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Deletion of gpUL132, a Structural Component of Human Cytomegalovirus, Results in Impaired Virus Replication in Fibroblasts

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The coding capacity of human cytomegalovirus (HCMV) for glycoproteins by far exceeds that of other herpesviruses. Few of these proteins have been characterized so far. We have investigated the gene product of reading frame UL132. The putative protein product of UL132 is a glycoprotein with a theoretical mass of 29.8 kDa. Transcription analysis revealed that the gene is transcribed with a true late kinetics from the laboratory-adapted strain AD169 and the low-passage isolate TB40E. Two proteins of 22 to 28 kDa and 45 to 60 kDa were detected in virus-infected cells as well as in extracellular virions. The larger protein carried N-linked carbohydrates. Both protein forms were present in laboratory-adapted strains as well as in low-passage isolates of HCMV. Recombinant viruses with the UL132 gene deleted were constructed in the low-passage HCMV isolate PAN as well as the high-passage isolate AD169. Deletion of UL132 from either genome resulted in a pronounced replication deficit with a reduction of approximately 100-fold for HCMV strain AD169. Thus, the protein product of the UL132 reading frame represents a structural viral glycoprotein of HCMV that has an important function for viral replication in tissue culture.

The *Herpesviridae* form a large and diverse family comprising three subfamilies, designated alpha-, beta-, and gammaherpesviruses. Structurally, all herpesviruses are composed of a large DNA genome encased in a capsid, which in turn is coated with a layer of proteins called the tegument and an envelope composed of approximately a dozen viral glycoproteins in a lipid membrane. The envelope glycoproteins of herpesviruses have a number of different functions during the replication cycle. During the initial phase of infection they serve as mediators of attachment and fusion with the target cell (reviewed in reference 42). During later stages of infection, the glycoproteins have roles in processes such as assembly of infectious virions, envelopment, and cell-to-cell spread (29).

The structural glycoproteins that mediate these processes can be divided into two broad classes: those that are conserved throughout the herpesvirus family, and those that are not. Among the first class are the glycoproteins gB, gH, gL, gM, and gN, with gB exhibiting the highest degree of sequence similarity (30). The gB proteins on some herpesviruses form homodimers or homotrimers (3). Heterodimerization of gH and gL is also a conserved feature, and in some cases, such as human cytomegalovirus (HCMV), a third glycoprotein (gO) is also a component of the gH/gL complex (19, 23, 24). During the initial phase of the infection, these proteins serve as mediators of attachment and fusion with the target cell (reviewed in reference 42). The functions of gM and gN, which form a heterodimeric complex, are less well defined. Studies of alphaherpesviruses suggest that they may have a role in virus-induced fusion/penetration and also in cytoplasmic envelopment (7, 9). For betaherpesviruses they may represent the binding molecules for heparan sulfate proteoglycans (21).

Structural glycoproteins that are not conserved between herpesviruses potentially add specific functions to individual genera or viruses and viral ligands that bind to cell surface receptors differ between the viruses and may thus in part contribute to the differences in cell and tissue tropism. For example, Epstein-Barr virus uses the two nonconserved glycoproteins gp350 and gp220 for initial interaction with the target cell (33). In addition, gp42, a component of the tripartite complex gH/gL/gp42, mediates B-cell entry, whereas entry into epithelial cells requires complexes without gp42 (6). Another striking example is the receptor binding capacity of gD of herpes simplex virus (41).

HCMV, a betaherpesvirus, is the most complex herpesvirus with respect to its glycoprotein genes. It has been estimated that the laboratory-adapted strain AD169 codes for as many as 57 reading frames with predicted features of glycoproteins (12). Low-passage isolates of HCMV contain 13 additional reading frames potentially coding for glycoproteins (10). The glycoprotein content of the virion envelope remains incompletely defined. A recent analysis using mass spectrometry has identified 19 structural glycoproteins as constituents of extracellular virions (47).

As a member of the herpesvirus family, HCMV contains the glycoproteins gB, gH, gL, gM, and gN (30). Whether these

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proteins serve identical functions as in the other herpesvirus subfamilies is not clear. Similarities involve the role of gB and gH in attachment and penetration (25). In addition, gB has been reported to bind the epidermal growth factor receptor and thus might contribute to entry into target cells (48). However, clear differences have been noted for the requirements of the individual proteins for viral replication. Deletion of gM and gN from the genomes of some alphaherpesviruses results in only minimal effects on in vitro virus assembly and replication, whereas these genes are essential for replication of HCMV, indicating that the proteins or protein complexes serve different functions in different herpesviruses (2, 17, 20, 27).

Little is known about envelope glycoproteins which are specific for betaherpesviruses or HCMV. The products of the readings frames UL4 (gp48) and TRL10 (gpTRL10) have been reported to be structural components of the virion (11, 39). Both proteins are dispensable for replication of HCMV in fibroblasts, but no function has been attributed to either one (17, 40). In order to understand the infectious process in more detail, an increased knowledge of the envelope glycoproteins of HCMV and their function during the replication cycle is needed.

Here we describe the identification of the protein product of open reading frame (ORF) UL132 as a structural glycoprotein and we show that deletion of the gene in laboratory-adapted HCMV strains as well as in low-passage isolates results in a virus with reduced replication capacity in fibroblasts.

MATERIALS AND METHODS

Cells and viruses. HCMV strain AD169 and recombinant viruses were propagated in primary human foreskin fibroblasts (HFF) grown in minimal essential medium (Invitrogen, Karlsruhe, Germany) supplemented with 5% fetal calf serum, glutamine (100 mg/liter), and gentamicin (350 mg/liter). Virions were isolated by glycerol-tartrate gradient centrifugation as described (44). 293T cells were cultured in Dulbecco′s modified Eagle′s medium supplemented with 10% fetal calf serum, glutamine and gentamicin. Cos7 cells were passaged in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, glutamine, and gentamicin.

Constructions of DNA vaccine plasmids expressing UL132. Three different UL132 DNA inserts were PCR amplified from template DNA plasmid pcUL132. The first insert, wt-UL132, encoding amino acids 1 to 271 of gUL132 protein, was amplified with primers prUL132.1 (5′-ACACTGAAGCTTATGCCGGCCTTG CGGGGGTCTC-3′) and prUL132.2 (5′-ACACTGGGATCCCTAGTCGTACTC GGGATCTCT-3′). The second insert, N-UL132, encoding the N-terminal segment (amino acids 23 to 80) of gUL132, was amplified with primers prUL132.3 (5′-ACACTGGCTAGCTACATGGACTTCAGCGACGCT-3′) and prUL132.4 (5′-ACACTGGGATCCCTATTTCATGATTTCATCATTGCG-3′). The third insert, d81/105-UL132, encoding a modified gUL132 which had a deletion from amino acids 81 to 105, was produced by religating two PCR fragments generated by using two overlapping primer pairs, prUL132.3 and prUL132.7 (5′-CACGA GGAGTATTTCATGATTTCATCATTGCG-3′), to generate the first fragment encoding amino acids 23 to 80, and prUL132.6 (5′-AAATCATGAAATACTC CTCGTGTTGCAAGCAC-3′) and prUL132.2 to generate the second fragment, encoding amino acids 106 to 271. A human tissue plasminogen activator leader was added to both the second and the third inserts. These inserts were individually subcloned into the DNA vaccine vector pJW4303. The resulting UL132 DNA vaccine constructs were confirmed by restriction enzyme analysis and large DNA preparations of these plasmids were conducted using the Mega plasmid purification kit (QIAGEN).

UL132 specific polyvalent sera from New Zealand White rabbits. Female New Zealand White rabbits weighing 2 kg were purchased from Millbrook Breeding Lab (Amherst, MA). Animals were housed in the facility managed by Department of Animal Medicine at the University of Massachusetts Medical School following Institutional Animal Care and Use Committee-approved protocols. Rabbits received four DNA immunizations by a Helios gene gun (Bio-Rad, Hercules, CA). A mixture of all three UL132 DNA vaccine plasmids was coated onto the 1.0-μm gold beads at 2 μg of DNA/mg of gold so that each shot delivered 1 μg of DNA. At each immunization, the rabbits were anesthetized, and a total of 36 nonoverlapping shots were delivered to the shaved abdomen. The serum was collected 2 weeks after the final immunization and tested for specificity against UL132 by Western blot analysis.

Preparation of eukaryotic expression constructs and DNA transfection. The entire UL132 reading frame was amplified from HCMV strains TB40E or AD169 with the following primers that contain BamHI and HindIII restriction sites (underlined): UL132 forward (5′-TTATGGATCCATGCCGGCCTTGCG GGGT-3′) and UL132 reverse (5′-ATCGAAGCTTGTCGTACTCGGGATCT CTG-3′). The PCR product was cleaved with the appropriate restriction enzymes and inserted into the expression vector pcDNA3.1myc/His (Invitrogen, Carlsbad, Calif.). The resulting plasmid, pcUL132myc/his, encoded full-length pUL132 fused to a C-terminal Myc/His tag. The integrity of pcUL132myc/his was confirmed by DNA sequencing.

293T cells were transfected with the respective DNAs using Lipofectamine Plus reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's suggestion except that the transfection mixture consisted of 1 μg of DNA, 95 μl of Dulbecco's modified Eagle's medium, and 6 μl of Lipofectamine Plus reagent. The mixture was added to a cell culture dish (3.5-cm diameter, seeded with 2×10^5 cells 1 day before). After 48 h, cells were harvested, washed three times with phosphate-buffered saline (PBS), and stored at −20°C until used.

Image analysis. Cos7 cells grown on glass coverslips in 24-well plates were transfected with 1 to 2 μg of plasmid DNA using Lipofectamine. Fibroblasts, also grown on glass coverslips in 24-well plates, were infected with virus at a multiplicity of infection of 0.4. Two to three days later, the coverslips were washed and fixed in 2.0% paraformaldehyde in PBS. The fixed cells were permeabilized with Triton X-100 containing buffer and then blocked with PBS–1% bovine serum albumin (26). Primary antibodies, including a Myc-specific monoclonal antibody, rabbit anticalreticulin (endoplasmic reticulum marker; Dianova, Hamburg, Germany) and rabbit anti-gm130 (Golgi marker; Dianova) were then added. Following washing, antibody binding was detected with the appropriate secondary antibody conjugated with either fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate (Dianova). Images were collected using a Zeiss Axioplan 2 fluorescence microscope fitted with a Visitron Systems charge-coupled device camera (Puchheim, Germany). Images were processed using MetaView software and Adobe Photoshop.

SDS-PAGE and immunoblotting. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 to 15% polyacrylamide gels under standard conditions. Proteins were transferred to nitrocellulose membranes, and membranes were blocked with PBS containing 0.1% Tween 20 and 5% powdered milk. Antibodies and sera were diluted in PBS containing 0.1% Tween 20. For detection of primary antibody binding, horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G antibody and the enhanced chemiluminescence detection system (Pharmacia Biotech) were used according to the manufacturer's instructions. Removal of N-linked oligosaccharides was carried out using recombinant peptide *N*-glycosidase F (PNGase F) and endoglycosidase H (New England Biolabs, Beverly, Mass.) according to the manufacturer's specifications.

RNA methods. For Northern blot analysis, whole-cell RNA was isolated from noninfected or infected cells at 7, 24, 48, and 72 h postinfection using an RNA isolation kit (QIAGEN, Hilden, Germany). For gel electrophoresis, 20 μg of each RNA preparation was denatured with glyoxal and separated in 1% agarose gels containing 40% formaldehyde. Northern blot analysis was carried out according to standard procedures. The UL132-specific probe was ^{32}P labeled in a random priming reaction (New England Biolabs, Beverly, Mass.) using full-length UL132 as the template. As an internal control, the filter was hybridized with a glyceraldehyde-3-phosphate dehydrogenase-specific probe.

For reverse transcription (RT)-PCR, infection was blocked with either 150 μg/ml cycloheximide or phosphonoformic acid at a concentration of 185 μg/ml. Total RNA was isolated using the High Pure RNA isolation kit (Roche Diagnostics, Indianapolis, Ind.) followed by an additional DNase I (Amersham Pharmacia Biotech, Freiburg) treatment for 2 h. RT-PCR was carried out with the Titan One-Tube RT-PCR System (Roche Diagnostics) according to the manufacturer′s specifications using the following specific primers: UL132s 5′-GGAT AAGCTTCTAGTCGTACTCGGGATC-3′; UL132as 5′-GATCGGATCCTTC AGCGACGCTACAAATATG-3′; GAPDHs 5′-GTACGTCGTGGAGTCCAC T-3′; and GAPDHas 5′-TCCACCACCCTGTTGCTGTA-3′ (46). To rule out possible DNA contamination, a control reaction without reverse transcriptase and containing RNA from all the preparations was carried out.

Construction of UL132 recombinant viruses. Recombinant viruses on the genetic background of strain AD169 were constructed using BAC-HB5 (5).

Deletion mutant RV-delUL132/AD was created by site-directed mutagenesis using a PCR-generated fragment electroporated into *Escherichia coli* strain DH10B harboring the HB5 bacterial artificial chromosome (BAC) and expressing bacteriophage λ functions *red*αβγ from plasmid pBADαβγ (32). The primers that were used to generate the recombination fragments represented hybrids in which the 5′ end was homologous to sequences flanking the HCMV region to be deleted (50 nucleotides) and 3′ ends were homologous to the kanamycin resistance gene as present in plasmid pcpo15-Link2 (30 nucleotides) (13). With respect to the HCMV nucleotide sequences, the 5′ primer (UL132-5-Mega) contained nucleotides 176930 to 176980 of strain AD169 and the 3′ primer (UL132-3-Mega) contained nucleotides 177772 to 177720. Thus, the resulting PCR product consisted of a kanamycin resistance gene flanked by short stretches of HCMV-specific sequences.

Following homologous recombination in *Escherichia coli*, colonies carrying kanamycin and chloramphenicol resistance were selected. The resulting BAC-delUL132/AD was used for transfection of fibroblasts and reconstitution of infectious virus RV-delUL132/AD. To create a revertant virus, the kanamycin gene was first removed from the BAC-delUL132/AD via Flp recombinase (13). This BAC was used for homologous recombination using plasmid pHB5PST-UL132, which consisted of ORFs UL131/UL132/IRL14 and IRL14 (nucleotides 176317 to 178332), inserted into the plasmid pST76K-SR (5). The plasmid was electroporated into *E. coli* harboring BAC-delUL132-kan. pST76K-SR contains the *sacB* gene that was utilized to select clones that had reinserted the correct UL131-IRL14 sequences.

To create BAC-UL132-M2 a PCR fragment was generated using HCMV/kanamycin hybrid primers as described above. The 5′ primer (UL132-5-M2) consisted of nucleotides 176840 to 1768880 and the 3′ primer (UL132-3-M2) consisted of nucleotides 176972 to 176934. The 3′ primer contained an additional sequence coding for the Flag epitope M2 (KDDDDKTD). Following recombination with BAC-HB5, the kanamycin gene was removed by Flp recombination. For all BACs the correct insertion of the recombination cassette into the HCMV genome was verified by restriction enzyme digest, Southern blot hybridization, and nucleotide sequencing of the flanking regions, including UL131 and IRL14.

For the generation of the UL132-deficient BAC-delUL132/PAN the 5′-flanking region of UL132 was amplified from BAC-PAN23 DNA (40) using the following primers containing either EcoRI or KpnI restriction sites (underlined): UL132-5′s (5′-CTATGAATTCGTCGGGCGGTGGCCGACAC-3′) and UL132-5′as (5′-CTTAGGTACCCTCGAGATGTGGCGACGTGG-3′). The PCR product (corresponding to nucleotides 10603 to 11671 of HCMV strain Toledo) was digested with both endonucleases and inserted upstream of the kanamycin resistance gene in pCP-o-15-LINK2. The 3′-flanking region of the UL132 ORF was amplified with primers containing either BamHI or PmeI restriction sites (underlined): UL132-3′s (5′-CATAGGATCCGATCCCGAGTACGACTAGG-3′) and UL132-3′as (5′-GGACGTTTAAACGGGACTCATGACGCGCGG-3′). The amplified product (nucleotides 12468 to 13801 of HCMV strain Toledo) was cleaved and ligated downstream of the kanamycin resistance gene in pCP-o-15-LINK2 resulting in the recombination plasmid pDeltaUL132rec.

Mutagenesis of BAC-PAN23 (40) was performed in one step using a linear fragment for homologous recombination. The fragment was excised from pDeltaUL132rec using the restriction enzymes EcoRI and PmeI and electroporated into *E. coli* JC8679 harboring the parental BAC plasmid PAN23. Bacterial colonies were selected on agar plates containing kanamycin (30 μg/ml) and chloramphenicol (30 μg/ml). Recombined BAC plasmids were analyzed by digestion with the appropriate restriction enzymes. To confirm the integrity of the resulting BAC, Southern blot analysis was carried out according to standard procedures. Successfully mutated BAC plasmids were electroporated into *E. coli* strain DH10B for stable maintenance.

Reconstitution of infectious virus. Infectious viruses were recovered by transfection of BAC DNA or BAC mutant DNA into fibroblast cells (MRC-5) as described (27). Briefly, MRC-5 cells were plated into six-well dishes at 70% to 80% confluence a day prior to transfection; 1 μg of HCMV BAC DNA, prepared using Nucleobond PC 100 columns (Macherey-Nagel, Düren, Germany), was added to 10 μl Superfect transfection reagent (QIAGEN, Hilden, Germany). Serum-free Dulbecco's modified Eagle's medium was added to the transfection mixture to a total volume of 100 μl and incubated for 30 min at room temperature. MRC-5 cells were serum starved for 30 min and subsequently 1 ml of Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum was added together with the transfection mixture for 4 h at 37°C. After removal of the transfection mixture the cells were incubated for 7 days in cell culture medium. The cells were split and cultured until the appearance of virus plaques.

Growth curves for PAN23-based viruses. Infectious supernatants were harvested from HFF cultures showing a cytopathic effect of 100%, and the cellular debris was removed by centrifugation. The cell culture supernatants were directly used to assay the growth kinetics of the viral mutants. To this end, 1 day after seeding 1.25×10^5 HFF per well in 12-well plates, cells were infected in quadruplicate with different dilutions of infectious supernatant (undiluted; 1:2; 1:4; and 1:8) in a volume of 500 μl. After incubation at 37°C for 3 h the infectious mixture was removed, cells were washed twice with PBS, and fresh medium was added. The next day, one of the four cultures of each dilution was taken to determine the percentage of infection via detection of immediate-early protein 1 (IE-1) expression by immunofluorescence analysis. The ratio of infected to uninfected cells was determined for each dilution, and those cultures containing approximately 60% infected cells were chosen to assay the viral replication kinetics. To this end, supernatant from the three remaining cultures was taken every 2 days and, again in different dilutions, used directly for infection of HFF in 96-well plates. The virus titer was determined as described above. Growth curves for AD169 derived viruses were done as described (27).

Analysis of virus entry. HFF were seeded on glass coverslips in 24-well dishes at a density of 6×10^4; 24 h after seeding, cells were infected with the viruses at a multiplicity of infection of 10 or 25 in a final volume of 250 μl culture medium. At different time points after infection cells were washed twice with PBS, fixed with paraformaldehyde, and processed for indirect immunofluorescence as described above. As the primary antibody, the pp65-specific mouse monoclonal antibody p65-8 was used. The secondary antibody was a Cy3-labeled anti-mouse immunoglobulin antibody (Dianova).

RESULTS

Genomic location of UL132 in different HCMV isolates and predicted features of the protein. HCMV AD169 and low-passage isolates differ in two major aspects (Fig. 1A). First, as originally reported for Toledo, low-passage isolates contain a DNA segment that is absent from AD169 (UL133 to UL151) (10, 31). Second, low-passage isolates lack an internal long repeat (IRL). A deletion and duplication produced these changes in AD169. Some of the ORFs missing in laboratory strains reside in inverted orientation in Toledo compared with other low-passage isolates. The inversion in Toledo resulted from recombination within sequences located between UL128 and UL129 at one end and UL133 to UL148 at the other end. The integrity of UL132 was conserved in all HCMV strains during these deletion/recombination/inversion events, suggesting an important function of UL132 for replication.

The ORF UL132 of AD169 codes for a protein of 270 amino acids with a theoretical molecular mass of 29.8 kDa. Computer predictions classify the UL132 polypeptide as a typical type I glycoprotein with a signal sequence between amino acids 1 and 24 and a membrane anchor between residues 84 and 106 (Fig. 1B). The extraluminal domain of UL132 also contains two motifs for addition of N-linked carbohydrate modification and several computer-predicted motifs for O-linked glycosylation. Comparison of the available amino acid sequences from different HCMV isolates reveals an overall identity of 86%. Differences in primary sequence are primarily found in the ectodomain of the protein, where identity is only 62%, whereas the transmembrane domain of this type I glycoprotein has an amino acid identity of >97% (data not shown).

Transcription of ORF UL132. In a first set of experiments, the transcription kinetics of ORF UL132 was analyzed using the low-passage HCMV isolate TB40E (38). To this end, total RNA was isolated from infected cells at immediate-early (IE), early (E), and late (L) times postinfection using appropriate inhibitors. Using RT-PCR with UL132-specific primers, a signal was obtained only in RNA preparations from late times after infection (Fig. 2). Northern blot analyses of RNA isolated at different time points after infection revealed a 3.6-kb RNA in cells infected with strain TB40E or AD169, indicating that

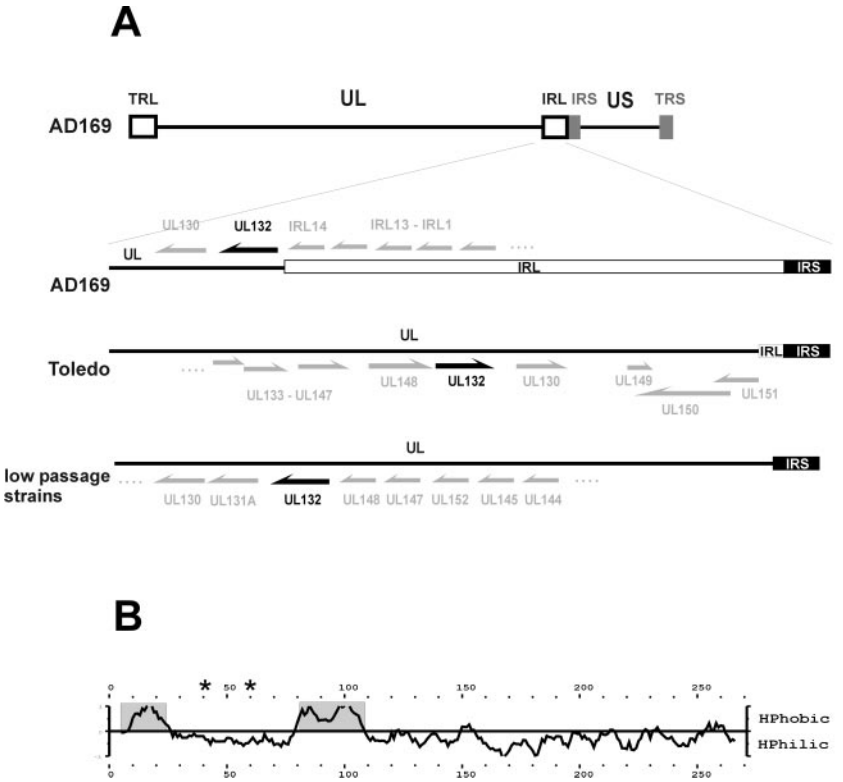

FIG. 1. Genomic localization of ORF UL132 and structural predictions of the protein product. (A) Localization of UL132 within the genomes of different HCMV strains. (B) Computer prediction of the hydrophilicity and potential N-linked glycosylation sites (*) of the predicted UL132 polypeptide. The potential signal and membrane anchor sequence is indicated by boxes.

the kinetics of transcription is similar between low-passage and high-passage HCMV isolates (Fig. 2). We conclude from these results that UL132 is a true late gene of HCMV.

UL132 protein in infected cells and in virions. Immunoblot analyses were carried out to characterize the expression of gpUL132 in infected cells and virions. Since low-passage HCMV strains produce less infectious virus and do not replicate to titers comparable to that of AD169 we used HCMV AD169, derived from the BAC HB-5 (5), for the initial analyses. Fibroblasts were infected with strain AD169 and cell lysates were prepared 96 h postinfection. In infected cells, two major signals were observed using a rabbit anti-UL132 serum. The larger protein migrated between 45 and 60 kDa and the smaller between 22 and 28 kDa (Fig. 3A). Digestion of the protein lysate with PNGase F, which removes complex N-linked sugars, reduced the molecular mass of the large protein to 35 to 45 kDa. Endoglycosidase H, which removes high-mannose carbohydrates, had no detectable effect on the migration of the protein.

The finding that digestion of the protein with endoglycosidase H resulted in a smear in PAGE indicated that additional modifications were present on the polypeptide, most likely O-linked carbohydrates. The extraluminal domain of UL132 contains a number of serine and threonine residues which could serve as substrates for addition of O-linked sugars. The small form of the protein was resistant to both glycosidases. Lysates from noninfected fibroblasts did not produce specific signals in immunoblots (Fig. 3A). Thus, our data demonstrated that the protein product of ORF UL132 represents a glycosylated protein and it was therefore designated gpUL132.

Immunoblot analysis using extracellular virions revealed bands identical to those in lysates from infected cells (Fig. 3B). The sensitivity of the virion-associated protein forms to endoglycosidase H and PNGase F was also identical to that of the polypeptides from infected cell lysates (data not shown). Extracellular virus particles from a number of low-passage HCMV isolates were also tested. As can be seen in Fig. 3C, all strains contained both forms of gpUL132. However, the relative ratios of the small and the large form of the gpUL132 differed between strains of viruses. In addition, since longer gels were used for this analysis, it became apparent that the diffuse migration of the large form shows considerable variation between strains and that the signal from the small gpUL132 consists of two to three different bands.

The theoretical molecular mass of UL132 is 29.8 kDa. Thus, the 45- to 60-kDa protein most probably represents fully modified gpUL132, carrying N-linked and O-linked carbohydrates. The nature of the 22- to 28-kDa protein was unclear. It could represent an unmodified or minimally modified form of gpUL132 with an aberrant migration in SDS-PAGE. The diffuse migration as well as the presence of multiple bands, however, indicated that the protein carried some modification. Alternatively, the protein could represent a posttranslationally processed form of the larger protein or be derived from initiation at an internal start site within the UL132 ORF and thus represent a shorter form of gpUL132.

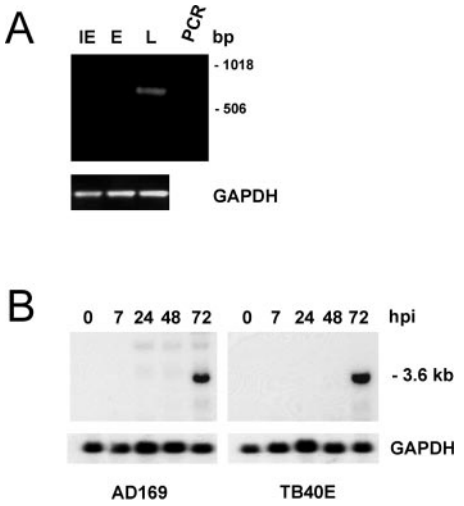

FIG. 2. Kinetics of transcription of UL132. (A) Total RNA was isolated from infected fibroblasts and RT-PCR was carried out using primers specific for UL132 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Amplification products were separated on a 1% agarose gel and stained with ethidium bromide. For immediate-early RNA preparation (lane IE), protein synthesis was inhibited using cycloheximide and RNA was prepared 7 h after infection. For preparation of early RNA (lane E), phosphonoformic acid was used to block viral DNA synthesis and the RNA was prepared 24 h after infection. Late RNA (Lane L) was prepared 72 h postinfection. Lane PCR represents a control PCR without reverse transcription. (B) Northern blot analysis of total RNA isolated from fibroblasts infected with HCMV strain AD169 or TB40E. RNA was isolated at the indicated hours postinfection (hpi) and subjected to Northern blot analysis. A strand-specific probe from the UL132 ORF was used to detect the RNA. Detection of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA was used as a control.

To obtain more information about the nature of the 22- to 28-kDa protein we performed an immunoblot analysis using extracellular viral particles of a recombinant virus (RV-UL132-M2) expressing gpUL132 containing an M2 epitope tag at the carboxy terminus (the construction of recombinant viruses is described collectively below). As shown in Fig. 3B, the proteins recognized by an anti-M2 monoclonal antibody were identical to the signals obtained with the polyclonal anti-UL132 rabbit serum. This indicates that the 22- to 28-kDa form of gpUL132 contains an intact carboxyl terminus and that the difference in molecular mass is the result of alterations within the amino terminus of the protein.

A hallmark of HCMV structural glycoproteins that have been identified thus far is the formation of disulfide-linked homomeric or heteromeric protein complexes. We therefore analyzed complex formation of gpUL132 in extracellular virions. Western blot analysis of viral lysates in the absence of reducing agents showed a migration pattern of the two forms of gpUL132 that was unchanged compared to that under reducing conditions, indicating that gpUL132 is not part of a disulfide-linked complex (Fig. 3B). The diffuse high-molecular-weight signals that are present in the immunoblot shown in Fig. 3B were not consistently observed and were considered nonspecific aggregation. Control analysis using a monoclonal antibody specific for gB showed the expected high-molecular-weight complex (8).

To define the intracellular localization of gpUL132 in infected cells we utilized fibroblasts infected with recombinant virus RV-UL132-M2. Since gpUL132 is expressed very late after infection, analysis using the anti-UL132 rabbit serum gave less clear results due to the binding of rabbit immunoglobulin G to virus-encoded Fc receptors (1). The cells were infected for 72 h and used for fluorescence imaging with antibodies directed against viral proteins or against cellular markers of the secretory pathway. Localization of gpUL132 showed little overlap with calrecticulin, a marker for the endoplasmic reticulum. In contrast, almost complete colocalization was observed of gpUL132 with markers for the trans-Golgi network (Fig. 4). Markers for the Golgi apparatus and gpUL132 showed partial overlap (data not shown). gpUL132 also showed colocalization with other viral envelope glycoproteins such as gB (Fig. 4) and gH (not shown).

Taken together, these data indicated that in infected cells gpUL132 accumulated in a juxtanuclear location that contains markers specific for the trans-Golgi network and which has been argued to constitute the virus assembly compartment (36). In addition, our findings also demonstrated that gpUL132 is a component of extracellular HCMV particles.

Recombinant expression of the UL132 polypeptide. To analyze the protein product(s) of ORF UL132 in the absence of viral infection, the coding sequence from strain TB40E was inserted into the vector pcDNA3myc/his, allowing the synthesis of a gpUL132 protein containing an epitope-tagged carboxyl terminus. Following transient transfection in 293T cells, the protein was analyzed in immunoblots using a Myc-specific monoclonal antibody. In immunoblots, multiple protein species were detected as a smear migrating between 30 kDa and 50 kDa, with two predominant bands at 35 and 42 kDa (Fig. 5). In contrast to the results obtained with infected cells, digestion of the protein lysate with endoglycosidase H reduced the molecular mass of the major bands to 30 to 35 kDa. PNGase F did not further reduce the molecular mass of the smallest form of the UL132 protein. However, some of the larger forms were reduced in mass, indicating that transiently expressed protein carries mainly high-mannose carbohydrates (Fig. 5). Similar results were obtained using the rabbit anti-UL132 (data not shown). Despite multiple attempts, the small-protein form of gpUL132 was not detected in transiently transfected cells.

The intracellular localization of transiently expressed gpUL132 was analyzed in indirect immunofluorescence analyses. Similar to the protein from infected cells, transiently expressed gpUL132 was found to colocalize with markers specific for the Golgi apparatus and the trans-Golgi network, and not with the endoplasmic reticulum-localized protein calreticulin (data not shown). In cotransfections, transiently expressed UL132 was also found to colocalize with HCMV gB (data not shown).

Construction of UL132 recombinant viruses. In order to investigate the role of gpUL132 in the replication of HCMV, we generated a series of recombinant viruses using a strategy that included the use of an infectious clone of HCMV maintained as a bacterial artificial chromosome in *E. coli*. To generate a UL132 null mutant in strain AD169 (RV-UL132del/AD) we utilized a kanamycin resistance cassette that was flanked by 50 nucleotides of ORFs UL130 and IRL14. To generate a null mutant in the low-passage HCMV isolate PAN,

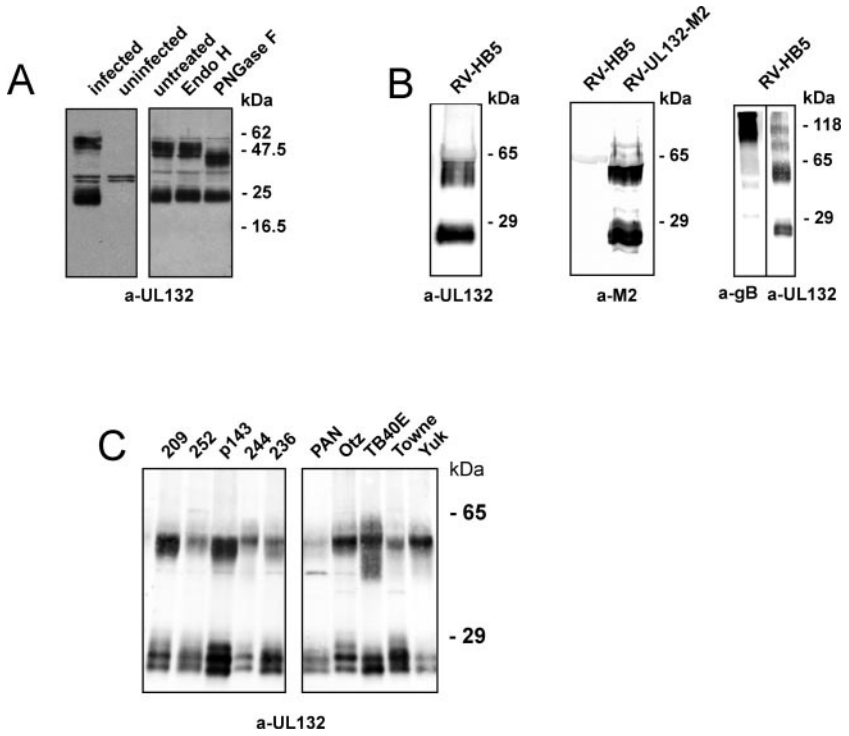

FIG. 3. Detection of gpUL132 in HCMV infected cells and extracellular virus particles. (A) Fibroblasts were infected for 10 days with HCMV-AD169 and lysates were subjected to digestion with endoglycosidase H or PNGase F followed by immunoblot analysis using an anti-UL132 rabbit serum (a-UL132). (B) Extracellular virus particles from RV-HB5 (representing strain AD169) or a recombinant virus expressing an M2 epitope-tagged gpUL132 (RV-UL132-M2) were used for immunoblot analysis using either an anti-UL132 rabbit serum (a-UL132), an anti-M2 monoclonal antibody (a-M2), or the gB-specific monoclonal antibody 27-287 (a-gB). The analysis shown in the right panel was carried out in the absence of reducing agents. (C) Immunoblot analysis of extracellular HCMV particles purified from the tissue culture supernatant. The individual strains are indicated. The anti-UL132 rabbit serum was used for detection of gpUL132.

a kanamycin resistance cassette was constructed that was flanked by ORFs 131 and 148. In both cases the predicted recombination product would have the entire ORF UL132 deleted and replaced by the kanamycin resistance gene.

The cassette was recombined into the corresponding HCMV BACs, pHB5 (5) and RV-PAN23 (40), and kanamycin-resistant colonies were selected. For strain AD169 a revertant virus (RV-AD132rev) as well as a recombinant virus expressing gpUL132 containing an M2 epitope at the carboxyl terminus (RV-UL132-M2) were also constructed. The DNA of the resulting BACs was analyzed for overall integrity by restriction analysis, Southern blot, PCR analysis, and nucleotide sequencing in the respective genomic regions and found to be as predicted (data not shown). BAC DNA was transfected into MRC-5 cells to reconstitute recombinant viruses. A cytopathic effect was observed for all viruses approximately 3 weeks after transfection.

Analysis of cells infected with RV-delUL132/AD or RV-delUL132/PAN viruses using a UL132-specific rabbit serum in an indirect immunofluorescence assay failed to detect expression of UL132 (data not shown). To verify the absence of gpUL132 from extracellular virions, Western blot analyses were performed with recombinant viruses derived from the AD169 BAC. Whereas the two protein forms of gpUL132 were detected in virions from RV-HB5 and the mutant RV-UL132-M2, no protein was seen in lysates from RV-delUL132/AD (Fig. 6). Reprobing the Western blot with an antibody against

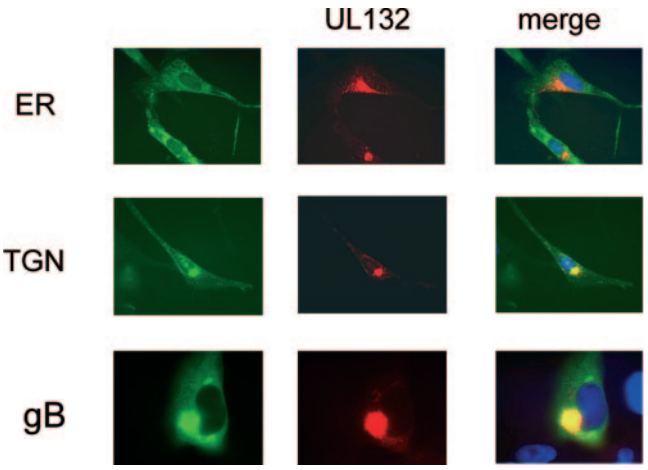

FIG. 4. Intracellular localization of gpUL132 in infected cells. Fibroblasts were infected with the recombinant virus RV-UL132-M2 for 72 h. The intracellular localization of the individual proteins was determined by comparing the signal from Flag-specific antibody M2 (UL132) with those of antibodies or lectins specific for HCMV gB or components of the secretory pathway (endoplasmic reticulum [ER], anticalreticulin; trans-Golgi network [TGN], wheat germ agglutinin). The cellular markers and gB were developed with fluorescein isothiocyanate, and gpUL132 with Cy3-coupled anti-mouse immunoglobulin G. Yellow indicates colocalization of the signal. In the merge panel, cell nuclei are also stained blue. Magnification: panels ER and TGN, ×400; panel gB, ×630.

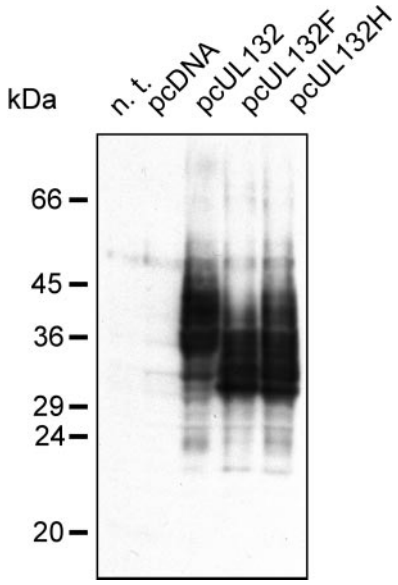

FIG. 5. Recombinant expression of UL132. 293T cells were transfected with a plasmid coding for UL132-Myc and cell lysates were prepared 48 h later. Lysates were treated with either PNGase F (pcUL132F) or endoglycosidase H (pcUL132H) or left untreated (pcUL132). Immunoblots were carried out and proteins were visualized using an anti-Myc antibody. Lanes: pcDNA, cell lysate prepared following transfection with plasmid pcDNA3; n.t., lysate from nontransfected 293T cells.

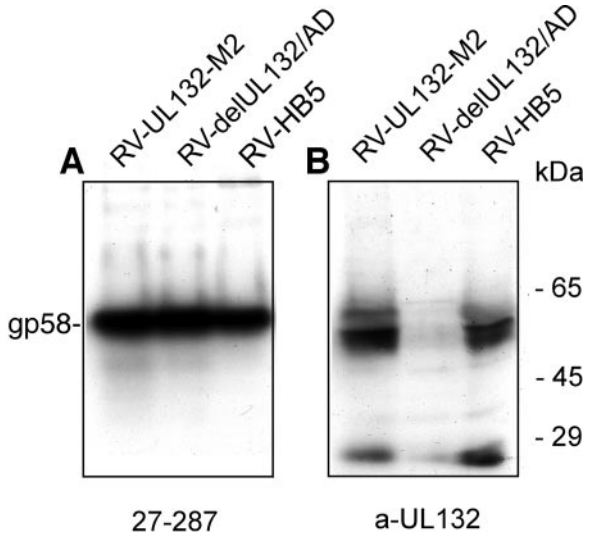

FIG. 6. Immunoblot analysis of virus particles lacking gpUL132. Extracellular virus particles were subjected to immunoblot analysis. Proteins were visualized using the gB-specific monoclonal antibody 27–287 (panel A) or the rabbit anti-UL132 serum (a-UL132) (panel B). Lanes: RV-HB5, recombinant virus representing AD169; RV-delUL132/AD, recombinant virus lacking ORF UL132; RV-UL132-M2, recombinant virus expressing M2-tagged gpUL132. The signal identifying the transmembrane unit of gB (gp58) is indicated in panel A.

gB demonstrated that comparable amounts of protein from each virus strain were analyzed (Fig. 6).

To investigate possible effects of UL132 deletion on the kinetics of virus replication, both single-step and multistep growth curve analyses were performed. In the case of RV-delUL132/AD, fibroblasts were infected with identical amounts of infectious virus from either the parental RV-HB5 or the mutant and the virus titers were determined in the tissue culture supernatants over a period of 11 days. As can be seen in Fig. 7A, the UL132 deletion resulted in an attenuated growth phenotype in cell culture. The final virus yield, obtained after 11 days of infection, was reduced approximately 100-fold. The RV-delUL132/AD-viruses also exhibited a severely reduced plaque size (data not shown). The revertant virus RV-UL132rev had replication kinetics identical to those of RV-HB5, indicating that second-site mutations did not contribute to the growth defect of the UL132 null virus.

Quantitative real-time PCR showed that the number of HCMV genome copies in the supernatants of RV-UL132del/AD-infected cells were reduced in parallel to the reduction in infectious titers, indicating that fewer viruses were produced by the deletion mutant (data not shown). We then performed a single-step growth curve utilizing a multiplicity of infection of 0.1 for both RV-HB5 and RV-delUL132/AD and titered the yield of infectious virus over 6 days using a conventional plaque assay. The yield of infectious RV-del132/AD virus was approximately 100-fold less than that of the wild-type RV-HB5 virus (data not shown). From these data we have concluded that the deletion of the UL132 open reading frame results in a growth-impaired phenotype that appears to be associated with a decrease in the production of infectious virus.

The replication kinetics of RV-delUL132/PAN was determined differently owing to the fact that this low-passage virus released small amounts of infectious virus into the tissue culture supernatant. In addition, the infectivity of the virus released into the tissue culture medium was unstable, as has also been observed with other low-passage HCMV isolates (40). Because a variety of methods reported for preservation of viral infectivity failed to maintain the infectivity of the PAN virus, a virus stock that contained a stable titer of virus for use in titration assays was impossible to obtain. As a consequence, virus titration and analysis of replication kinetics were performed during the same experiment as described in the Materials and Methods section. Experiments were done in triplicate and the results are shown in Fig. 7B. At day 6 postinfection, the parental virus RV-PAN23 reached a titer of 2×10^4 PFU/ml, whereas RV-delUL132/PAN reached titers of approximately 5×10^3 PFU/ml.

We conclude from these results that deletion of ORF UL132 from the genome of high- and low-passage HCMV strains results in a significant replication deficit in fibroblasts in cell culture.

Entry of UL132 recombinant viruses. As a component of the virion envelope, gpUL132 might be involved in the early steps of infection, i.e., attachment and/or penetration, and the observed replication deficit of gpUL132-negative viruses might be a consequence of the inhibition or delay of these early stages of infection. To test a potential role of gpUL132 in attachment and/or penetration we analyzed the entry of the viruses into fibroblasts. Experimentally, we made use of the fact that during the first minutes after penetration, large amounts of the tegument protein pp65 (ppUL83) are translocated to the cell nucleus (37). This process can be monitored

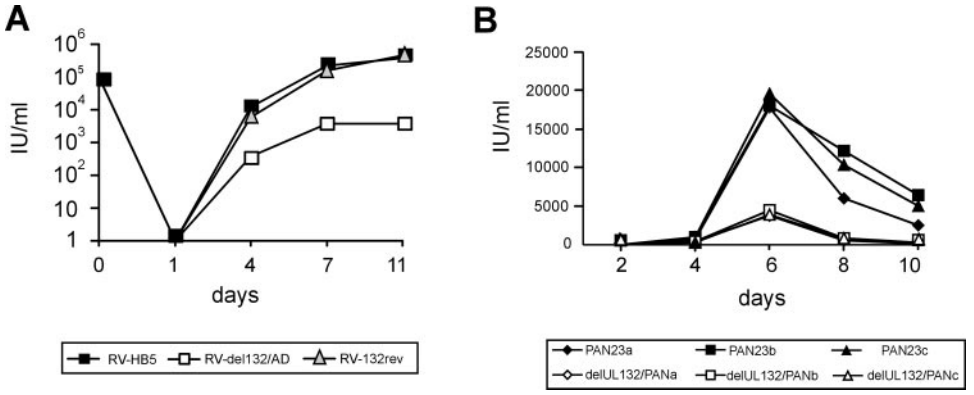

FIG. 7. Replication of UL132 mutant viruses. (A) Fibroblasts were seeded in six-well dishes and infected with the indicated virus strains (multiplicity of infection, 0.1). At the indicated days postinfection, supernatants from the infected cultures were harvested and infectious virus was titrated using indirect immunofluorescence with an antibody directed against the immediate-early antigen 1. Each data point represents the mean of three independent wells. RV-HB5: virus representing AD169; RV-delUL132/AD: recombinant virus lacking ORF UL132; RV-132rev: revertant virus with UL132 reintroduced into RV-HB5. (B) HFF were infected with a multiplicity of infection of 1. At the indicated time points postinfection (days), virus titers in the supernatant of three independently infected cultures (a to c) were determined as described in Materials and Methods. PAN23: recombinant virus PAN; delUL132/PAN: recombinant PAN virus lacking ORF UL132. IU: infectious units.

by indirect immunofluorescence and represents a sensitive test for the very early events during infection.

Fibroblasts were infected with RV-HB5 or RV-delUL132/AD viruses for 5, 10, 20, or 30 min and processed for indirect immunofluorescence using monoclonal antibody 65-8 specific for pp65 (36). RV-delUL132/PAN could not be tested in this assay due to the low titer of extracellular virus. As can be seen in Fig. 8, there was no difference in the delivery of pp65 to the cell nuclei between RV-HB5 and RV-delUL132/AD. As soon as 5 min after infection, a faint pp65 signal was detectable in the nuclei of most cells. The signal intensity increased at 10 and 20 min postinfection (not shown) and at 30 min after infection the cell nuclei of most cells were stained intensively, indicating that viral particles had infected the target cells and delivered the pp65 protein to the nucleus (Fig. 8). The similar kinetics of entry of RV-HB5 and RV-delUL132/AD was seen in assays using either preadsorption of virus to the target cells at 4°C or infecting the cells at 37°C (data not shown). Thus, we conclude that deletion of gpUL132 from the viral particle has no major impact on the entry of HCMV.

DISCUSSION

Although a great deal is known about the envelope of alphaherpesviruses, our understanding of the composition of the HCMV envelope is still incomplete. The recent analysis by Varnum et al. (47) identified 19 virally encoded glycoproteins as components of the viral particle. In that study, mass spectrometry was used to characterize the HCMV proteome and gpUL132 was found to be a component of the virus. The abundance was calculated to be around 0.4%, which classifies gpUL132 as a low-abundance structural component of HCMV. While mass spectrometry provides valuable information on the overall protein composition of viral particles, it also has limitations, e.g., for the identification of protein complexes or posttranslational modification of proteins.

In this report we have shown that gpUL132 exists in two forms, 45 to 60 kDa and 22 to 28 kDa, in infected cells as well as in extracellular virions from low-passage and high-passage HCMV strains. The sensitivity of the protein to PNGase F indicated that the 45- to 60-kDa form of gpUL132 in infected cells and virions carries complex N-linked sugars. The sensitivity of the 45- to 60-kDa form of gpUL132 to digestion with PNGase F also provided proof that gpUL132 is a type I glycoprotein with its amino-terminal domain being on the outside of infected cells or virions. The two potential consensus sequences for the addition of N-linked carbohydrates (NMT_{34} and NMT_{63}) are both found between the predicted signal sequence (amino acids 1 to 24) and the transmembrane anchor (amino acids 84 to 106).

In addition to complex N-linked carbohydrates, gpUL132 most probably carries O-linked sugars. This is indicated by the diffuse migration of the protein after removal of the N-linked carbohydrates. According to computer-based predictions,

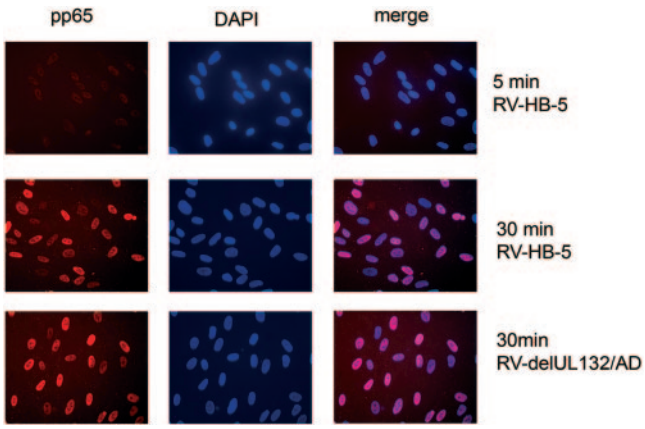

FIG. 8. Entry of UL132 mutant viruses into fibroblasts. Fibroblasts were infected (multiplicity of infection, 10) with particles of the respective viruses at 37°C for the indicated times and processed for indirect immunofluorescence using a pp65-specific monoclonal antibody (pp65). Cell nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI). Magnification, ×400.

gpUL132 carries 17 sites for addition of O-linked glycosylation (NetOGlyc 3.1 server; http://www.cbs.dtu.dk). Additional modifications such as phosphorylation, which has also been detected on other HCMV structural glycoproteins such as gB, could further add to the diffuse migration pattern seen in PAGE (34).

The 22- to 28-kDa form of gpUL132 did not appear to carry N-linked sugars based on our finding that digestion with endoglycosidase H or PNGase F failed to yield a more rapidly migrating protein. The origin(s) of this smaller form of gpUL132 form is unclear at present. Splicing of the mRNA and thus the generation of a smaller ORF is unlikely since we found only a single 3.6-kb RNA in infected cells; however, we cannot eliminate the possibility that the smaller form of gpUL132 arose from a minor population of spliced RNA that was not detected in this analysis. From data derived with RV-UL132-M2 it is clear that the carboxy-terminal domain of the protein is intact, indicating that the difference in mass between the two forms of gpUL132 must reside in the amino-terminal part of the protein.

Several explanations for the origin of the smaller forms gpUL132 are possible. The theoretical molecular mass of the unmodified UL132 polypeptide of strain AD169 is 29.9 kDa. Thus, it is conceivable that the small protein could represent a minimally modified form of gpUL132 showing a slightly aberrant migration in PAGE. However, the presence of some modification on this form of the molecule is indicated by its diffuse migration in PAGE as well as the multiple forms recognized by the anti-UL132 serum in a number of HCMV strains. Alternatively, the protein might represent a proteolytic cleavage product or originate from an internal in-frame start codon, resulting in a truncated form of the protein. Internal initiation has also been observed for other HCMV structural proteins (43). Interestingly, the smaller gpUL132 form was seen only in infected cells and extracellular virions but was not produced in transfected cells, indicating that the context of virus infection is prerequisite for the generation of the small forms of the gpUL132. In addition, a pulse-chase experiment of AD169-infected cells failed to reveal a precursor-product relationship between the larger and smaller forms of gpUL132, suggesting that the smaller form was not a proteolytic cleavage product of the larger form of gpUL132 (data not shown). Although the nature of the protein and the underlying mechanism(s) responsible for the generation of the small form of gpUL132 have yet to be defined, it is important to note that both forms are incorporated into virus particles in nearly equivalent amounts. This observation raises the possibility that both forms of gpUL132 could have roles in the replication and infectivity of HCMV.

Deletion of ORF UL132 from the genome of the laboratory strain AD169 as well as the low-passage isolate PAN resulted in a pronounced replication deficit. This effect could not be attributed to second-site mutations within our recombinant viruses because different and independent strategies were used to generate the two mutant viruses RV-delUL132/AD and RV-delUL132/PAN, making it unlikely that in both cases an additional mutation could result in the observed replication deficit, and a revertant virus that was constructed for strain AD169 showed replication kinetics identical to the wild type. Finally, strains AD169 and PAN contain ORF UL132 in a different genomic context, making it highly unlikely that disturbance in transcription or translation of adjacent genes accounts for the altered replication.

In addition, the replication deficit that was observed in our study using RV-delUL132/AD is in agreement with data generated during genomewide transposon-mediated insertional mutagenesis in strain AD169 (14). In contrast, a recent study using the low-passage clinical isolate FIX came to the conclusion that deletion of ORF UL132 had no influence on the replication of the mutant virus in fibroblasts, although the virus replicated more slowly in endothelial cells (16). We also noted that deletion of the UL132 ORF in a clinical virus isolate, PAN, had a lesser effect on virus replication in fibroblasts. This finding is somewhat difficult to interpret directly because none of these three viruses is isogenic and additional viral genes could influence the growth phenotype of these viruses in fibroblasts. However, a possible explanation for these discrepant findings could be a compensation in strain FIX of the defect introduced by the deletion of UL132 by additional gene products.

HCMV FIX represents a genetically complete virus since it encodes a number of functions, including endothelial cell tropism and transmission to leukocytes, that have been lost by AD169 and PAN. The genetic basis of the loss of function for these two strains has not been completely determined, but genes from the Ulb′ region, in particular UL128 to UL132 and UL146/147, are most probably key to these functions (16). The genome of strain AD169 does not contain the Ulb′ region and consequently this virus has lost endothelial cell tropism as well as the capacity to be transmitted to leukocytes. The PAN virus represents an intermediate between FIX and AD169 in that it contains a Ulb′ region but has lost the endothelial cell tropism and leukocyte transfer potential. It is conceivable that the intermediate phenotype of PAN compared to FIX and AD169 with respect to replication of the mutant viruses might be the result of a partial compensation of the UL132 defect by a function provided by the Ulb′ region.

We can only speculate on the function of gpUL132 in the viral replication cycle, but at this point, it appears to have an important role in production of infectious progeny. This assumption is based on several observations.

(i) Quantitative real-time PCR showed that the number of HCMV genome copies in the supernatants of RV-UL132del/AD-infected cells was reduced in parallel to the reduction in infectious titers, indicating that smaller numbers of DNA-containing virions were produced by the deletion mutant.

(ii) The multistep growth curves showed that in the case of the RV-HB5 and RV-delUL132/AD viruses, titers in the tissue culture supernatant reached a plateau within the observation period of 11 days, indicating that the defect in the UL132 mutant virus is not simply a delay in replication. For strain PAN and the respective UL132 deletion mutant, peak virus titers were reached at the same day postinfection, again pointing to the fact that production of infectious virions was not delayed in the UL132 mutant virus.

(iii) The entry of UL132 mutant virus was not impaired compared to wild-type RV-HB5, indicating that gpUL132 has no major role in attachment and/or penetration of HCMV to fibroblasts.

The current model of herpesvirus morphogenesis postulates

that capsids obtain a primary envelope as they pass through the inner nuclear membrane. On exit from the outer nuclear membrane, they undergo deenvelopment. Capsids that are released in the cytoplasm are subsequently coated with tegument proteins and finally wrapped by membranes of a post-Golgi compartment that has been termed the assembly compartment (36). This compartment has been shown to contain proteins found in the trans-Golgi network and late endosomes (18, 29). Thus, the structural glycoproteins must be targeted to this compartment and, late in infection, become concentrated within this organelle. This proposed assembly pathway appears to be a default assembly pathway for all herpesviruses (29). However, it should be emphasized that the number of proteins required for assembly of an infectious particle and their specific function(s) in virion morphogenesis likely vary significantly between individual families of the herpesviruses (29). Thus, it could be difficult to extrapolate findings from studies of the assembly pathway of a specific herpesvirus to other members of this diverse family.

The finding that recombinant gpUL132 can be localized to the trans-Golgi network in the absence of other viral functions demonstrates that, similar to other structural glycoproteins of HCMV such as gB, gpUL132 contains all of the *cis*-acting elements necessary for trans-Golgi network localization. This is in contrast to a number of other HCMV structural glycoproteins, including gH, gM, gN, and gpTRL10, which require complex formation with other viral proteins in order to reach the more distal parts of the secretory system (22, 26, 39). Inspection of the gpUL132 cytoplasmic domain for the presence of trafficking motifs that are known to function in these processes reveals a number of motifs that may be involved in this intracellular trafficking. Three tyrosine-based motifs, YXXΦ (where Y is tyrosine, X is any amino acid, and Φ is any bulky hydrophobic amino acid), are found at positions $YQRL_{162}$, $YVSV_{231}$ and $YDEL_{235}$. YXXΦ motifs mediate the incorporation of membrane proteins into transport vesicles due to interaction with cellular adaptor proteins (reviewed in reference 4). In addition, gpUL132 contains acidic cluster motifs which potentially interact with the connector protein PACS-1 (phosphofurin acidic cluster sorting protein 1), which redirects proteins from the endosomes to the trans-Golgi network, a proposed site of tegument assembly and virion envelopment (15). Finally, gpUL132 contains a dileucine-based sorting signal ($DEEAVNLL_{127}$) which is potentially also involved in binding to cellular adaptor protein complexes. Corresponding motifs in alphaherpesviruses are responsible for direct transport of glycoproteins to membranes that are involved in cell-cell contact (28, 35, 45). Thus, it seems likely that trafficking of gpUL132 involves transport to the plasma membrane and recycling from there to the endosomal compartment involved in virus envelopment. Future experiments will test this hypothesis.

In summary, we have shown that the protein product of ORF UL132 of HCMV represents a structural viral component and that deletion of the reading frame results in a drastic replication deficit in low-passage and laboratory-adapted strains of HCMV. Further studies will be aimed at clarifying the function of gpUL132 during the replication of HCMV.

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