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Characterization of the human herpesvirus 6A U23 gene

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ABSTRACT

Human herpesvirus 6 (HHV-6), which replicates abundantly in T cells, belongs to the *Roseolovirus* genus within the betaherpesvirus subfamily. Members of the *Roseolovirus* genus encode seven unique genes, U20, U21, U23, U24, U24A, U26, and U100. The present study focused on one of these, U23, by analyzing the characteristics of its gene product in HHV-6A-infected cells. The results indicated that the U23 protein was expressed at the late phase of infection as a glycoprotein, but was not incorporated into virions, and mostly stayed within the trans Golgi network (TGN) in HHV-6A-infected cells. Furthermore, analysis using a U23-defective mutant virus showed that the gene is nonessential for viral replication in vitro.

Introduction

Human herpesvirus (HHV)6 is closely related to HHV-7, and both viruses belong to the *Roseolovirus* genus of the betaherpesvirus subfamily, which is characterized by growth in T lymphocytes, high prevalence, and association with febrile illness (Caselli and Di Luca, 2007; Nicholas, 1996). HHV-6 has been classified into two variants, HHV-6A and HHV-6B, based on cell tropism and genetic and antigenic differences (Aubin et al., 1991; Campadelli-Fiume et al., 1993; Salahuddin et al., 1986; Wyatt et al., 1990). More recently, they were re-classified into two species, HHV-6A and HHV-6B, on the basis of distinct biological features (see the Virus Taxonomy List 2011), although the sequence identity between the HHV-6A and -6B is almost 90% over their entire genome (Dominguez et al., 1999; Gompels et al., 1995; Isegawa et al., 1999). HHV-6B causes exanthem subitum in primary infections (Yamanishi et al., 1988), and is ubiquitous, with more than 90% of adults having antibodies against it (Okuno et al., 1989; Ward et al., 1993). However, the diseases caused by HHV-6A are unknown. *Roseolovirus* genomes encode seven unique genes, U20, U21, U23, U24, U24A, U26, and U100. The U100 gene encodes the gQ protein, which is important for virus entry (Akkapaiboon et al., 2004; Kawabata et al., 2011; Maeki et al., 2013; Mori, 2009; Mori et al., 2003a, 2003b; Tang et al., 2010,2011). HHV-7 U21 binds to class I major histocompatibility complex (MHC) molecules and down-regulates their expression on the cell surface (May et al., 2010). HHV-6 A U24 mediates a rapid relocalization of cluster-determinant 3 (CD3) and T-cell receptor (TCR) proteins from the cell surface to early endosomes (Sullivan and Coscoy, 2010). Furthermore, U24 mediates the downregulation of TCR and also the transferrin receptor (TfR), through a PPXY motif near the amino terminus of U24, suggesting a general block in early endosomal recycling (Sullivan and Coscoy, 2008, 2010). U20 is responsible for HHV-6B inhibition of TNF receptor-dependent signaling and apoptosis (Kofod-Olsen et al., 2012). The U23 gene, however, has remained largely uncharacterized. Therefore, in this study, we focused on the HHV-6A U23 gene, and characterized the gene product in HHV-6 A-infected cells. We found that U23 is a glycoprotein expressed at the late phase of infection that localizes mainly to the trans Golgi network (TGN), but is not expressed in virions. Analysis of U23-defective mutant viruses showed that this gene is nonessential for viral replication in vitro.

Results

As shown in Fig. 1a, U23 is encoded in the unique region of the HHV-6A genome (Dominguez et al., 1999; Gompels et al., 1995; Isegawa et al., 1999). The predicted protein sequence encoded by the U23 ORF is 236 amino acids long, and contains two hydrophobic regions, possibly a signal sequence and a transmembrane domain.

To analyze the U23 gene product, we first produced a monospecific antibody (ab) against the U23 protein, as described in the "Materials and methods" section. The specificity of the obtained ab was confirmed using U23-expressing plasmid-transfected cells (Fig. 1b). Next, its expression was examined in HHV-6A-infected cells.

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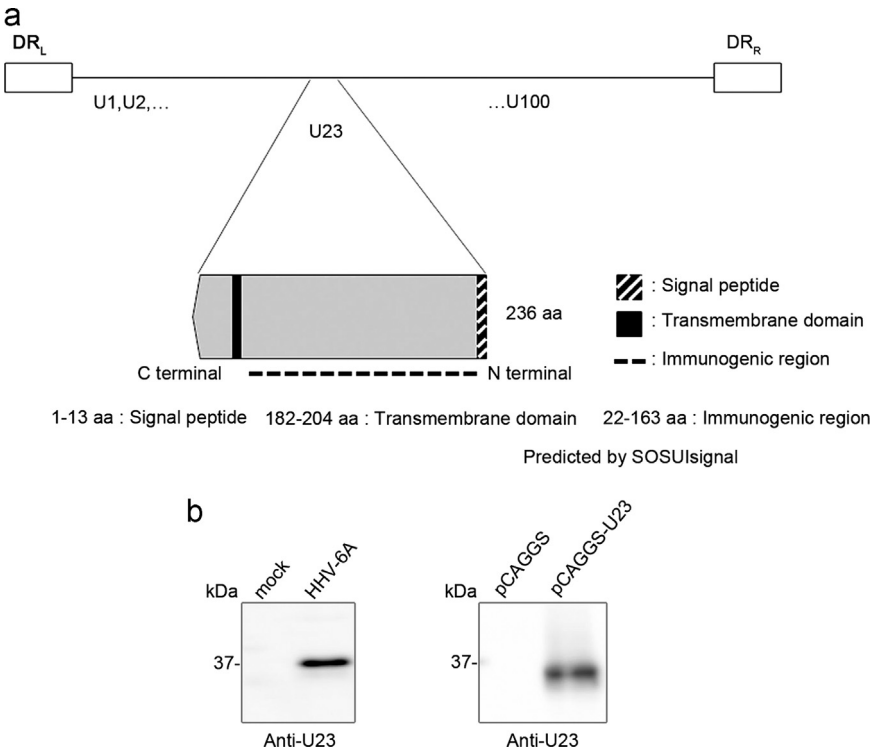

Fig. 1. HHV-6A-specific reactivity of anti-U23 antibody. (a) Schematic interpretation of the structure of HHV-6A U23. The arrow-shaped box represents the ORF, including the N and C terminals. The dotted line represents the position of the immunogenic region used to produce the antibodies. The black-and-white-banded square represents the predicted signal peptide and the dark square represents the predicted transmembrane domain. SOSUI was used to conduct sequence analyses (⟨http://bp.nuap.nagoya-u.ac.jp/sosui/sosuisignal/sosuisignal_submit.html⟩). (b) HSB-2 cells were mock-infected or infected with HHV-6A strain GS. The cells were lysed in RIPA buffer (containing 0.1% SDS, 1% NP40) at 48 h post-infection. The 293T cells were transfected with plasmid pCAGGS-U23, or plasmid pCAGGS as a negative control. The cells were lysed in TNE buffer (containing 1% NP40) at 48 h post-infection. Lysates were resolved by SDS-PAGE with 13% polyacrylamide under non-reducing conditions, and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The blots were then incubated with anti-U23 polyclonal antibody.

A protein of approximately 37 kDa was detected by the anti-U23 ab in HHV-6A-infected cells, but not in mock-infected cells (Fig. 1b).

Next, we examined the kinetics of U23 protein expression. U23 was detected at 1 day post-infection (PI) and its expression was gradually increased over 3 days PI (Fig. 2a). HHV-6A gQ1 was used as a positive control for infection (Fig. 2a). To examine the kinetics of infection in more detail, HHV-6A was infected with or without phosphonoformic acid (PFA). In the presence of PFA, U23 protein was not detected, and neither was gQ1, which is a late protein. However, two other proteins, IE1 (immediate early) and U27 (early), were detected (Fig. 2b), indicating that U23 is a late protein.

U23 is predicted to be a glycoprotein. Therefore, HHV-6A-infected cell lysates were digested with endo H or PNGase F. As expected, U23 expressed in HHV-6A-infected cells was digested with both endoglycosidases, indicating that U23 is an N-linked glycoprotein (Fig. 3). The gQ1 protein is also shown, as a positive control for the digestion (Fig. 3).

As we found that U23 is a glycoprotein, we next examined whether U23 is expressed in virions, because generally viral glycoproteins that are expressed at the late phase are incorporated into viral particles.

Unexpectedly and surprisingly, whereas gQ1-80K was detected in virions, U23 was not, indicating that U23 is not an envelope glycoprotein (Fig. 4).

Because U23 was not expressed in virions, even though it is a late-phase glycoprotein, we examined its localization in HHV-6A-infected cells. As predicted, it rarely colocalized with gB, which is an envelope glycoprotein, nor did it colocalize with CD63 and Lamp1. It mainly colocalized with TGN46 and, to a lesser degree, with Calnexin. These results indicate that U23 is mainly found at the TGN in infected cells (Fig. 5).

Next, we examined whether U23 is essential for virus growth. To study this, we constructed a HHV-6A bacterial artificial chromosome (BAC) with a U23 gene mutated to substitute tagg for methionine residues (HHV-6ABACΔU23) (Fig. 6a), and transfected it into the cells. The virus assembled from HHV-6ABACΔU23 appeared to be identical to virus assembled from HHV-6ABAC (Fig. 6c). Its revertant genome (HHV-6ABACΔU23rev) was also constructed, and virus was reconstituted from this genome. To confirm whether U23 could have been knocked out, a Western blot was performed using the virus-infected cells. U23 was not detected in rHHV-6ABACΔU23-infected cells, but it was detected in rHHV-6ABAC-infected cells, indicating that U23 was not expressed in rHHV-6ABACΔU23-infected cells (Fig. 6b). These results indicate that U23 is nonessential for virus replication. Next, we compared the growth of rHHV-6ABACΔU23 with that of the original virus and its revertant virus. As shown in Fig. 7, there were no differences in virus growth between rHHV-6ABACΔU23, rHHV-6ABAC, and rHHV-6ABACΔU23rev, indicating that U23 is not necessary for virus growth in vitro.

Discussion

The genus *Roseolovirus* encodes several unique genes (Caselli and Di Luca, 2007; Nicholas, 1996), including the previously uncharacterized HHV-6A U23. Bioinformatic predictions suggested that it was a type I membrane-spanning protein. First, we investigated whether the U23 ORF product was expressed in HHV-6A-infected cells by producing ab against U23. U23 was detected as an approximately 37 kDa late-phase protein expressed in HHV-6A-infected cells. Furthermore, complete digestion with endo H and PNGase F indicated that U23 was an N-linked glycoprotein, although it was not incorporated into viral particles. Generally, herpesvirus glycoproteins expressed at late phase

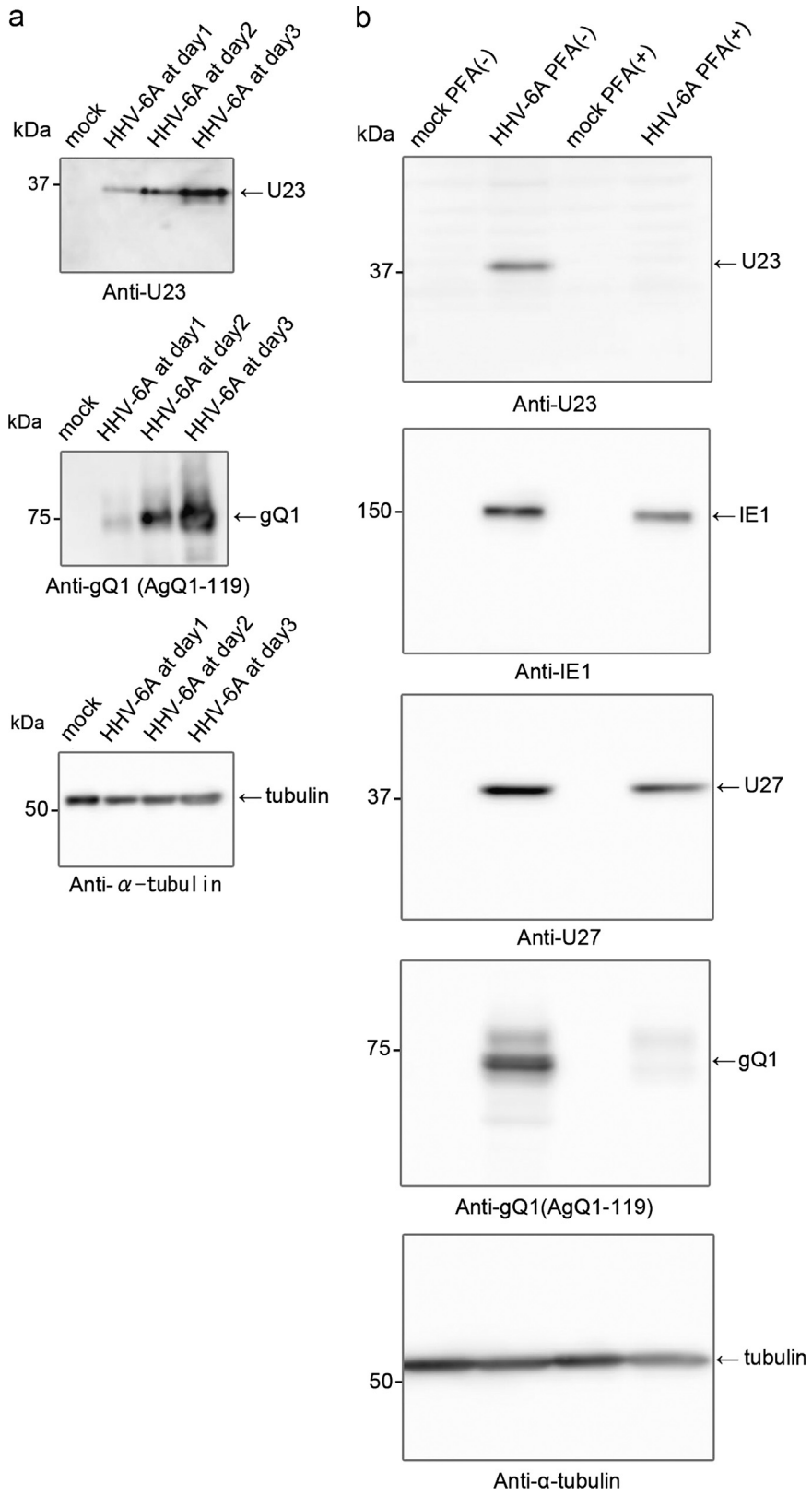

Fig. 2. Kinetic analysis of U23 expression. (a) HSB-2 cells were infected with strain GS and then harvested at 1, 2, and 3 days post-infection. The cells were lysed in RIPA buffer. Lysates were resolved by SDS-PAGE with 13%, 7.5% or 10% polyacrylamide under non-reducing or reducing conditions, and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The blots were incubated with anti-U23, anti-gQ1, or anti-α-tubulin antibodies. (b) HSB-2 cells were infected with HHV-6A strain GS in the presence of PFA (300 μg ml^{-1}) or in the absence at 48 h post-infection. The cells were lysed in RIPA buffer. Lysates were resolved by SDS-PAGE with 13% or 7.5% polyacrylamide under non-reducing or reducing conditions, and electrotransferred to PVDF membranes. The blots were then incubated with anti-U23 polyclonal antibody or anti-U27, anti-IE1, anti-gQ1, or anti-α-tubulin monoclonal antibodies.

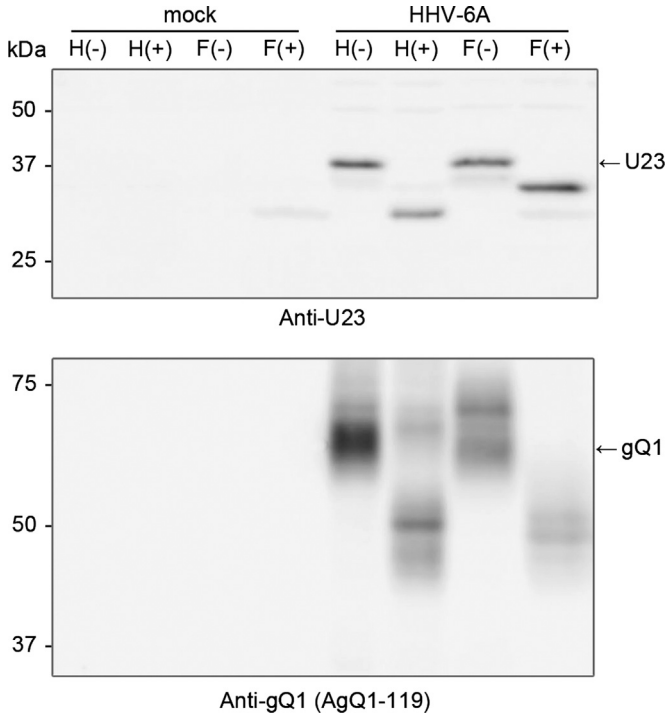

Fig. 3. Glycosylation analysis of U23. HSB-2 cells were mock-infected or infected with HHV-6A strain GS. The cells were harvested at 72 h post-infection. The cells were lysed in RIPA buffer and digested with endo H (H) or PNGase F (F), resolved by SDS-PAGE with 13% or 7.5% polyacrylamide under non-reducing conditions, and electrotransferred to PVDF membranes. The blots were incubated with anti-U23 polyclonal antibody or anti-gQ1 monoclonal antibody.

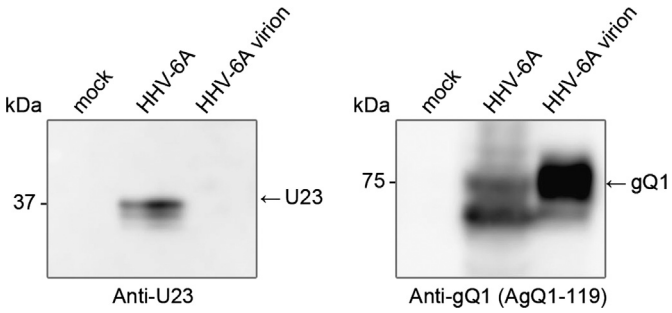

Fig. 4. Western blot of purified HHV-6A virions. Purified HHV-6A virions were resolved by SDS-PAGE with 13% or 7.5% polyacrylamide under non-reducing or reducing conditions, and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The blots were then incubated with anti-U23 polyclonal antibody or anti-gQ1 monoclonal antibody. Strain GS-infected HSB-2 cells lysed at 48 h post-infection were used as a positive control (HHV-6A).

of infection, are incorporated into virions, where they work as envelope glycoproteins. Interestingly, U23 did not appear to be an envelope glycoprotein. Furthermore, we found that it did not colocalize with gB and CD63, which are incorporated into viral particles, nor did it colocalize with lamp 1, which is usually detected at endosomes. It is interesting that U23 stays in the TGN and is not transported to viral budding sites as are almost all of the other viral proteins. Since it was found to be localized mainly to the TGN, U23 may be involved in the modification of the cellular machinery in HHV-6A-infected cells, perhaps in the modification of sorting or recycling of cargo proteins.

As the U23 gene is unique to the *Roseolovirus* genus, it was thought not to be a core gene for virus replication. As predicted, U23-defective mutant viruses were reconstituted from the genome and growth of the mutants appeared to be identical to that of the original type virus in vitro.

However, the function of U23 in HHV-6A infection is still unknown. U23 may play an important role in *in vivo* virus replication and *Roseolovirus*-specific pathogenesis. Recently, an animal model for HHV-6A has been shown (Tanner et al., 2013). The model would be useful to analyze HHV-6A pathogenesis.

Materials and methods

Cells and virus strains

The human T-cell lines JJhan and HSB-2 were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with 8% fetal bovine serum, and 293 T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 8% fetal bovine serum. Umbilical cord blood mononuclear cells (CBMCs) were prepared as previously described (Dhepakson et al., 2002). CBMCs were provided by K. Adachi (Minoh Hospital, Minoh, Japan) and H. Yamada (Kobe University Graduate School of Medicine, Kobe, Japan) and purchased from the Cell Bank of the RIKEN BioResource Center, Japan. The HHV-6 A strain GS was propagated and titrated in HSB-2 cells. Virions were purified as described previously (Mori et al., 2008). Phosphonoformic acid (PFA) inhibits viral DNA synthesis. HSB-2 cells were infected with HHV-6A strain GS, cultured in medium with PFA (300 $\mu g\,ml^{-1}$), and harvested at 48 h PI. Regarding CBMC usage, it was approved by the ethical committees of Kobe University Graduate School of Medicine.

Antibodies

Rabbit antisera specific for HHV-6A protein U23 were generated from rabbits injected with glutathione S-transferase (GST)–U23 fusion protein, and boosted with U23 protein that GST-tag had been cleaved. The primer pair AU23bamF64 (5′-ACCGGATCCACTACAGAAGTTTCGGC-3′) and AU23xhoR489 (5′-ACCCTCGAGGACTTGAACAGTATCAG-3′) was used for PCR amplification. The PCR product was then inserted in-frame into the pGEX6P-1 bacterial expression vector (GE Healthcare Bio-Sciences, Piscataway, NJ) via the BamHI and XhoI sites (underlined). The resultant GST-U23 fusion protein was expressed in *Escherichia coli*, affinity purified, and used to produce abs in rabbits. For cleavage of GST-tagged protein, PreScission Protease was purchased from GE Healthcare. Eluted GST-tagged U23 protein was digested with PreScission Protease as specified by the manufacturer.

The monoclonal antibodies (Mabs) for HHV-6A gB (OHV-1), gQ1 (AgQ-119), IE1, and U27 were described previously (Akkapaiboon et al., 2004; Huang et al., 2006; Okuno et al., 1992). Mabs were purchased for α-tubulin (Sigma-Aldrich, Japan), calnexin (Abcam, Cambridge, United Kingdom), CD63 (Sanquin Blood Supply, Amsterdam, The Netherlands), and Lamp1 (Santa Cruz Biotechnology (SCBT), Dallas, Texas). Polyclonal antibodies (Pabs) for TGN46 were purchased from AbD Serotec, Oxford, United Kingdom. The following secondary abs were used: Alexa Fluor 488 or 594-labeled donkey anti-mouse, -sheep, or -rabbit immunoglobulin G (Molecular Probes-Eugene, Oregon).

Immunofluorescence assays

Indirect immunofluorescence assays (IFA) were performed as described previously (Dhepakson et al., 2002). Specific immunofluorescence was observed with a confocal laser scanning microscope (OLYMPUS FV1000D).

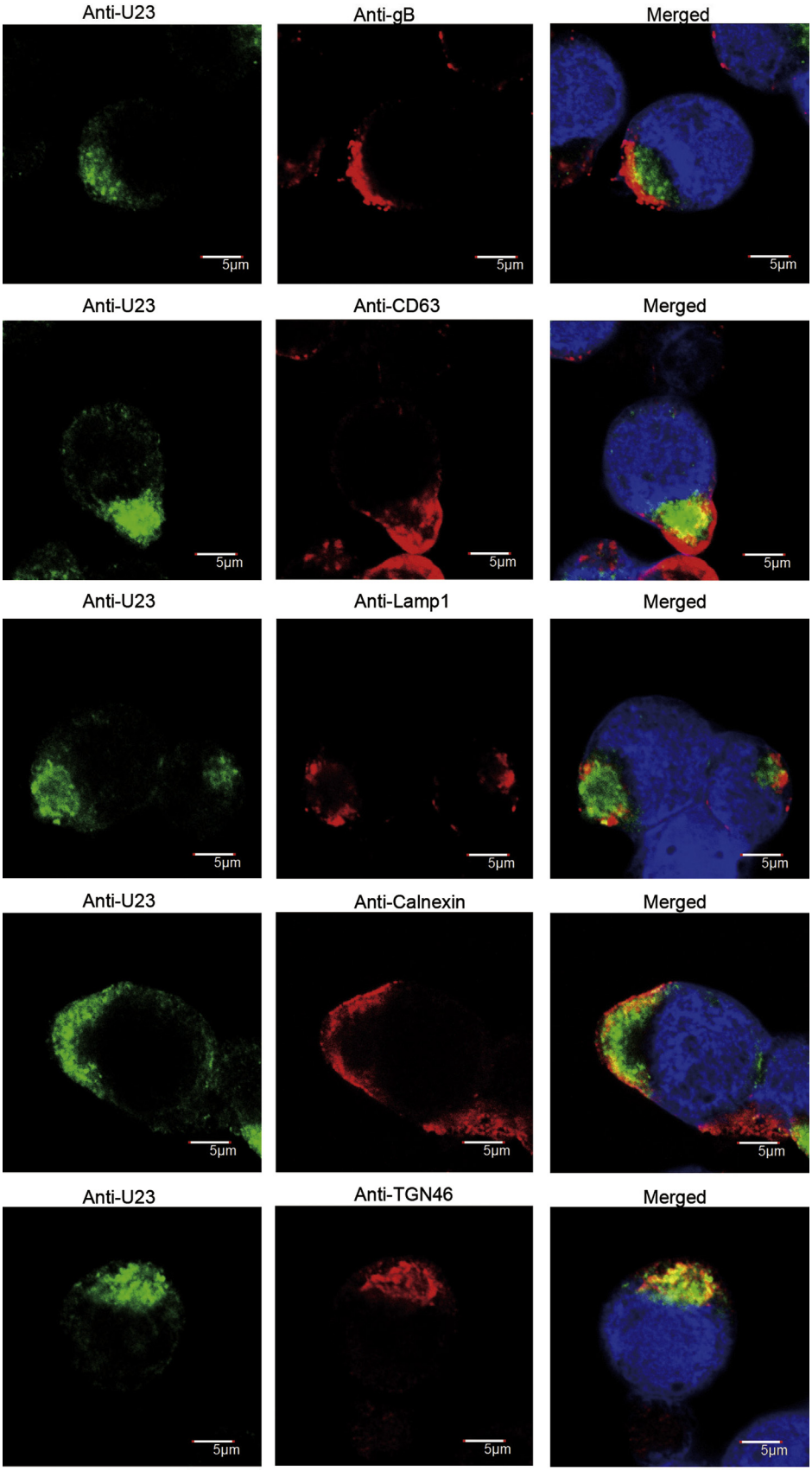

Fig. 5. C**ellular localization of the U23 protein in HHV-6A-infected cells.** HSB-2 cells were infected with strain GS. At 48 h post-infection, the cells were harvested for immunofluorescence analysis. Antibodies for U23, gB, CD63, Lamp1, Calnexin, or TGN46 were used, along with Hoechst 33342 for staining. Single sections are shown. Scale bar: 5 μm.

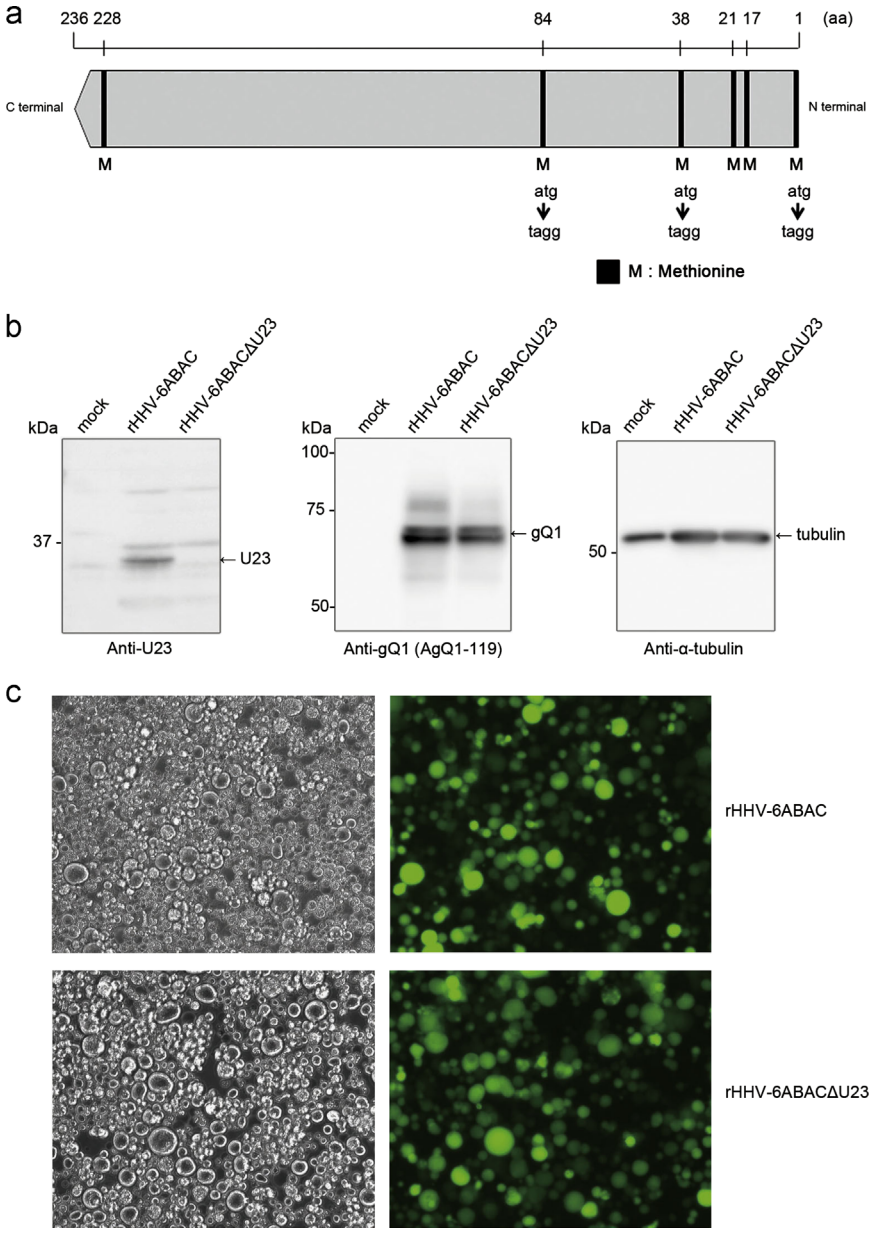

Fig. 6. Cytopathic effect and green fluorescent protein (GFP) expression in reconstituted virus-infected cells. (a) At the top, the scale bar indicates the full length of the HHV-6A U23 gene. The mutated parts of the methionine (M) site of the U23 gene sequence are also shown. (b and c) Umbilical cord blood mononuclear cells were infected with rHHV-6ABAC or rHHV-6ABACΔU23 and then cocultured with JJhan cells to permit cell-to-cell infection. Cells were harvested 3 days after the start of cell-to-cell infection. (b) The samples were resolved by SDS-PAGE with 13% or 7.5% polyacrylamide under non-reducing or reducing conditions, and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The blots were reacted with anti-U23 polyclonal antibody, anti-gQ1 monoclonal antibody, or anti-α-tubulin monoclonal antibody. (c) Light microscopic images are shown at the left, and GFP fluorescence images in the same microscopic field are shown at the right; rHHV-6ABAC (upper panel) and rHHV-6ABACΔU23 (lower panel).

Western blotting

Western blotting was performed as described previously (Akkapaiboon et al., 2004). The bound antibodies were detected by chemiluminescence using Western blotting detection reagents (Nacalai Tesque) and were visualized with the LAS minidetection system (GE Healthcare).

Glycosidase digestion

For endoglycosidase digestion, endoglycosidase H (endo H) and peptide *N*-glycosidase F (PNGase F) were purchased from New England Biolabs. Lysed cells were resuspended in digestion buffer and digested with endo H or PNGase F as specified by the manufacturer (Akkapaiboon et al., 2004).

Plasmid constructions

HHV-6A U23 expression vectors were constructed by using plasmid pCAGGS. Plasmid pCAGGS was kindly provided by Dr. Jun-ichi Miyazaki, Osaka University, Japan (Niwa et al., 1991). A DNA fragment comprising the full-length U23 gene open reading frame was amplified by PCR and inserted in-frame into pCAGGS via the NotI and XhoI sites.

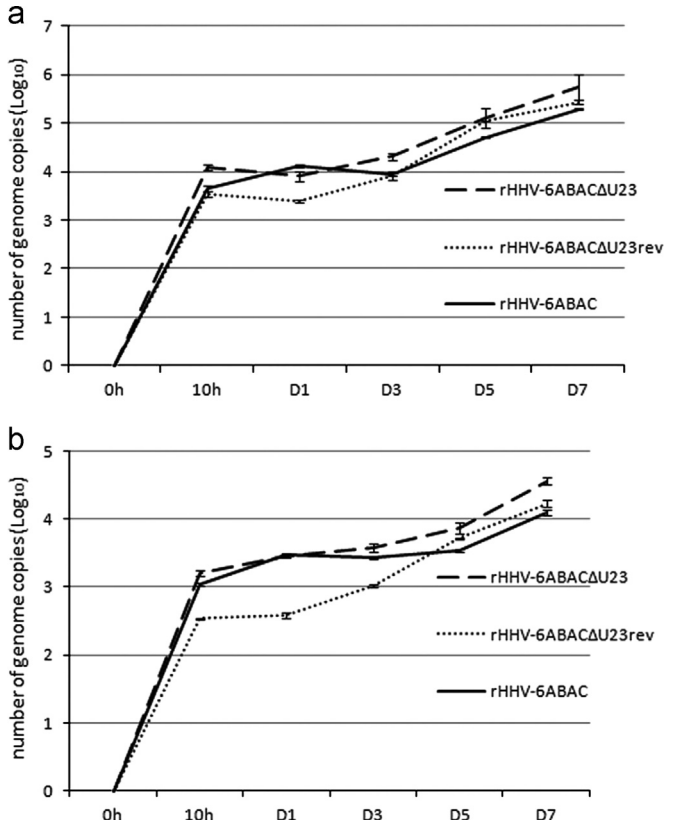

Fig. 7. Comparison of growth kinetics of rHHV-6ABAC, rHHV-6ABACΔU23, and rHHV-6ABACΔU23rev. CBMCs were infected with rHHV-6ABAC, rHHV-6ABACΔU23, or rHHV-6ABACΔU23rev at MOI (multiplicity of infection) of approximately 0.03 (a and b). The cells and culture supernatants were harvested at 0 h, 10 h, 1 day, 3 days, 5 days, or 7 days post-infection. The viral genome copy number in cells (a) or culture supernatants (b) in each sample was quantified by real-time PCR. The data shown represent one of three independent experiments.

Plasmid transfections

293 T cells were transfected by the calcium phosphate method as described previously (Koshizuka et al., 2010).

Construction of HHV-6ABACΔU23 and its revertant

HHV-6ABACΔU23 and its revertant were constructed by using modified two-step Red-mediated mutagenesis (Oyaizu et al., 2012; Tang et al., 2010; Tischer et al., 2006). First, U23 was amplified from HHV-6ABAC using the primers of 5′-accggatccgatccccctcggacgccgc-3′ and 5′-acagtcgacttagtaaaccgagatacag-3′. Next the PCR product was digested with BamHI and SalI enzymes and cloned into pBluescript SK (−) vector. Then, the codons for three methionine residues (No. 1, 38, and 84) in the U23 gene were mutated using the QuikChange lightning multi site-directed mutagenesis kit (Agilent Technologies) and the primers: 5′-GGGCAATCGCAAAAACTAGGTTGTTCTTGTC-3′, 5′-GCGTCTAAAAACTAGGGAAACGGATGTATCG-3′ and 5′-ATCAGTCTTTTTAGGGTGACCTCTACG-3′, according to the manufacturer's protocol. Next, the first Red-recombination was conducted by transforming GS1853 *E. coli* harboring the G-1 clone of HHV-6ABAC with ampicillin and sacB gene fragment amplified from pST76A-SR plasmid using the primers: 5′-GCTATCTTGGTGTGGGTGTGATTAAAAAACCGCTGCACTTGGGCAATCGCGGATCTTCACCTAGATCC-3′ and 5′-TCATCTGCAAGTCTTTACATCCCTCCTCATTTGTACATCCGGTTTTTTCTCCACCCCAGGCTTTACAC-3′. This recombination resulted in the replacement of the U23 gene in HHV-6ABAC with ampicillin and sacB genes. Then, the secondary Red-recombination was performed by transforming the resultant *E. coli* with the U23 mutant gene fragment, which had been amplified from pBluescript SK(−)U23m (described above), using the primers: 5′-CTGCTATCTTGGTGTGGGTGTG-3′ and 5′-TCATCTGCAAGTCTTTACATCCCTCCTC-3′. The *E. coli* were selected on LB plates containing chloramphenicol and 5% sucrose. The resultant BAC was designated HHV-6ABACU23m. Its revertant was similarly constructed, as follows. First, Red-recombination was performed by transforming GS1783 harboring HHV-6ABACΔU23 with ampicillin and sacB gene fragment. Then, the resultant *E. coli* were transformed with the U23 gene, which was amplified from pBluescript SK(−)U23 (described above). The resultant BAC was designated HHV-6ABACΔU23rev.

Viral growth assay

The viral growth assay was performed as described previously (Oyaizu et al., 2012).

Acknowledgments

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