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Expression of Human Cytomegalovirus Immediate Early US3 Gene in Human Fibroblast Cells

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US3 gene is a member of the human cytomegalovirus (HCMV) immediate early gene. Although the precise functions of the US3 gene in HCMV replication and pathogenesis are not known, it has been reported to play a role in inhibiting major histocompatibility class I antigen presentation. For further knowledge of US3 gene expression, rabbit polyclonal antiserum of the US3 gene product was used for indirect immunofluorescence assay. In permissive human foreskin fibroblast (HFF) cells, US3 gene expression was detectable as crescent or half-moon shape in the perinuclear region at immediate early times after virus infection. HFF cells infected with mutant HCMV lacking US3 open reading frames were negative for US3 immunofluorescence assay. Double immunofluorescence assay using monoclonal antibody to gamma adaptin (specific for the Golgi complex) and rabbit anti-US3 antiserum revealed that US3 gene product could be localized to the Golgi complex. At later time after HCMV infection, US3 gene products were detected as globular aggregates in the cytosol. These aggregates were positive for gamma adaptin and stained with preimmune serum, suggesting a nonspecific reaction to the Golgi complex. Northern blot analysis revealed that transcription of US3 was observed only during immediate early times after virus infection (until 6 h postinfection). Therefore US3 gene expression appears to be confined to immediate early time and its gene products are localized to the Golgi complex as crescent shaped forms in the perinuclear cytoplasm.

Key words: Human cytomegalovirus, US3 gene, immunofluorescence

Human cytomegalovirus (HCMV) is a member of the beta-herpesvirus causing clinically significant diseases in immunocompromised people. HCMV contains the biggest genome among animal viruses infecting humans and codes for 203 open reading frames (10). Gene expression of HCMV is divided into three classes according to the time in which genes are expressed: immediate early (IE), early (E) and late (L) genes (12, 20). IE genes are the first set of genes expressed in HCMV-infected cells. Of the IE genes, major immediate early (MIE) genes are regarded as the most important in the regulation of HCMV replication. MIE genes are transactivators of other IE genes or E genes (14).

US3 is another IE gene and codes for three proteins produced from alternatively spliced RNAs (24). US3 contains an enhancer region located between -55 and -300 bp and a silencer located between -340 and -600 bp (9, 25). The enhancer and silencer appear to be responsible for cell-type specific expression of US3 (7). Another mechanism for US3 gene expression is mediated by a *cis*-repressive region (CRS) which is located between the TATA box and the transcription initiation region (6). CRS reduces US3 gene expression at early times after virus infection. Although US3 is dispensable for HCMV replication in cultured cells (15, 18), transactivation of HCMV E gene promoters by MIE genes are more efficient if the US3 gene is expressed (11). Recently functional studies of US3 have focused on the role of US3 in viral evasion of immune response.

Major histocompatibility complex (MHC) class I is required for cytotoxic T lymphocyte (CTL)-related immune response. CTLs recognize foreign antigens in the context of self MHC class I. Thus, HCMV develops a way to escape an attack from CTL by reducing expression of MHC class I on the surface of virus-infected cells. Several genes are involved in this process. HCMV gpUS3 is known to associate with the MHC class I molecule and beta 2-microglobulin in the luminal space of the endoplasmic reticulum (ER), resulting in retention of the complex in the ER lumen (1). HLA class I molecules are then

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thought to be dislocated from the ER to the cytosol followed by rapid degradation by the proteosome. HCMV gpUS2 and gpUS11 are involved in this process (16, 27). HCMV blocks transport of cytosolic peptides into the ER lumen by expressing gpUS6 which associates with a transient assembly complex composed of transporter associated proteins, HLA class I, beta 2-microglobulin, calreticulin, and tapasin (2, 13).

In this study, we attempted to understand the expression of US3 in permissive human fibroblast cells. We found that the US3 gene was expressed at immediate early times after virus infection and the gene product was localized to the Golgi complex.

Materials and Methods

Cells and virus

Primary human foreskin fibroblast (HFF) cells were used in this study. Cells were propagated in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) under 5% CO_2 atmosphere at 37°C. The Towne strain of HCMV and mutant HCMV with deletion of US3 region [HCMV (US3-)] were used. To prepare virus stocks, HFF cells were infected with HCMV at a multiplicity of infection (MOI) of approximately 0.01 plaque forming units (PFU) per cell. The medium was changed 4 to 5 days postinfection and the virus was harvested at 8 to 10 days after infection by two cycles of freezing and thawing. The cell extract was sonicated, and stored at 70°C.

Plaque assay

To determine the infectious titer of virus, a serially diluted (10-fold) virus sample was inoculated (0.2 µl per 35 mm culture dish) onto a confluent monolayer of HFF cells and incubated for 1 h with gentle rocking every 15 min. Cells were followed by a wash with phosphate buffered saline (PBS) and overlayed with a semisolid medium consisting of DMEM with 2% FBS, 0.25% agarose (Type II, medium EEO, Sigma Chemical Co., St. Louis, MO, USA), 100 units/ml penicillin, 100 µg/ml streptomycin, and 1 µg/ml Fungizone (Flow Lab., McLean, VA, USA). After 7 days, a second overlay was added and cells were fixed with 10% formalin in 0.85% saline about 2 weeks after virus infection. The cell monolayer was then stained with 0.03% methylene blue and the number of formed plaques was counted.

Indirect immunofluorescence assay (IFA)

Cells were seeded on 4 well chamber slides. At an appropriate time after infection, cells on the slide were washed in Tris-Saline solution and fixed with cold methanol for 8 min at -20°C, dried and stored at -20°C. The stored slides were rehydrated in Tris-Saline solution. Monoclonal antibody MAb810 (Chemicon, Temecula, CA, USA), rabbit antiserum against US3 or rabbit preimmune serum, and mouse monoclonal anti-γ adaptin antibody (Transduction Laboratory, Lexington, KY, USA) were used for immunostaining of HCMV MIE, US3, and Golgi complex, respectively. Rabbit anti-US3 serum was kindly provided by Dr. Gary S. Hayward of Johns Hopkins University (Baltimore, MD, U.S.A.) It was raised against the synthetic oligopeptide corresponding to amino terminus of predicted US3 protein. After incubation for 1 h at 37°C, cells were washed three times in Tris-Saline solution, 5 min each, and binding of the primary antibody was detected with an FITC-conjugated anti-mouse IgG or rhodamine-conjugated anti-rabbit IgG antibody for 45 min at 37°C. After washing two times in Tris-Saline, cells were mounted with PBS/glycerol, examined, and then photographed with a laser scanning confocal microscope (Bio-Rad Laboratories, Hercules, CA, USA).

Northern hybridization

Cellular RNA was extracted from cells by using RNAzol B (Tel-Test Inc., Friendswood, TX, USA), according to the supplier's protocol. Isolated RNA (25 µg) was denatured in denaturation solution (2.4 µl 10×MOPS, 4.2 µl 37% formaldehyde, 12 µl 100% formamide) and reacted for 10 min at 65°C and for 10 min on ice, respectively. The RNA loading dye and ethidium bromide were then added into the denatured RNA sample. The RNA sample was analyzed by electrophoresis in a 1% agarose gel containing 0.92 M formaldehyde, transferred to a nitrocellulose membrane, Gene Screen Plus (NEN™ Life Science Products, Boston, MA, U.S.A.), and fixed by UV cross-linking (Ultra-Violet Products, Cambridge, U.K.) and baking for 2 h at 80°C. The membrane was prehybridized overnight at 42°C in a hybridization solution (50% formamide, 10% dextran sulfate, 7% SDS, 25 M $NaHPO_4$ pH 7.2, 25 M NaCl, 1 mM EDTA, 100 µg/ml denatured salmon sperm DNA). Probe DNA including the US3 region was synthesized by the polymerase chain reaction, and denatured for 5 min in boiling water. After cooling for 5 min on ice, 5× OLB solution (0.25 M Tris pH 8.0, 0.5 mM each dATP, dGTP, dTTP, 1 M HEPES pH 6.6, 30 OD units/ random hexanucleotides, 25 mM $MgCl_2$, 50 mM beta-mercaptoethanol), bovine serum albumin (BSA, 10 mg/ml), 5 µl ^{32}P-dCTP (3,000 ci/mM), and 1 µl Klenow enzyme (200 units) were added into probe DNA and the DNA was reacted for 2 h at 37°C for *in vitro* transcription. Unincorporated nucleotides were removed by a Sephadex G-50 column. Hybridization was performed overnight at 42°C in the renewed hybridiza- tion solution as described above. The membrane was washed with 2× SSC, 0.2% SDS at room temperature and once with 1× SSC, 0.2% SDS at room temperature for 15 min each. After washing with 0.5× SSC, 0.2% SDS for 15 min at room temperature, the membrane was exposed to X-ray film.

Results and Discussion

Expression of US3 gene in human fibroblast cells
Human foreskin fibroblast (HFF) cells were grown in 4-well chamber slides and infected with HCMV at an MOI of 1. The expression of US3 was determined at 4 h after HCMV infection by double immunofluorescence assay (IFA) using rabbit anti-US3 antiserum and mouse monoclonal antibody MAb810 to the common region of IE1 and IE2 proteins. As expected MIE gene products were detected in the nucleus of HCMV-infected cells (Fig. 1A). US3 gene products were detected in the cytoplasm surrounding the nucleus in the shape of a crescent or half-moon (Fig. 1B). Rabbit pre-immune serum failed to detect US3 gene products (Fig. 1D). In order to confirm that the fluorescence detected with anti-US3 antiserum is specific to US3 gene products, HFF cells were infected with HCMV mutants lacking US3 and IFA was performed. While MIE gene products were easily detected with MAb810 (Fig. 1E), anti-US3 antiserum failed to detect any fluorescence in HFF cells infected with the mutant virus. Therefore, US3 gene expression in HFF cells appears to be responsible for the crescent or half-moon shapes near the nucleus.

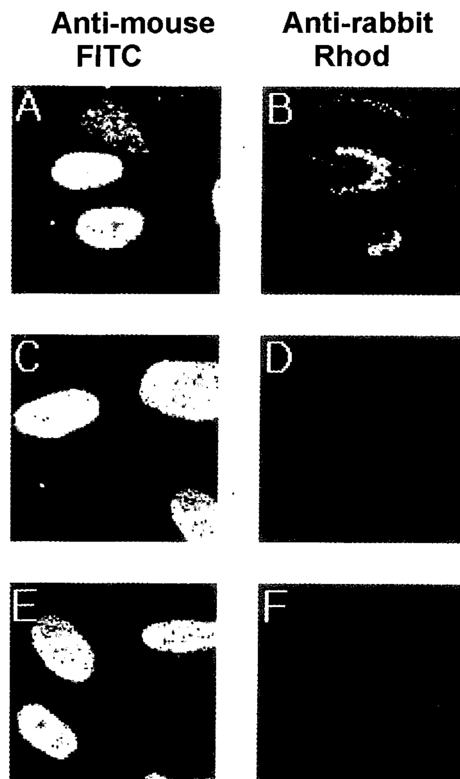

Fig. 1. Immunocytochemical localization of MIE and US3 gene products in HCMV-infected HFF cells. At 4 h post infection, HCMV-infected and US3(-) HCMV-infected HFF cells were double stained with mouse monoclonal Ab against MIE (A, C, E), and rabbit polyclonal Ab against US3 (B, F) or rabbit preimmune serum (D), followed by FITC-conjugated anti-mouse IgG and Rhodamine-conjugated anti-rabbit IgG. Cells were examined with a confocal scanning laser microscope. A, B, C and D infected with HCMV; E and F infected with US3(−) HCMV.

Time-course of US3 gene expression in HFF cells
Although classified as IE genes, MIE genes are expressed until late times after virus infection. Thus, it was of interest to determine whether US3 gene expression is confined to immediate early times or extended to later times after HCMV infection. HFF cells were infected with HCMV and indirect immunofluorescence assay was performed at 4, 12, and 24 h postinfection. Cells were double-labeled with rabbit anti-US3 antiserum and MAb810 to MIE gene products. As shown in Fig. 2, MIE gene expression was detected until 24 h postinfection. HCMV-infected cells were also positive for IFA with antiserum to US3 until 24 h postinfection. However, US3 gene expression was detected as crescent or half-moon shaped at 4 h postinfection (Fig. 2B) while at 24 h postinfection US3 gene expression was detected as globular aggregates in the cytoplasm adjacent to the nucleus of HCMV-infected cells (Fig. 2F). Crescent or half-moon shapes were not detectable in HCMV-infected HFF cells at 24 h postinfection. A mixture of crescent shape and globular aggregates was detected at 12 h postinfection (Fig. 2D).

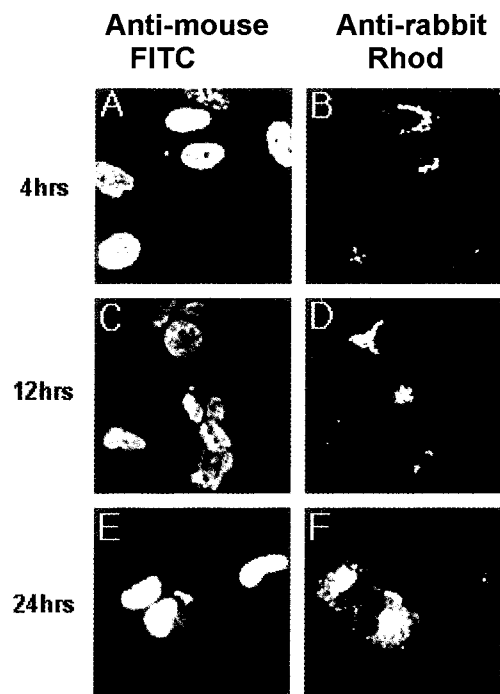

Fig. 2. The time-dependent expression of MIE and US3 gene products in HCMV-infected HFF cells. HCMV-infected HFF was stained with mouse monoclonal Ab against MIE (A, C, E), and rabbit antiserm raised against US3 (B, D, F), followed by FITC-conjugated anti-mouse IgG and Rhodamine-conjugated anti-rabbit IgG.

Since the patterns o immunostaining with anti-US3 antiserum at early times differed (4 h postinfection) from those at late times (24 h postinfection), we needed to determine whether the late pattern (cytoplasmic globular aggregate) was due to US3 gene function or nonspecific interaction to HCMV infection. HFF cells were infected with HCMV and double IFA using MAb810 to MIE gene products and rabbit preimmune serum was performed at various times after virus infection. As shown in Fig. 3, HFF cells were negative for preimmune serum at early times after virus infection, while at late times after infection all HCMV-infected cells were stained with preimmune serum. The staining pattern with preimmune serum was similar to that with anti-US3 antiserum, showing globular aggregates in the cytoplasm. Crescent or half-moon shapes were not observed in HCMV-infected HFF cells stained with preimmune serum. Furthermore, preimmune serum was able to stain globular aggregates in HFF cells infected with a HCMV(US3-) mutant (data not shown). Thus, it appears that the globular aggregates observed in HFF cells at late times after HCMV infection was due to nonspecific interaction to virus infection and that the crescent or half-moon shape detected with anti-US3 antiserum was specific to US3 gene products.

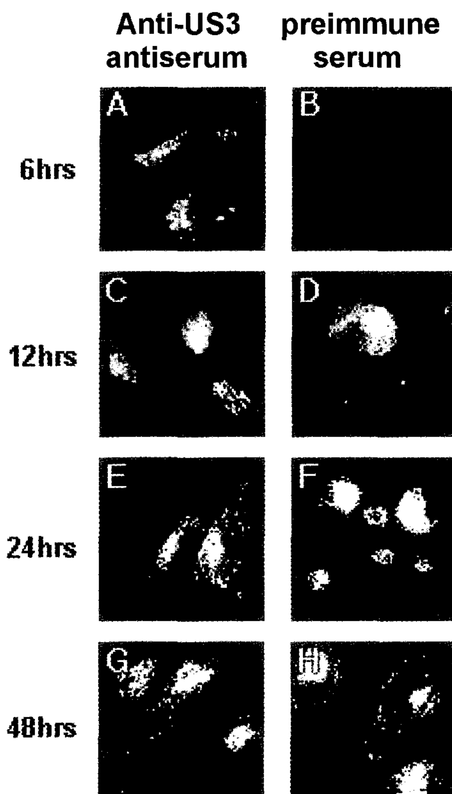

Fig. 3. Immunocytochemical analysis of US3 gene products in HCMV-infected HFF cells. At 6, 12, 24, 48 h postinfection, HCMV-infected HFF cells were stained with rabbit polyclonal antiserum against US3 (A, C, E, G)or rabbit preimmune serum (B, D, G, H) followed by Rhodamine-conjugated anti-rabbit IgG. Cells were examined with a confocal scanning laser microscope.

The above observation was confirmed by northern blot analysis using the *Sma*I fragment of HCMV genomic DNA containing the US3 gene. As shown in Fig. 4, US3 RNA transcription was detected at 3 and 6 h postinfection, while at later times after infection (12, 24, and 48 hr postinfection) US3 RNA synthesis was not detected. Therefore, the globular aggregates detected with both anti-US3 antiserum and preimmune serum may not result from US3 gene expression. However, it is possibe that the US3 gene products are long-lived and present in the globular aggregates.

Continued or over-expression of US3 gene may be cytotoxic because it interacts with the MHC class I molecule and remains in the inner lumen of the ER. This may interfere with the normal functioning of the ER and the proper processing of early and late viral glycoproteins as well as cellular glycoproteins. Accordingly US3 gene expression needs to be downregulated as the successful virus infection proceeds. IFA and northern blot analysis by this study suggest that the US3 gene is expressed at immediate early times after HCMV infection (3 and 6 h postinfection). As infection progresses US3 gene expression was no longer detectable at later times (24 and 48 h postinfection). Furthermore, the turning-off point of US3 gene expression appears to be around 12 h postinfection when HCMV E genes begin to be expressed. The mechanism for down regulation of the US3 gene at the onset of HCMV E gene expression might be due to the presence of the *cis*-repressive region (CRS) located upstream from the US3 open reading frame (6, 8, 9). The CRS element regulates trancription from the enhancer-containing promoter presumably by binding a repressor protein at early and late times after HCMV infection (7). Using recombinant HCMV with a mutant US3 gene without the CRS region, it was

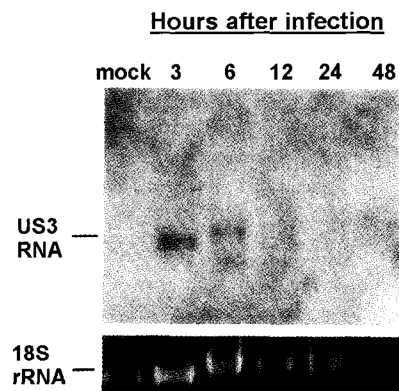

Fig. 4. Northern blot analysis of US3 transcripts. Whole cell RNA was harvested from mock-infected or HCMV-infected HFF cells at 3, 6, 12, 24, 48 h. postinfection. RNA was hybridized to ^{32}P-labeled US3 specific probe (nt 193421 to 195108).

shown that the US3 gene expression continued to increase throughout the viral replication cycle to levels 20- to 40-fold higher than for the wild type. On the other hand, the maximum level of wild type US3 gene expression was detected within 6 h postinfection (19).

Localization of US3 gene products to the Golgi complex It was reported that US3 retains MHC class I molecule in the luminal space of the ER (1) and the normal pathway of MHC class I transport to the surface of cells is through the Golgi complex. Since the pattern of immunofluorescence stained with anti-US3 antiserum obse-rved in previous experiments was reminiscent of the Golgi complex, we examined the possibility of localization of US3 gene products to the Golgi complex. HFF cells were infected with HCMV and double IFA was performed with monoclonal antibody to γ-adaptin (specific for *cis*-Golgi) and anti-US3 antiserum (Fig. 5). All HFF cells were stained with γ-adaptin whether infected with HCMV (Fig. 5A), uninfected (Fig. 5C), or infected with the HCMV(US3-) mutant (Fig. 5E). On the other hand, only wild type HCMV-infected cells were immunostained with anti-US3 antiserum (Fig. 5B). Arrows shown in Fig. 5A indicate HFF cells which were not infected with HCMV. HFF cells uninfected with HCMV or infected with the HCMV(US3-) mutant were negative for the anti-US3 antiserum (Fig. 5D and 5F). Comparison of Fig. 5A and 5B reveals that the regions stained with anti-US3 antiserum overlap the regions stained with monoclonal antibody to γ-adaptin.

Our data shown above suggest that the structure of the Golgi complex may be altered in HFF cells infected with HCMV. Initially the Golgi complex as immunostained by antibody to γ-adaptin appears to be crescent to half-moon shape in the perinuclear region of the cytoplasm. As infection progressed the Golgi complex seemed to alter as globular aggregates (Fig. 6). Electron microscopic study suggests that the ultrastructure of the Golgi complex was disrupted in HCMV-infected cells (data not shown). Cellular proteins destined for secretion or membrane presentation are synthesized in rough ER and transported through the Golgi complex (5, 23). There is also a report

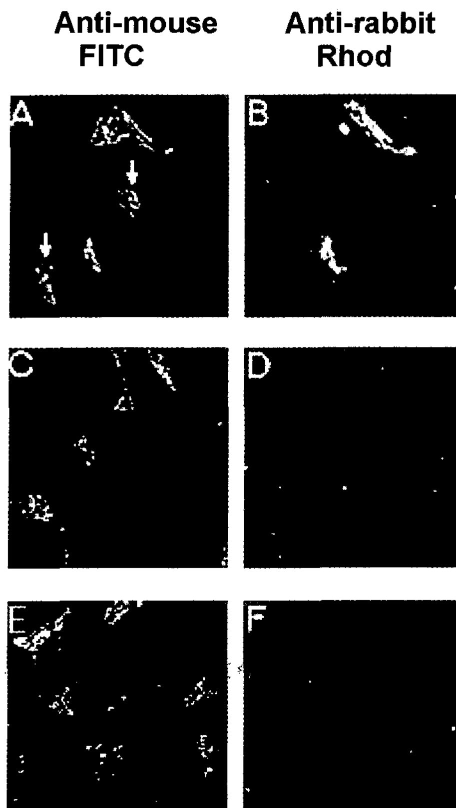

Fig. 5. Intracellular localization of US3 gene products to the Golgi apparatus in HCMV-infected HFF cells. At 4 h post infection, HCMV-infected (A, B, C, D) and US3(–) HCMV-infected (E, F) HFF cells were stained with mouse monoclonal Ab against MIE, MAB810 (A, C, E), and rabbit polyclonal Ab against US3 (B, F) or rabbit preimmune serum (D), followed by FITC-conjugated anti-mouse IgG and rhodamine-conjugated anti-rabbit IgG. Cells were examined with a confocal scanning laser microscope.

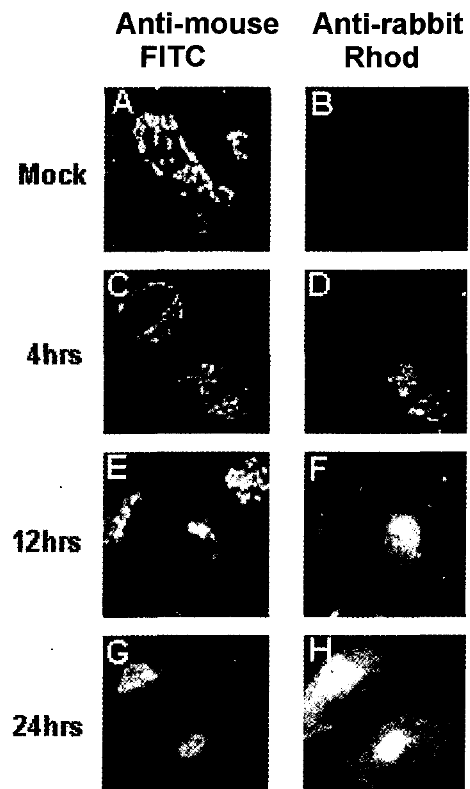

Fig. 6. The time-dependent expression of US3 gene products to the Golgi apparatus in HCMV-infected HFF cells. Mock-infected and HCMV-infected HFF cells were fixed at 4, 8, 12, 24 h postinfection. Cells were permeabilized, and incubated with mouse monoclonal Ab against γ-adaptin (specific for Golgi apparatus), and rabbit polyclonal Ab against US3, followed by FITC-conjugated anti-mouse IgG and rhodamine-conjugated anti-rabbit IgG.

suggesting that the structure of Golgi complex is altered by infection with HCMV (22). Alteration of the Golgi complex structure has also been observed with other virus infection, such as the herpes simplex virus (3, 4), Epstein Barr virus (28), pseudorabies virus (26), vaccinia virus (21), and corona virus (17). Alteration of the Golgi complex structure may affect the normal transport of cellular proteins such as MHC class I molecules and of viral envelope glycoproteins. It is of interest that the above mentioned viruses are all enveloped viruses. Therefore, structural alteration of the Golgi complex by these viruses could be a common mechanism to facilitate the transport of viral glycoproteins to the surface of infected cells to be incorporated as viral envelopes. Further studies are needed to elucidate the relationship between the structural alteration of the Golgi complex and HCMV replication.

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