Maturation of Human Herpesvirus 6A Glycoprotein O Requires Coexpression of Glycoprotein H and Glycoprotein L

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Glycoprotein O (gO) is conserved among betaherpesviruses, but little is known about the maturation process of gO in human herpesvirus 6 (HHV-6). We found that HHV-6 gO maturation was accompanied by cleavage of its carboxyl terminus and required coexpression of gH and gL, which promoted the export of gO out of the endoplasmic reticulum (ER). Finally, we also found that gO was not required for HHV-6A growth in T cells.

Human herpesvirus 6 (HHV-6) is an enveloped DNA virus of the betaherpesvirus subfamily (1). Initially, HHV-6 was divided into two variants, HHV-6A and HHV-6B, based on differences in the biology, immunology, and epidemiology of isolated strains of the virus (2–5). Recently, the virus was officially classified as two different species, namely, HHV-6A and HHV-6B, by the International Committee on Taxonomy of Viruses (6). Two glycoprotein complexes, gH/gL/gQ1/gQ2 and gH/gL/gO, are expressed on the HHV-6 envelope (7–9). The former complex binds to CD46 (HHV-6A) (10, 11) or CD134 (HHV-6B) (12), which plays a key role in viral entry into target cells.

Viral glycoproteins mature after they are transported from the endoplasmic reticulum (ER) to the Golgi or beyond. This process is accompanied by modification of glycans and/or other moieties and, in some cases, by cleavage of the glycoproteins (13). When glycoproteins are transported from the ER to the Golgi, some (or all) of the N-linked oligosaccharides on the glycoproteins are modified from high-mannose oligosaccharides into complex oligosaccharide. These two types of oligosaccharides are differentially resistant to endo- β -N-acetylglucosaminidase H (Endo H; releasing high-mannose glycans only) and peptide-N-glycosidase F (PNGase F) digestion (releasing both high-mannose and complex glycans), resulting in digested glycoproteins of different molecular weights; thus, it is easy to analyze the maturation process of glycoproteins by monitoring the N-linked oligosaccharide modifications they carry.

The HHV-6 U47 open reading frame (ORF) encodes gO. HHV-6A gO is predicted to be a 651-amino-acid protein with a polypeptide backbone of 73 kDa. Previously, we reported that two forms of gO with different molecular masses, around 120 kDa and 75 kDa, are expressed in HHV-6-infected cells. The large gO modified with high-mannose oligosaccharide is not a component of the gH/gL/gO complex, and the small gO is modified with complex N-linked glycans, forms a complex with gH/gL, and is incorporated into HHV-6 virions (9); however, the orientation and maturation processes of HHV-6 gO proteins are unknown. Maturation of human cytomegalovirus (HCMV) gO depends on the presence of gH and gL (14). Similarly, maturation of gQ1 and gQ2 in HHV-6A depends on the coexpression of gH and gL (15).

Therefore, we tested whether HHV-6A gH and gL also function in gO maturation. To this end, we transfected 293T cells (Fig. 1A and B) and HEK293SGnTI[−] (16) cells (Fig. 1C and D) (in HEK293SGnTI[−] cells, glycoproteins could not be modified with complex-type glycans) with a gO-expressing plasmid alone or

with gO in combination with gH- and gL-expressing plasmids and then analyzed the N-linked glycans on gO. As shown in Fig. 1A, when gO was coexpressed in 293T cells with gH and gL, a fraction of gO (the mature form of gO, indicated with stars) was resistant to Endo H digestion, indicating that this portion of gO had been transported from the ER to the Golgi. In contrast, Endo H-resistant gO digestion was not detected when gO was expressed alone. In HEK293SGnTI[−] cells, even the mature form of gO was sensitive to Endo H digestion because there is no complex-type-glycan modification (Fig. 1C). We also digested gO in the gH/gL/gO complex, immunoprecipitated from gH-, gL-, and gO-expressing cells, and found that the mature form of gO was resistant to Endo H digestion in 293T cells (Fig. 1B) and sensitive to Endo H digestion in HEK293SGnTI[−] cells (Fig. 1D). All these data indicated that the mature form of gO was transported in gH/gL/gO complex to the Golgi. As to the premature forms of gO (indicated with triangles in Fig. 1A and C), they were visible as two different-size bands around 80 to 90 kDa in immunoblots before Endo H or PNGase F digestion and became a single band after the digestion, indicating that these two forms of gO N-linked glycans might be slightly different.

When gO, gH, and gL were expressed in HEK293SGnTI[−] cells, a band smaller than 75 kDa could be detected in cell lysate (Fig. 1C) and more clearly in the undigested immunoprecipitate (Fig. 1D). And after PNGase F digestion of cell lysates from gH-, gL-, and gO-expressing 293T cells and the immunoprecipitate from the same lysates, we found that gO with complex glycans had a lower molecular weight than gO with high-mannose glycans (Fig. 1A and B). This observation suggested that gO was cleaved during its maturation process. Because the anti-gO antibody used in our experiments recognized the N terminus of gO, we hypothesized that the carboxyl terminus of gO was cleaved away during the

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FIG 1 gH and gL are required for gO maturation. 293T cells (A) and HEK293SGnTI⁻ cells (C) were transfected with a gO-expressing plasmid, either alone or in combination with gH- and gL-expressing plasmids (indicated at the top). Cells were harvested, lysed, and digested with Endo H (H) or PNGase F (F) for Western blot analysis using an anti-gO monoclonal antibody (Mab) on day 2 posttransfection. 293T cells (B) and HEK293SGnTI⁻ cells (D) transfected with gH-, gL- and gO-expressing plasmids were harvested and lysed as described for panels A and C. The lysates were subjected to immunoprecipitation using an anti-gH antibody. The precipitant was digested with Endo H (H) or PNGase F (F) and then subjected to Western blot analysis using an anti-gO Mab. C, control. Different gO forms are indicated with different markers (triangles and stars) on their left.

maturation process. To test this idea, we constructed a plasmid for expression of gO C-terminally tagged with the hemagglutinin (HA) epitope and expressed gOHA alone or with gH and gL in 293T cells (Fig. 2A, top) and in HEK293SGnTI⁻ cells (Fig. 2A,

bottom). As shown in Fig. 2A, the mature form of gO (around 50 kDa after PNGase F digestion) could not be detected in the cell lysates (Fig. 2A, lanes 2 to 7) or anti-gH immunoprecipitates by using anti-HA antibody (Fig. 2A, lanes 8 to 10); however, this

FIG 2 Cleavage of gO during its maturation process. (A) 293T cells (top) and HEK293SGnTI⁻ cells (bottom) were transfected with a plasmid expressing hemagglutinin (HA)-tagged gO (AgOHA), either alone (lanes 2 to 4) or in combination with gH- and gL-expressing plasmids (lanes 5 to 13). The cells were harvested on day 2 posttransfection, lysed, and subjected to immunoprecipitation by using anti-gH antibody. The cell lysates (lanes 1 to 7) and precipitants (lanes 8 to 13) were digested with Endo H (H) and PNGase F (F) and then subjected to Western blot analysis using anti-HA and anti-gO antibodies. C, control. Black triangles indicate the mature form of gO after digestion. (B) Summary of the gO maturation process. (Top) Expression of gO alone; (bottom) coexpression of gO with gH and gL.

form of gO could be detected using anti-gO antibody (Fig. 2A, lanes 11 to 13). Thus, the smear (around 68 kDa) that was seen in the cell lysate or, more obviously, in the Endo H-digested immunoprecipitate from 293T cells expressing gH, gL, and gO (Fig. 1 and 2A) represented the cleaved gO product carrying N-linked complex-type glycans, which were removed by PNGase F but not by Endo H. This form of gO could be more easily detected in HEK293SGnT1⁻ cells, because this form of gO could not be modified with complex glycans in this type of cells. And, as a result, the uncleaved (immature form) and cleaved (mature form) gOs were modified with similar high-mannose glycans, showing the totally different molecular weights, which could be easily confirmed in Western blot analysis. All these results indicated that when gO was expressed along with gH and gL, the carboxyl terminus of gO was cleaved away, probably during the maturation process. We tried to detect the cleaved C-terminal gO (tagged with HA) by Western blotting using high-percentage SDS-PAGE and anti-HA antibody; however, no such small fragment could be detected (data not shown). This might be because of rapid degradation of the fragment. We summarized the gO maturation process with and without gH and gL in a schematic diagram (Fig. 2B).

For both HCMV gH/gL/UL128-131 and HHV-6A gH/gL/gQ1/gQ2, coexpression of all components of each complex is required for the transport and expression of the complex to the cell surface (15, 17). Furthermore, coexpression of gO with gH and gL in HCMV promotes gO transport to the Golgi but not to the cell surface (14). We analyzed the cell surface expression of gO when it was expressed alone or with gH and gL in 293T cells, and the expression of each glycoprotein was confirmed by immunofluorescence assay (IFA) (data not shown). As reported previously, when gH and gL were coexpressed with gQ1 and gQ2, cell surface expression of gH and gL was dramatically upregulated (Fig. 3) (15); however, when gH and gL were coexpressed with gO, no further upregulation of gH and gL on the cell surface could be detected (Fig. 3, left). Furthermore, coexpression of gO with gH, gL, gQ1, and gQ2 had little influence on cell surface expression of gH and gL relative to coexpression of gH, gL, gQ1, and gQ2 (Fig. 3, left). Very little gO was detected on the cell surface when it was expressed alone; however, coexpression of gO with gH and gL affected the cell surface expression of gO slightly in transfected cells (Fig. 3, right), because of gO interaction with gH and gL.

Next, we analyzed the role of gO in the context of HHV-6A infection. Recently, we succeeded in cloning the HHV-6A genome as a bacterial artificial chromosome (BAC) (18). For this study, we constructed a BAC in which the gO gene was deleted (HHV-6ABACΔgO) and its revertant (HHV-6ABACΔgOrev) by two-step Red recombination (Fig. 4A), as described previously (19). We successfully reconstituted infectious viruses from both variant BACs. No gO was detected in cord blood mononuclear cells (CBMCs) infected with the gO deletion-containing virus (Fig. 4B), indicating that gO is not essential for HHV-6A propagation in CBMCs. Some of the CBMCs were purchased from RIKEN (the Institute of Physical and Chemical Research; Japan). The ethical committee of Kobe University Graduate School of Medicine approved the use of CBMCs in this study.

We then compared the growth kinetics of these viruses in CBMCs, using a previously described method (20). As shown in Fig. 4C, rHHV-6ABAC, rHHV-6ABACΔgO, and rHHV-6ABACΔgOrev ("r" indicates a recombinant virus) all exhib-

FIG 3 Expression of glycoproteins on the cell surface during coexpression. 293T cells were transfected with the glycoprotein expression plasmids indicated under the individual histograms. Cells were stained with anti-gH/gL antibody (left) or anti-gO antibody (right). Histograms show fluorescence intensity, measured in arbitrary units, on a log scale (x axis) and relative cell number on a linear scale (y axis).

FIG 4 Construction and analysis of gO deletion-containing human herpesvirus 6A (HHV-6A). (A) Illustration of gO deletion in the HHV-6A genome (HHV-6ABAC). The HHV-6 genome consists of three major internal repeat elements (R1 to R3), the origin of replication (oriLyt), and the direct repeats (DRL and DRr). gO (U47) deletion results in the tail-to-tail connection of U46 and U48 (gH). (B) Viruses reconstituted from HHV-6A bacterial artificial chromosome (BAC) genomes (HHV-6ABAC [wild type], HHV-6ABACΔgO, and HHV-6ABACΔgOrev [revertant]) were confirmed by Western blot analysis using lysates from infected cord blood mononuclear cells (CBMCs). Each immunoblot was performed using the antibody indicated below the corresponding blot image. (C) Comparison of growth kinetics of rHHV-6ABAC, rHHV-6ABACΔgO, and rHHV-6ABACΔgOrev. CBMCs were infected with each virus at an MOI (multiplicity of infection) of 0.01. The cells were harvested at 0 h, 8 h, 1 day, 3 days, 5 days, or 7 days postinfection. Viral genome copy number in each sample was quantitated by real-time PCR. Data shown here represent one of three independent experiments.

ited similar growth kinetics in CBMCs, indicating that gO deletion did not affect viral growth in CBMCs. gO contributes to the cell tropism of both HCMV and murine cytomegalovirus (21, 22); in those viruses, gO is required for the entry into fibroblasts, but not endothelial and epithelial cells (14, 22). Because HHV-6A has broad cell tropism (23), HHV-6A gO may support the function of the gH/gL/gQ1/gQ2 complex, which facilitates viral entry by binding to the ubiquitously expressed cell surface marker CD46.

In summary, we analyzed the maturation process of HHV-6A gO in detail. gO is a viral glycoprotein that is cleaved during its maturation process, although the biological meaning of gO cleavage still needs to be elucidated. Because gO is abundantly expressed in HHV-6A-infected cells (9), gO could play a role in HHV-6A infection. Although gO is not required for the growth of HHV-6A in CBMCs (mainly in CD4⁺ T) *in vitro*, it may play a role in HHV-6A infection of cells other than T cells and function during HHV-6A infection *in vivo*.

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