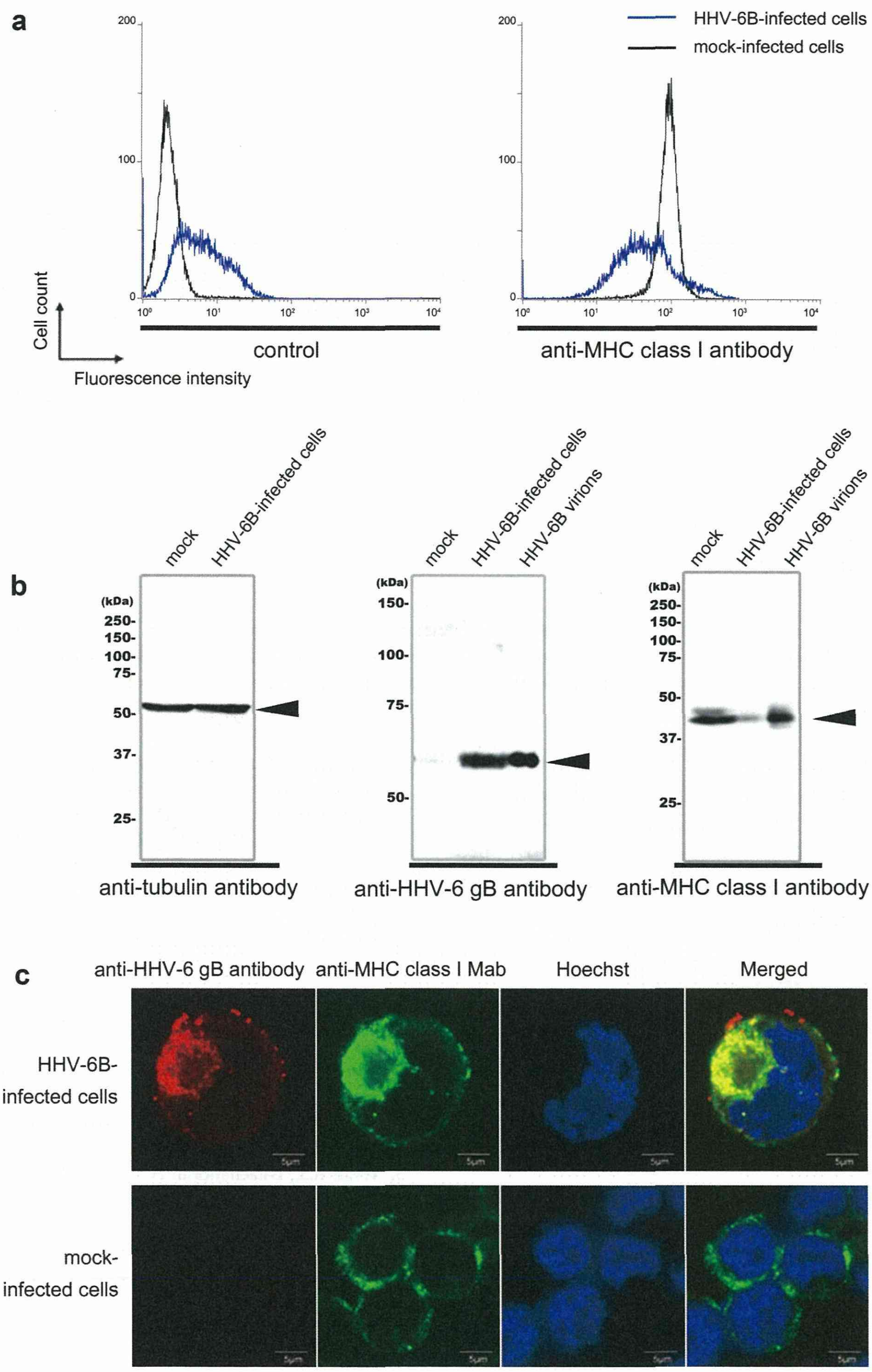

Fig. 2. Expression of MHC class I in HHV-6B-infected cells. (a) Expression of MHC class I on the surface of HHV-6B-infected cells is downregulated. HHV-6B-infected or mock-infected cells were harvested at 72 hr post-infection and fixed with 4% (w/v) paraformaldehyde. Fixed cells were stained with an 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.