Rhesus and Human Cytomegalovirus Glycoprotein L Are Required for Infection and Cell-to-Cell Spread of Virus but Cannot Complement Each Other[∇]

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Received 16 September 2010/Accepted 17 December 2010

Rhesus cytomegalovirus (RhCMV), the homolog of human cytomegalovirus (HCMV), serves as a model for understanding the pathogenesis of HCMV and for developing candidate vaccines. In order to develop a replication-defective virus as a vaccine candidate, we constructed RhCMV with glycoprotein L (gL) deleted. RhCMV gL was essential for viral replication, and virus with gL deleted could only replicate in cells expressing RhCMV gL. Noncomplementing cells infected with RhCMV with gL deleted released intact, noninfectious RhCMV particles that were indistinguishable from wild-type RhCMV by electron microscopy and could be rescued by treatment of cells with polyethylene glycol. In addition, noncomplementing cells infected with RhCMV with gL deleted produced levels of gB, the major target of neutralizing antibodies, at levels similar to those observed in cells infected with wild-type RhCMV. Since RhCMV and HCMV gL share 53% amino acid identity, we determined whether the two proteins could complement the heterologous virus. Cells transfected with an HCMV bacterial artificial chromosome with gL deleted yielded virus that could replicate in human cells expressing HCMV gL. This is the second HCMV mutant with an essential glycoprotein deleted that has been complemented in cell culture. Finally, we found that HCMV gL could not complement the replication of RhCMV with gL deleted and that RhCMV gL could not complement the replication of HCMV with gL deleted. These data indicate that RhCMV and HCMV gL are both essential for replication of their corresponding viruses and, although the two gLs are highly homologous, they are unable to complement each other.

Human cytomegalovirus (HCMV) is the causative agent of several life-threatening diseases in immunocompromised persons and is the leading viral cause of birth defects in the United States. Given the medical and economic burden of HCMV-related disease, the Institutes of Medicine has given the development of an HCMV vaccine “priority one” status (39). At present, the vaccine candidate that is farthest along in clinical trials, soluble HCMV glycoprotein B (gB), reduced the rate of CMV infection in healthy women by 50% (30). While promising, more effective vaccines, including those that might stimulate higher levels of cellular immune responses, would be desirable.

With the exception of chimpanzee CMV, rhesus CMV (RhCMV), is the closest homolog to HCMV and has been used as a model for studies of pathogenesis and vaccine development (45). From 42 to 60% of RhCMV genes have functional and positional homologs in HCMV, depending on the

strain of RhCMV analyzed and the criteria used for identifying open reading frames (12, 34). RhCMV encodes at least 21 homologs of HCMV glycoproteins, including gB, gH, gL, gM, gN, and gO and UL128, UL130, and UL131, all of which are essential for efficient replication of the virus in the host.

All human herpesviruses encode a homolog of gL. While gL is thought to be essential for viral replication, all known functional properties of gL are directly associated with its dimerization with gH. The most extensively studied gH/gL complex is that of herpes simplex virus (HSV). Although gH and gL are expressed as separate open reading frames, maturation, transport, and function of both proteins are dependent on each other's expression; failure to express either gH or gL results in mislocalization and improper folding of the other (3, 8, 17, 32). Absence of gL, resulting in the lack of a functional gH, also results in a defect in virus entry (35) and an inability to initiate membrane fusion (7). Although gH/gL has been postulated to function as a fusogen (33, 40), the cocrystal structure of gH/gL does not resemble a classical type 1, 2, or 3 fusion protein (5). These results suggest that gH/gL does not act directly as a fusogen but rather modulates the function of gB, a known type 3 fusogen (13). The cocrystal structure also demonstrates the extensive contacts between gH and gL, highlighting the dependence of both glycoproteins on each other to maintain gH/gL structure and function (5). Not surprisingly, HSV gL deletion mutants are deficient for

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[∇] Published ahead of print on 29 December 2010.

virus entry but can be propagated in cell lines that provide gL *in trans* (35).

HCMV gH and gL, like their HSV homologs, interact extensively (15, 19, 38). In the absence of gL, HCMV gH does not fully mature and is not transported to the plasma membrane (19, 38). Unlike HSV, however, HCMV gH and gL form larger complexes with either gO or the UL128/UL130/UL131 proteins, the latter of which are required for entry into epithelial and endothelial cells (36, 44). While HCMV gL has been deleted from bacterial artificial chromosomes (BACs) containing the entire HCMV genome, virus was not recovered when the mutated BACs were transfected in human fibroblasts (10, 14). These data suggest that HCMV gL is essential for viral replication; however, since the mutant BACs were not complemented *in trans*, it was not proven that the failure to produce virus was not due to another mutation in the BAC or that the deletion of gL affected a neighboring gene that might be required for virus replication. To date, only one essential HCMV glycoprotein, gB, has been deleted and complemented in mammalian cells (18).

We describe here the construction and characterization of RhCMV and HCMV gL deletion mutants that can be propagated in cells stably transduced to express the corresponding gL. Noncomplementing cells infected with the RhCMV gL deletion mutant express high levels of RhCMV gB and produce virions indistinguishable from wild-type virus by electron microscopy; however, these virions are not infectious. Despite their homology, cells expressing HCMV gL and RhCMV gL were not able to complement RhCMV with gL deleted or HCMV with gL deleted, respectively.

MATERIALS AND METHODS

Cell lines and viruses. Cos-1, telomerase-immortalized rhesus fibroblasts (Telo-RF cells) (22) and human foreskin fibroblasts (HFFs) were grown in Dulbecco modified Eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; HyClone), penicillin, and streptomycin (complete medium). Recombinant RhCMV BAC strain 68.1, a gift from Peter Barry (University of California, Davis), was propagated in Telo-RF cells as previously described (4). Wild-type HCMV was isolated from a patient at the NIH Clinical Research Center and amplified twice in human foreskin fibroblasts.

Retrovirus production and transduction. A retrovirus vector expressing RhCMV gL was derived by amplifying RhCMV gL from RhCMV BAC 68.1 using primers 5'-AAGGAAAAAGCGGCCGACTGAGTAAGAGACAACCGATGTGG-3' and 5'-ACCTGGTAGTCGACTCCTTAACAGACAAGAGCGAG-3', cutting the PCR product with NotI and SalI and ligating the DNA containing RhCMV gL to retrovirus vector CMMPL115-IRES-GFP (20) that had been digested with NotI and XhoI. The resulting plasmid was termed pCMMPL RhgL-IRES-GFP and expresses both RhCMV gL and green fluorescent protein (GFP). Plasmid pMD-gagpol expresses murine leukemia virus Gag and Pol proteins (20), and pMD-G expresses vesicular stomatitis virus G (29). Recombinant retrovirus expressing RhCMV gL was generated by transfecting 293T cells with plasmids pCMMPL RhgL-IRES-GFP, pMD-gagpol, and pMD-G (20) using Lipofectamine 2000 in 100-mm dishes. Recombinant retrovirus expressing HCMV gL was generated by transfecting 293T cells with plasmids CMMPL115-IRES-GFP, pMD-gagpol, and pMD-G. At 24 h after transfection, the medium was replaced with 5 ml of complete medium containing 100 mM HEPES (collection medium). At 48, 72, and 96 h posttransfection, the supernatant was harvested and replaced with fresh medium. Supernatants were combined, filtered through 0.45- μ m-pore-size filters, and stored at -80°C.

For the transduction of fibroblasts, recombinant RhCMV gL or HCMV gL retrovirus was incubated overnight with subconfluent Telo-RF or HFF cells in the presence of 5 μ g of Polybrene/ml in DMEM containing 10% FBS. The next day, the medium was replaced, and when the cells were confluent they were treated with trypsin, replated, and transduced with retrovirus a second time. The resulting cultures—Telo-RF:RhGL, Telo-RF:HgGL, HFF:RhGL, and HFF:HgGL—

were monitored for GFP expression to ensure that >80% of cells were GFP positive prior to use in any assays.

LIPS assay. The luciferase immunoprecipitation system (LIPS) was used to measure antibody levels to RhCMV proteins (1). The open reading frames of RhCMV gB, gL, and pp65.2 proteins were each fused to a plasmid encoding *Renilla* luciferase (*Ruc*). Portions of the genes encoding RhCMV gB (amino acids 374 to 636), gL (amino acids 31 to 259), and pp65.2 (amino acids 1 to 543) were amplified by PCR with the primer pairs 5'-CCGGATCCACTGATTCCG CATCGGAC-3' and 5'-CCCTCGAGTTACTTCCCTGCAGAAC-3', 5'-CCG AATTCCGTCGATGCTTTACTGGG-3' and 5'-CCGTCACCTACAGGAATG TTTTCCAAC-3', and 5'-CCCTCGAGCTAACTACGGTGTCTTTT-3' and 5'-CCGGATCCATGGCCTCGCGACCG-3', respectively. The gB and pp65.2 PCR products were digested with BamHI and XhoI, and the gL PCR product was digested with EcoRI and KpnI and inserted into the corresponding site of plasmid pREN2 (2), C-terminal to *Ruc*. The final constructs were sequenced, and expression was verified by immunoblot analysis with antibody to *Ruc*.

Plasmids expressing RhCMV proteins were transfected into Cos-1 cells and, 48 h later, whole-cell lysates were prepared as described previously for other herpesvirus proteins (2). Plasma was obtained from rhesus macaques found to be naturally infected or uninfected using a serologic assay that measures antibody to RhCMV-infected cells (Oregon National Primate Research Center). Portions (1 μ l) of a 1:10 dilution of plasma from RhCMV-seropositive or -seronegative rhesus macaques in buffer A (20 mM Tris [pH 7.5], 150 mM NaCl, 5 mM MgCl₂, 1% Triton X-100), 40 μ l of buffer A, and 50 μ l of 10⁷ light units (LU) of *Ruc*-RhCMV antigen Cos1 cell extract were incubated at room temperature in each well of a 96-well plate. After 1 h, 7 μ l of a 30% suspension of protein A/G-beads was added to the bottom of each well of a 96-well filter HTS plate (Millipore, Bedford, MA). Then, 100 μ l of the antibody-antigen complexes was added to the filter plate, followed by incubation for 1 h on a shaker, and the plates were washed 10 times in protein A and twice in phosphate-buffered saline (PBS) using a vacuum manifold. The LU were measured by using coelenterazine substrate mixture (Promega, Madison, WI) in a Berthold LB 960 Centro microplate luminometer (Berthold Technologies, Bad Wilbad, Germany). The LU data shown are the averages of two independent experiments and are corrected for background LU values of *Ruc* Cos-1 cell extract added to protein A/G beads but not incubated with plasma.

RhCMV BAC mutagenesis. RhCMV BAC strain 68.1 DNA was harvested from *Escherichia coli* strain DH10B by a Nucleobond BAC 100 kit (Macherey-Nagel, Inc., Bethlehem, PA) and electroporated into *E. coli* strain SW102 (which lacks the galactose kinase enzyme and contains heat-inducible phage recombination genes), using previously described methods (43). RhCMV gL was deleted and replaced with GalK using the RED recombination method as described previously (43). The *galK* gene from plasmid pGalK (a gift from Neil Copland) was amplified by PCR using the primers 5'-TGCTCGTATGGTTAAGCATA CAAGCTTTATTATCAGGAATGTTTTCCAACATCCACTGCTTTAAGGC CATCTGTGACAAATTAATCATCGGCA-3' and 5'-CCCTATAATCCTGC AAGGACAGTAGAGTATCGAGACTGGTATACTACTAGTAAAGAGA CAACCGATGTGGTCAGCACTGCTGCTCTT-3' (underlined nucleotides correspond to *galK* sequences) that have flanking sequences that correspond to RhCMV 68.1 (12), nucleotides 150387 to 150457 and 151191 to 151261, respectively, in the region within and adjacent to RhCMV gL. PCR products were digested for 1 h at 37°C with DpnI to fragment the methylated plasmid template, and the resulting PCR product was gel purified by using a QIAquick gel extraction kit (Qiagen, Valencia, CA). To induce recombination, *E. coli* strain SW102 containing RhCMV BAC 68.1 was heat shocked at 42°C for 15 min and then electroporated with 50 ng of the DNA fragment containing *galK* with gL flanking sequences. After electroporation, the cells were incubated in 1 ml of L broth for 1 h at 30°C, washed three times in 1 $\times$ M9 buffer, and plated onto M63 minimal medium plates with chloramphenicol and galactose as the sole carbon source. After incubation for 3 days at 30°C, bacterial colonies were picked and screened for *galK* inserts by PCR. Positive clones were then streaked onto MacConkey plates with galactose and chloramphenicol to ensure *galK* expression. Red colonies were streaked twice onto M63 minimal medium plates with chloramphenicol and galactose to ensure clonality. BAC DNA was isolated from the bacteria and PCR was used to verify that RhCMV gL had been deleted.

To remove the *galK* cassette from the BAC, double-stranded oligonucleotides (5'-TATGTAGTAAAGCGGGTGTGTTTGTAGCGGCTGTTTCCACTCG TCTGGCTCCACATCGGTTGTCTTACTCAGTAGTATAACAGTCTCG ATACTCA-3' and 5'-TAGAGTATCTAGACTAGTGTACTACTAGTAGTAA GAGACAACCGATGTGGAGCCAGACGAGTGGAAACAGCCGCTAACA AACAACCCGCTTTACTACATA-3') containing RhCMV gL sequences flanking the *galK* cassette with the 5' region corresponding to RhCMV 68.1 nucleotides 150387 to 150457 and the 3' region corresponding to RhCMV 68.1

nucleotides 151191 to 151261 were synthesized and annealed to each other. The double-stranded oligonucleotide was electroporated into SW102 bacteria containing RhCMV Δ gL/*galK* that had been heat shocked at 42°C for 15 min. After electroporation, the bacteria were incubated in 10 ml of L broth for 4.5 h at 30°C, washed three times in 1× M9 buffer, and plated onto M63 minimal medium plates with chloramphenicol and 2-deoxy-galactose and glycerol as the carbon sources. After incubation at 30°C for 3 days, colonies were picked and restreaked three more times on plates with M63 minimal medium, 2-deoxy-galactose, chloramphenicol, and glycerol to ensure clonality. Clones were screened for the loss of *galK* and deletion of gL by PCR and FspI restriction enzyme digestion. The junction of the gL deletion was sequenced to ensure that the construct was correct.

The construction of HCMV BAC DNA with gL (UL115) deleted was previously described (10).

Southern blots. BAC DNA was harvested from *E. coli* strain SW102 using a Nucleobond BAC 100 kit. RhCMV virion DNA was harvested from infected cell supernatant as previously described (4). A total of 200 ng of RhCMV-virion DNA and 5 μ g of RhCMV-BAC DNA were digested with BspI, separated on a 0.7% agarose gel, and transferred to a nitrocellulose membrane. The membrane was hybridized with a [³²P]dCTP-labeled probe specific for RhCMV gL and its upstream region. The probe was generated by PCR using a forward primer upstream from RhCMV gL (nucleotides 150293 to 150316) and a reverse primer within RhgL (nucleotides 150872 to 150892).

Viral replication assays. Telo-RF cells and Telo-RF cells transduced with retrovirus expressing RhCMV gL were plated into six-well plates. At 18 h after plating, cells were infected with 2.5 PFU of either wild-type RhCMV or RhCMV Δ gL/cell for 1 h at 37°C. Cells were washed three times with PBS to remove residual inoculum and incubated at 37°C in complete medium. At 1, 2, 3, 6, and 8 days after infection, the supernatant was removed, and virus titers were determined by plating dilutions of supernatant on Telo-RF cells transduced with RhCMV gL.

PEG fusion assay. Recombinant RhCMV harvested from noncomplementing Telo-RF cells was incubated with Telo-RF and Telo-RF:RhgL cells for 1 h at 37°C. The virus inoculum was removed, and the cells were washed three times in serum-free DMEM or in medium with decreasing concentrations of polyethylene glycol (PEG) 6000 (50, 25, and 12.5%). After PEG treatment, the cells were washed with DMEM and incubated at 37°C in complete medium. At 5 days after PEG treatment, viral replication, as measured by development of cytopathic effect (CPE), was monitored by light microscopy.

gL antibody production. Two synthetic peptides consisting of amino acids E208 to D229 and D229 to N241 of RhCMV gL were conjugated to keyhole limpet hemocyanin (KLH). Each peptide-KLH conjugate was injected into two rabbits at days 1, 14, 28, and 42. Serum was collected at 56 days after the first inoculation. For immunofluorescence experiments, the preimmune and immune sera were precleared on Telo-RF cell lysates to reduce nonspecific antibody binding.

Immunoblots. Telo-RF cells were plated into six-well plates at a density of 300,000 cells per well. At 16 h after plating, the cells were mock infected or infected with 10 PFU of wild-type RhCMV 68.1 or RhCMV Δ gL/cell. At 72 h after infection, cell monolayers were washed with PBS and lysed in 400 μ l of 2× SDS-PAGE loading buffer containing 5% β -mercaptoethanol for 5 min at room temperature. Lysates were boiled for 5 min and centrifuged to remove insoluble debris. Lysates containing 20,000 cell equivalents were separated on 4 to 20% SDS-PAGE gels, transferred to polyvinylidene difluoride membranes, and blocked for 1 h at room temperature with Tris-buffered saline supplemented with 0.1% Tween 20 (TBST) and 5% nonfat milk. Mouse anti-HCMV gB AD-1 (purified and concentrated from hybridoma cells kindly provided by William Britt) was diluted 1:1,000 and incubated overnight at 4°C in TBST and 5% nonfat milk. Blots were washed three times with TBST at room temperature, incubated with goat anti-mouse antibody conjugated to horseradish peroxidase (1:10,000) (Millipore), washed three times with TBST, and developed by using enhanced chemiluminescence (Pierce).

Immunofluorescence. Telo-RF and Telo-RF:RhgL cells were plated onto glass coverslips. One day after plating, cells were either mock infected or infected with 1 PFU of wild-type RhCMV or RhCMV Δ gL/cell. Three days after infection, cells were washed with PBS and fixed with acetone for 5 min at -20°C. Coverslips were washed with PBS and blocked for 1 h at room temperature with 10% FBS in PBS. Coverslips were incubated with preimmune rabbit serum, mouse anti-HCMV gB antibody (1:1,000; hybridoma provided by William Britt), or serum from rabbit 93 immunized with RhCMV gL peptide E208-D229 (1:100) overnight at 4°C, washed three times with PBS, incubated with secondary antibodies (1:1,000 donkey anti-rabbit Texas Red and donkey anti-mouse Alexa 488), and washed three times with PBS. Coverslips were placed onto slides with DAPI

(4',6'-diamidino-2-phenylindole) in the mounting medium. Images were taken on a Leica SP5 microscope, and images processed with ImageJ software (National Institutes of Health, Bethesda MD).

Transmission electron microscopy. Telo-RF cells were grown on Thermanox coverslips (Nalge-Nunc International, Rochester, NY) and, when confluent, the cells were infected with recombinant RhCMV at an MOI of 3. Three days after infection, cells were fixed for 1 h at 4°C with 2.5% glutaraldehyde, 4% paraformaldehyde, and 0.1 M sodium cacodylate buffer (pH 7). Samples were then fixed for 1 h with 0.5% osmium tetroxide-0.8% potassium ferricyanide, followed by 1 h in 1% tannic acid and overnight in 1% uranyl acetate at 4°C, and finally dehydrated with a graded ethanol series prior to embedding in Spurr's resin. Thin sections were cut with an RMC MT-7000 ultramicrotome (Ventana, Tucson, AZ) and stained with 1% uranyl acetate and Reynold's lead citrate prior to viewing them at 80 kV on a Hitachi H-7500 transmission electron microscope (Hitachi, Tokyo, Japan). Digital images were acquired with a bottom-mount AMT digital camera system (AMT, Chazy, NY).

RESULTS

RhCMV gL fails to elicit detectable antibody responses in naturally infected rhesus macaques. If a CMV gL-null virus were to be used as a prophylactic vaccine, it is important to determine whether gL is a major target of antibody production in the blood. Therefore, RhCMV-specific antibody levels in the plasma of known RhCMV-seropositive and RhCMV-seronegative macaques (as determined by an RhCMV-specific enzyme-linked immunosorbent assay) were measured by the LIPS assay. Portions of RhCMV pp65.2, gB, and gL were fused to the C terminus of *Renilla* luciferase (*Ruc*), and the levels of antibodies to RhCMV gB, pp65.2, and gL were quantified by using the LIPS assay. As expected, high levels of antibody to RhCMV gB were detected in all 11 RhCMV-seropositive monkeys and in none the 17 RhCMV-seronegative animals (Fig. 1A). Low levels of antibody to RhCMV pp65.2 was detected in 10 of 11 RhCMV-seropositive macaques and in none of 17 seronegative animals (Fig. 1B), consistent with prior observations that antibody responses to pp65.2 are not sustained over time (46). In contrast, we were unable to detect antibody to the RhCMV gL fusion protein in any of the RhCMV-seropositive animals (Fig. 1C). To ensure that the lack of gL-specific antibodies in rhesus macaque plasma was not due to masking of epitopes by fusing gL to the C terminus of *Ruc*, we also fused gL to the N terminus of *Ruc*; however, we were unable to detect gL antibodies in animals using the LIPS assay (data not shown). To verify that the LIPS assay detects antibody to gL, sera from rabbits immunized with gL peptides were tested, as well as sera prior to immunization. Although preimmune sera did not precipitate the gL fusion protein, sera obtained after immunization with gL peptide precipitated the fusion protein (Fig. 1C). In addition, serum from three other rabbits immunized with RhCMV gL peptides also precipitated a gL-*Ruc* fusion protein (data not shown). These data suggest that antibodies specific for gL may be produced at very low levels in naturally infected RhCMV rhesus monkeys and that deletion of gL from RhCMV may not have as large of an effect on neutralizing antibody titers as the deletion of other essential glycoproteins such as gB. It is unclear whether the deletion of gL would affect antibody production to gH or the UL128/UL130/UL131 proteins. Therefore, measurement of antibody levels to these proteins would be important if animals were infected with a replication-defective RhCMV vaccine with gL deleted.

FIG. 1. Naturally infected rhesus monkeys do not produce detectable levels of antibody to RhCMV gL. Antibody levels to RhCMV gB, pp65.2, and gL were measured by LIPS assay with 17 RhCMV-seronegative and 11 RhCMV-seropositive animals. The data are presented as the amount of light units (LU) produced after immunoprecipitation. The long horizontal bars indicate the mean luminescence, and the short horizontal bars represent the standard deviations. Serum obtained from preimmune rabbits or rabbits immunized with gL peptides were included as a negative and positive controls, respectively, for the gL LIPS assay.

Construction of RhCMVΔgL BAC. An RhCMV BAC with gL deleted (RhCMVΔgL BAC) was created by using the galactose kinase (GalK) selection system (43). RhCMV gL is encoded by nucleotides 150420 to 151196 of the RhCMV genome (12) (Fig. 2A). Initially, the majority of the RhCMV gL open reading frame was replaced with a GalK cassette (see Materials and Methods); thereafter, the GalK cassette was removed, resulting in a BAC (RhCMVΔgL) with nucleotides 150459 to 151190 of RhCMV deleted (codons 3 to 245 of RhCMV gL, Fig. 2B). To ensure that gL was deleted, wild-type RhCMV BAC and RhCMVΔgL BAC DNA were digested with BspI (which cuts within the gL open reading frame), and Southern blotting was performed with a probe corresponding to sequences upstream and within the gL open reading frame (Fig. 2B). Digestion of wild-type RhCMV BAC DNA with BspI resulted in the detection of a 6.1-kb fragment, while

FIG. 2. Map and Southern blot of the region of RhCMV with gL deleted. (A) Map of the RhCMV genome in the region containing gL and adjacent genes. Nucleotide positions of the BspI sites and the gL open reading frame (ORF) are indicated. The white arrow indicates the orientation and position of the gL ORF. The gray arrows indicate the positions and orientation of the adjacent ORFs. The dotted line denotes the location of the probe used for Southern blot analysis. (B) Map of the RhCMVΔgL genome in the region of the gL deletion. The nucleotide positions of the beginning and end of the deletion are noted. Loss of the majority of the gL ORF results in loss of a single BspI site. (C) Southern blot of RhCMV BAC DNA, RhCMV virion DNA, RhCMVΔgL BAC DNA, and RhCMVΔgL virion DNA digested with BspI and hybridized with a radiolabeled probe specific for the region overlapping RhCMV gL and the upstream neighboring gene. The probe hybridizes to a 6.1-kb BspI fragment in RhCMV BAC DNA and a 9.2-kb BspI fragment in RhCMVΔgL DNA. Size markers are in kilobase pairs.

digestion of RhCMVΔgL BAC resulted in a 9.2-kb fragment due to the loss of the BspI restriction site in the RhCMVΔgL BAC (Fig. 2C).

Transduction of Telo-RF cells with retrovirus expressing RhCMV gL. To produce rhesus fibroblasts that can complement replication of RhCMV with gL deleted, we constructed a replication-defective retrovirus that expressed RhCMV gL and GFP (see Materials and Methods). Telo-RF cells were transduced twice with retrovirus encoding RhCMV gL and GFP. After the second transduction, more than 80% of the cells were GFP positive, and GFP expression was stably maintained for 6 months (Fig. 3A). These data suggest that the majority of the cells transduced with the RhCMV gL retrovirus stably express RhCMV gL.

FIG. 3. Expression of GFP and RhCMV gL in Telo-RF:RhGL cells. (A) Telo-RF cells were mock transduced or transduced twice with retrovirus expressing RhCMV gL and GFP. RhCMV gL expression was indirectly measured by expression of GFP from the IRES following the gL gene in the retroviral transcript. Images of GFP expression were taken 6 months after transduction. (B) gL expression in Telo-RF:RhGL cells. Telo-RF and Telo-RF:RhGL cells were fixed and stained for gL using rabbit polyclonal antibody raised against gL peptide, followed by anti-rabbit Texas Red-conjugated antibody. Cells were costained with DAPI to visualize nuclei.

To confirm expression of gL in the Telo-RF cells transduced with retrovirus (Telo-RF:RhGL cells), we performed immunofluorescence assays with RhCMV gL antibody. Wild-type Telo-RF and Telo-RF:RhGL cells were fixed and stained for gL using rabbit immune serum specific for gL. As expected, gL was not detected in Telo-RF cells stained with anti-gL antibody (Fig. 3B) or in Telo-RF:RhGL cells stained with preimmune sera (data not shown). In contrast, the majority of Telo-RF:RhGL cells stained with anti-gL antibody were positive for gL (Fig. 3B).

Complementation of RhCMVΔgL by rhesus fibroblasts expressing RhCMV gL. gL has been postulated to be essential for HCMV replication as an HCMV BAC with the gL ORF deleted did not produce virus when transfected into fibroblasts (10, 14). Since the HCMV BAC with gL deleted was not transfected into complementing cells and the gL deletion mutant was not rescued, it is unclear whether gL is actually essential for CMV infection. To determine whether virus could be recovered from the RhCMVΔgL BAC by expression of RhCMV gL *in trans*, Telo-RF and Telo-RF:RhGL cells were transfected with wild-type RhCMV BAC or RhCMVΔgL BAC using Lipofectamine 2000. Wild-type RhCMV BAC resulted in

plaques 10 to 12 days after transfection of Telo-RF or Telo-RF:RhGL cells (data not shown).

Transfection of Telo-RF cells with the RhCMVΔgL BAC resulted in single refractile (infected) cells, but the infection did not spread to form plaques, and the monolayer returned to a normal appearance. Transfection of Telo-RF:RhGL cells with the RhCMVΔgL BAC resulted in single refractile cells by 4 days after infection and plaques by 10 to 12 days. The resulting virus was termed RhCMVΔgL. About 4 weeks after transfection with the RhCMVΔgL BAC, supernatants from Telo-RF and Telo-RF:RhGL cells were harvested, clarified twice by low-speed centrifugation, and passaged onto fresh Telo-RF and Telo-RF:RhGL cells. Supernatant from Telo-RF:RhGL cells, but not from nontransduced Telo-RF cells, resulted in plaque formation when passaged onto fresh Telo-RF:RhGL cells (Fig. 4A, lower panels, and data not shown). Single infected cells were observed when supernatant from Telo-RF:RhGL cells was passaged onto Telo-RF cells not expressing gL, but these foci did not spread to form plaques (Fig. 4A, upper panels).

To confirm that replication of RhCMVΔgL was due to complementation *in trans*, and not to recombination of gL into the viral genome, DNA isolated from virus propagated on complementing cells was harvested, digested with BlnI, and Southern blotting was performed with a probe specific for sequences upstream and within the gL open reading frame. Digestion of wild-type RhCMV virion DNA with BlnI resulted in a 6.1-kb fragment, while digestion of the RhCMVΔgL virion DNA resulted in a 9.2-kb fragment due to the loss of the BlnI restriction site in RhCMVΔgL (Fig. 2C). These results indicate that RhCMVΔgL can be complemented by gL expressed *in trans* and that the mutation is stable after passage in Telo-RF cells expressing gL.

Growth properties of RhCMVΔgL on complementing cells.

To determine the efficiency of complementation of Telo-RF cells expressing gL, we infected Telo-RF:RhGL cells with wild-type RhCMV or RhCMVΔgL at an MOI of 2.5 PFU/cell. At various times after infection, the supernatant titers were determined on Telo-RF:RhGL cells to test for the release of infectious virus. At 2 days after infection, Telo-RF:RhGL cells infected with wild-type RhCMV or RhCMVΔgL released infectious virus into the supernatant (Fig. 4B). Virus titers increased on days 3 and 6, and the titer of the two viruses released into the supernatant was similar throughout the time course. Titers of wild-type RhCMV produced in Telo-RF cells not expressing gL were similar to levels observed in Telo-RF:RhGL cells; however, no virus was released from Telo-RF cells not expressing gL when infected with RhCMVΔgL (data not shown). These data indicate that the efficiency of replication of RhCMVΔgL in complementing cells is similar to that of wild-type RhCMV.

Telo-RF cells infected with RhCMVΔgL fail to express detectable levels of gL. To verify that gL was not expressed in cells infected with RhCMVΔgL, Telo-RF cells were mock infected or infected with RhCMV or RhCMVΔgL. Three days after infection, cells were fixed and stained with DAPI and with anti-gB or anti-gL antibodies. As expected, neither gB nor gL were detected in mock-infected Telo-RF cells (Fig. 4C). RhCMV-infected or RhCMVΔgL-infected Telo-RF cells stained for gB resulted in punctate staining of the periphery of the cell. RhCMV-infected Telo-RF cells stained for gL showed cyto-

FIG. 4. RhCMVΔgL forms plaques only in cells expressing RhCMV gL. (A) Telo-RF cells expressing RhCMV gL were transfected with RhCMVΔgL BAC DNA. When the cells showed 90% CPE, the supernatant was harvested, clarified by low-speed centrifugation, and then added to either nontransduced Telo-RF cells (RF, upper panels) or Telo-RF cells expressing RhCMV gL (Telo-RF:RhgL, lower panels). At 4 and 10 days postinfection (dpi), photomicrographs of cell monolayers were obtained. (B) Replication of RhCMVΔgL in complementing cells. Telo-RF cells stably transduced to express RhCMV gL were infected with RhCMV or RhCMVΔgL at an MOI of 2.5 PFU/cell. At the times indicated, supernatant virus was collected and assayed for infectious virus. The data presented are the averages of two experiments, and error bars indicate the standard deviations. (C) Expression of gB and gL in Telo-RF cells infected with RhCMV or RhCMVΔgL. Telo-RF cells were infected at an MOI of 1 PFU/cell. At 3 days after infection, the cells were fixed and stained for either gB or gL as indicated.

plasmic staining, a finding consistent with prior observations of HCMV gL localization in permeabilized cells (41). In contrast, gL-specific staining was not observed in RhCMVΔgL-infected cells. The inability to detect gL in RhCMVΔgL-infected cells is consistent with the Southern blot results that gL was deleted from cells infected with RhCMVΔgL.

RhCMVΔgL-infected Telo-RF cells express gB at similar levels and produce virions with a morphology similar to that of the wild-type virus. To determine whether Telo-RF cells express similar levels of viral proteins when infected with RhCMVΔgL and wild-type virus, we infected Telo-RF cells with the two viruses at an MOI of 2.5 PFU/cell. At 3 days after infection, whole-cell lysates were prepared and immunoblotting was performed with antibodies to HCMV gB. The levels of RhCMV gB were similar for cells infected with RhCMVΔgL and wild-type RhCMV (Fig. 5A). These experiments indicate that the absence of gL does not interfere with the expression of the late viral antigen, gB. Attempts to test other CMV antigens, such as pp65, were not successful, since the HCMV-

specific antibodies did not cross-react with RhCMV antigens (data not shown).

Virion morphology in Telo-RF cells not expressing gL was also analyzed in cells infected with RhCMVΔgL (produced in complementing cells) and wild-type virus. Telo-RF cells were infected with the two viruses at an MOI of 3 PFU/cell. At 5 days after infection, examination of cells by transmission electron microscopy showed that intracellular RhCMVΔgL and wild-type virions were morphologically indistinguishable (Fig. 5B, left panels). Similarly, extracellular virions from cells infected with the two viruses were similar (Fig. 5B, right panels). Thus, gL is not required for either virion assembly or morphogenesis.

Complementation of RhCMVΔgL with PEG. Although deletion of gL rendered RhCMV noninfectious, infection of non-complementing Telo-RF cells resulted in robust expression of gB and the release of virions into the supernatant. Since HCMV gH/gL is important for virus entry (42), we determined whether infection of virions lacking gL could be rescued by

FIG. 5. gB expression and virion morphogenesis in cells infected with RhCMVΔgL is similar to RhCMV. (A) Telo-RF cells were infected with 2.5 PFU of RhCMV or RhCMVΔgL/cell. At 72 h postinfection, cell lysates were prepared, and gB was detected by Western blotting. (B) Telo-RF cells were infected with either RhCMV (upper panels) or RhCMVΔgL (lower panels) at an MOI of 3. At 5 days postinfection the cells were fixed, and transmission electron microscopy was performed. RhCMV and RhCMVΔgL virions are detected in the cytoplasm (left panels) and outside the cell (right panels).

treatment with PEG, which promotes the fusion of membranes. Telo-RF cells not expressing gL were infected with RhCMVΔgL (that had been grown in complementing cells and therefore had gL in the virion). Ten days after infection, supernatant containing gL⁺ virions was harvested, clarified twice by centrifugation, and used to infect Telo-RF or Telo-RF:RhgL cells. One hour after infection, the cells were washed in the presence or absence of PEG to induce membrane fusion. After washing, complete medium was added, and the cells were monitored for the presence of CPE and/or plaque formation. In the absence of PEG, neither Telo-RF nor Telo-RF:RhgL cells were infected (Fig. 6, left panels). In the presence of PEG, infection of Telo-RF cells with RhCMVΔgL grown in non-complementing cells resulted in single infected cells (Fig. 6, top middle panel) that were unable to produce new infectious virus or form plaques (Fig. 6, top right panel). In contrast, in the presence of PEG, infection of Telo-RF cells expressing gL with

RhCMVΔgL virions released from noncomplementing cells resulted in multiple infected cells (Fig. 6, lower middle panel). Supernatant harvested from the PEG-treated Telo-RF:RhgL cells, which had been infected with RhCMVΔgL, was transferred to fresh Telo-RF:RhgL cells, and robust plaque formation was observed, indicating the production of new infectious virus (Fig. 6, lower right panel). These results demonstrate that RhCMVΔgL is competent for replication if barriers to entry are removed and that intact, but noninfectious, virions are released from Telo-RF cells infected with RhCMVΔgL.

HCMV with gL deleted can be rescued by the expression of HCMV gL in HFF cells. To verify whether HCMV gL is essential for HCMV replication and can be complemented in *trans*, HFF cells were transduced with retrovirus that stably expressed HCMV gL and GFP (HFF:RhgL cells). Similar to the Telo-RF:RhgL cells, greater than 80% of the cells stably expressed GFP after two rounds of transduction. GFP expression

FIG. 6. The infectivity of RhCMVΔgL can be rescued by treatment with PEG. RhCMVΔgL passaged in noncomplementing Telo-RF cells was used to infect Telo-RF cells or Telo-RF cells expressing RhCMV gL for 1 h at 37°C. The virus inoculum was removed, and the cells were washed three times in serum-free DMEM (left panels) or DMEM containing PEG (center panels). At 5 days postinfection, the supernatant from the PEG-treated cells was transferred to monolayers of complementing Telo-RF:RhgL cells (right panels).

FIG. 7. Rescue of HCMV Δ gL infectivity in HFFs expressing HCMV gL. (A) HFFs were mock transduced or transduced twice with retrovirus expressing HCMV gL. Expression of gL was indirectly confirmed by expression of GFP from the IRES:GFP cassette. (B) HCMV Δ gL replicates in cells expressing HCMV gL. HFF and HFF:HgL cells were infected with HCMV Δ gL, and photomicrographs were obtained 18 days after infection.

was stably maintained and detected at 6 months posttransduction in the majority of cells in culture (Fig. 7A). To produce HCMV with gL deleted, HFF:HgL cells (and HFF cells as a control) were transfected with an HCMV BAC with gL deleted (10) and monitored for CPE. At 3 weeks after transfection, plaques were present in HFF:HgL cells but not in nontransduced HFF cells. Supernatant from HFF:HgL cells transfected with the HCMV Δ gL BAC was harvested and used to infect fresh HFF or HFF:HgL cells. Plaques were observed in cells expressing HCMV gL but not in nontransduced cells (Fig. 7B and Fig. 8). These results indicate that HCMV Δ gL, like RhCMV Δ gL, cannot replicate unless complemented by gL provided *in trans*.

Heterologous gL proteins from RhCMV and HCMV are unable to complement gL-null viruses. Since the amino acid sequences of HCMV and RhCMV gL are highly conserved (53% amino acid identity), we determined whether gLs from different species could complement each other. Telo-RF and HFF cells were transduced with retroviruses expressing either HCMV gL or RhCMV gL, resulting in HFF:HgL, HFF:RhgL, Telo-RF:HgL, and Telo-RF:RhgL cells. Nontransduced Telo-RF and HFF cells, as well as retrovirus-transduced Telo-RF and HFF cells, were either mock infected or infected with 100 PFU of RhCMV, RhCMV Δ gL (produced in complementing cells), HCMV, or HCMV Δ gL (produced in complementing cells). At 14 days after infection, cells were monitored for CPE. Wild-type RhCMV formed plaques in all of the cells tested, a finding consistent with previous observations that RhCMV grows in

rhesus and human cells (Fig. 8, column 2) (25). Plaques were also observed in Telo-RF:RhgL and HFF:RhgL cells infected with RhCMV Δ gL but not in Telo-Rh:HgL or HFF:HgL cells (Fig. 8, column 3). Wild-type HCMV formed plaques in all of the HFF cells but not in any of the Telo-RF cells, which is consistent with observations that HCMV replication is restricted to only human cells (Fig. 8, column 4). Plaques were observed in HFF:HgL cells infected with HCMV Δ gL but not in HFF:RhgL cells (Fig. 8, column 5). These results indicate that while RhCMV and HCMV gLs are highly homologous, they are not able to complement one another.

DISCUSSION

In this report, we show that HCMV gL and RhCMV gL are essential for HCMV and RhCMV infection, respectively. The loss of infectivity due to the deletion of RhCMV gL was rescued by expressing gL *in trans* or by treating gL-null virions with PEG. Expression of the viral late protein gB was similar in RhCMV- and RhCMV Δ gL-infected cells, and virions produced from these cells were indistinguishable when examined by transmission electron microscopy. Finally, while HCMV and RhCMV gLs are highly homologous, they are unable to functionally complement replication of the heterologous deletion mutant.

HCMV and RhCMV gLs were essential for infectivity of their corresponding viruses. While this is the first time that gL deletion viruses have been constructed and characterized for a betaherpesvirus, other studies have characterized deletions of gL or gH in alpha- and gammaherpesviruses. gL⁻ or gH⁻ HSV is not infectious, but partial infectivity is recovered when infection is performed in the presence of PEG (11). Epstein-Barr virus and bovine herpesvirus 1 with gH deleted can bind to target cells but fail to enter the cells unless treated with PEG to induce membrane fusion (27, 37). Similarly, we found that PEG could restore infectivity of gL⁻ RhCMV, suggesting that RhCMV gL, and therefore the gH/gL complex, is important for an early step in virus entry. This conclusion is supported by the observation that gL⁻ virions produced in noncomplementing cells were indistinguishable from wild-type virions by transmission electron microscopy. This implies that gL (i.e., the gH/gL complex) plays little or no role in virus egress or virion morphogenesis. These findings are consistent with previous observations with HSV gL mutants (35). Similarly, cells infected with gH⁻ pseudorabies virus have virions both in the cytoplasm and outside of cells but not in neighboring cells (31).

Herpesvirus gH/gL complexes have been proposed to function as fusogens (6, 20, 40). Coexpression of HCMV gH and gL by retroviral vectors induced syncytium formation in the absence of gB in fibroblasts (20). In contrast, coexpression of HCMV gL and gH by adenovirus vectors in epithelial cells did not induce cell-to-cell fusion, but the combination of gL, gH, and gB did (42). While HSV gH/gL has also been postulated to be a fusogen, the crystal structure of the gH/gL complex indicates that putative gH fusion peptides are buried in beta-hairpins located in multistranded sheets and thus unlikely to participate in fusion (5). In addition, the crystal structure of HSV gH/gL does not resemble any known fusogen. Therefore, gH/gL, which binds gB, a known fusogen (13), may regulate

FIG. 8. HCMV and RhCMV gL is unable to complement heterologous gL-null viruses. Telo-RF and HFF cells were mock transduced or were transduced with retroviruses expressing either RhCMV or HCMV gL and subsequently infected with 100 PFU of the viruses indicated. At 14 days postinfection, photomicrographs were obtained.

gB-induced fusion rather than function as a direct inducer of membrane fusion.

HCMV gL forms a tripartite complex with gH and gO in mammalian cells (16, 21), and expression of all three proteins is necessary for efficient infection of fibroblasts in cell culture (44). In addition, HCMV gL forms a protein complex with gH, UL128, UL130, and UL131, which allows the virus to enter epithelial and endothelial cells (36, 44). The precise structure of this complex is unknown; however, HCMV gL binds to UL128 by noncovalent interactions and, while gL does not directly interact with UL131, UL131 does bind directly to gH in the presence of gL (36). The HCMV gH/gL/UL128/UL130/UL131 complex is a target for neutralizing antibodies (26) and should be considered when designing vaccines. Like HCMV, the entry of RhCMV into epithelial and endothelial cells is dependent on RhCMV UL128, UL130, and UL131, and it is assumed that RhCMV gH/gL/gO and RhCMV gH/gL/UL128/UL130/UL131 function in a manner similar to their HCMV orthologs (24, 25). Our data confirm the importance of gL, and therefore the gH/gL dimer, for entry of RhCMV and HCMV into fibroblasts. While the gL mutations were constructed in BACs lacking UL128/UL130/UL131, we have recently inserted the UL128/UL130/UL131 genes into RhCMVΔgL (unpublished results) and plan to use the latter virus in future studies.

Given the homology of the RhCMV and HCMV gLs we hypothesized that the two proteins might be able to complement each other. Heterologous gL proteins have been shown to complement gL function in part or in full. HSV-1 and HSV-2 gH and gL, which share 67% amino acid identity, can complement each other in cell-to-cell fusion assays (28). In addition, while gL of varicella-zoster virus and Epstein-Barr virus have only 14% amino acid identity and 40% amino acid similarity, respectively, the two gLs can act as a chaperone for the heterologous gH to reach the cell surface (23). We found that while RhCMV gL and HCMV gL share 53% amino acid identity, they were unable to complement replication of gL-null HCMV or gL-null RhCMV, respectively. This indicates that complementation of the gL function in CMV is highly specific for its corresponding gH, and it is unlikely that gL from other human herpesviruses will be able to complement HCMV gL-null mutants.

Currently, there is no licensed vaccine for prevention of HCMV infection. HCMV with gL deleted could function as a defective infectious single cycle (DISC) vaccine (reviewed in reference 9) since the virus can infect cells that do not express gL and undergo a single cycle of replication, resulting in the release of mature, noninfectious viral particles. RhCMVΔgL, grown in complementing cells, can infect noncomplementing

cells efficiently and produce levels of gB, the major target of neutralizing antibody to CMV, at levels similar to those seen in cells infected with wild-type virus. Unlike CMV glycoprotein subunit vaccines, CMV with gL deleted should present nearly all of the CMV proteins to the immune system in the context of MHC class I so that cellular immunity to a large number of viral proteins is induced. In addition, we report that naturally infected RhCMV-infected macaques do not produce detectable levels of antibody to gL, which suggests that loss of gL might have less of an effect on neutralizing antibody titers than deletion of gB. However, loss of gL might affect development of neutralizing antibodies to the gH/gL or gH/gL/UL128/UL130/UL131 complex. Since RhCMVΔgL and HCMVΔgL produced in complementing cells can infect noncomplementing cells, virus produced in complementing cells does contain functional gL complexes, which might be sufficient to elicit some neutralizing antibody to these complexes. Finally, the failure of the closely related HCMV or RhCMV gL to complement gL-null RhCMV or gL-null HCMV, respectively, suggests that gL encoded by human alpha- or gammaherpesviruses are unlikely to complement HCMV lacking gL. Thus, HCMV lacking gL is likely to be a safe vaccine candidate that might induce humoral and cellular immunity to nearly all of the viral proteins.

ACKNOWLEDGMENTS

This study was supported by the Intramural Research Programs of the National Institute of Allergy and Infectious Diseases and the National Institute of Dental and Craniofacial Research.

We thank William Chang and Peter Barry for RhCMV BAC and Telo-RF cells, Fenyong Liu for the HCMV BAC with gL deleted, Teresa Compton for the plasmids used to construct retroviruses, William Britt for the HCMV gB hybridoma cells, and Neal Copeland for SW102 cells and pGalK.

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