Guinea Pig Cytomegalovirus Protective T Cell Antigen GP83 Is a Functional pp65 Homolog for Innate Immune Evasion and Pentamer-Dependent Virus Tropism

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ABSTRACT The guinea pig is the only small animal model for congenital cytomegalovirus (CMV) but requires species-specific guinea pig cytomegalovirus (GPCMV). Tegument protein GP83 is the presumed homolog of HCMV pp65, but gene duplication in the *UL82-UL84* homolog locus in various animal CMVs made it unclear if GP83 was a functional homolog. A GP83 null deletion mutant GPCMV (GP83dPC+) generated in the backdrop of glycoprotein pentamer complex (PC)-positive virus, required for nonfibroblast infection, had normal growth kinetics on fibroblasts but was highly impaired on epithelial and trophoblast cells. GP83dPC+ virus was highly sensitive to type I interferon (IFN-I), suggesting that GP83 had an innate immune evasion function. GP83 interacted with cellular DNA sensors guinea pig IFI16 and cGAS, indicating a role in the cGAS/STING pathway. Ectopically expressed GP83 in trophoblast cells restored GP83dPC+ virus growth. Additionally, mutant virus growth was restored in epithelial cells by expression of bovine viral diarrhea virus (BVDV) N^{pro} protein targeting IRF3 as part of the cGAS/STING pathway, or alternatively, by expression of the fibroblast cell receptor platelet-derived growth factor receptor alpha (PDGFRA). HCMV pp65 is a T cell target antigen, and a recombinant adenovirus encoding GP83 was evaluated as a vaccine. In GPCMV challenge studies, vaccinated animals had various levels of protection against wild-type virus, with a protective response against the 22122 prototype strain but little protection against a novel clinical strain of GPCMV (TAMYC), despite 100% identity in GP83 protein sequences. Overall, GP83 is a functional pp65 homolog with novel importance for epithelial cell infection, but a GP83 T cell response provides limited vaccine efficacy.

IMPORTANCE Congenital CMV (cCMV) is a leading cause of cognitive impairment and deafness in newborns, and a vaccine is a high priority. The guinea pig is the only small animal model for cCMV but requires guinea pig cytomegalovirus (GPCMV). The translational impact of GPCMV research is potentially reduced if the virus does not encode functional HCMV homolog proteins. This study demonstrates that tegument protein GP83 (pp65 homolog) is involved in innate immune evasion and highly important for infection of nonfibroblast cells via the viral glycoprotein pentamer complex (PC)-dependent endocytic entry pathway. The PC pathway is highly significant for virus dissemination and disease in the host, including cCMV. A GP83 candidate adenovirus (Ad) vaccine strategy in animals induced a cell-mediated response but failed to provide cross-strain protection against a novel clinical strain of GPCMV. Results suggest that the pp65 antigen provides very limited efficacy as a stand-alone vaccine, especially in cross-strain protection.

KEYWORDS CMV vaccine, IFI16, IRF3, cGAS, congenital CMV, cytomegalovirus, epithelial cells, guinea pig, pentamer complex, pp65

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Human cytomegalovirus (HCMV), a betaherpesvirus, is a leading cause of congenital disease in newborns. Congenital cytomegalovirus (cCMV) infection occurs globally at a rate of 0.5 to 2% of births and can lead to serious symptomatic disease, including impaired vision, mental retardation, sensorineural hearing loss (SNHL), and microcephaly in newborns (1–4). In the United States, approximately 8,000 newborns each year will have permanent disabilities associated with cCMV, and up to 30% of hearing loss in children is attributed to cCMV (5, 6). It is estimated globally that a million babies are born each year with cCMV (4), and a vaccine against cCMV is considered a high priority (7).

A complication in the evaluation of intervention strategies against cCMV is that HCMV is species specific and preclinical animal model studies require the use of species-specific animal CMV (8–11). Although a number of animal CMVs are studied, there are only two models for cCMV (guinea pig and rhesus macaque). The guinea pig is the focus of this study, with cCMV causing disease, including SNHL, in newborn pups (12–15), and various intervention strategies against cCMV have been evaluated in this model (16–18). In HCMV, viral glycoproteins (gB, gH, gL, gM, gN, gO) form specific complexes on the virion that are required for fibroblast cell entry (gB; gM/gN; gH/gL/gO) and are neutralizing antibody targets (19–25). Guinea pig CMV (GPCMV) has glycoprotein complexes similar to those of HCMV, which are essential for GPCMV infection of fibroblast cells as well as neutralizing target antigens (26–30). Additionally, HCMV encodes a glycoprotein pentamer complex (gH/gL/UL128/UL130/131, or PC) that is necessary for infection of nonfibroblast cells via an endocytic pathway rather than via the PC-independent pathway of direct cell membrane fusion that occurs in fibroblasts (31). Platelet-derived growth factor receptor alpha (PDGFRA), present in fibroblasts, has been identified as the cell receptor necessary for the HCMV direct fusion pathway of cell entry (32). A similar requirement of PDGFRA has recently been demonstrated for GPCMV fibroblast infection (29, 33, 34). Several receptors/coreceptors have been identified for PC-dependent cell entry of HCMV, including neuropilin-2 (NRP2), OR1411, CD147, CD46, and beta-integrins (35–40), but the process of PC-dependent cell entry is only partially understood and receptors are likely cell type dependent. The PC is an important neutralizing antibody target and is being explored as a candidate vaccine (41). Importantly, GPCMV encodes a similar functional pentamer complex (gH/gL/GP129/GP131/GP133), which is necessary for virus infection of nonfibroblast cells, including renal epithelial cells, placental trophoblasts, amniotic sac membrane cells, and macrophage (26, 27, 29, 42, 43). The PC is therefore highly important for GPCMV dissemination and congenital infection (27, 29, 43).

In both HCMV and GPCMV, the gB glycoprotein is an immunodominant neutralizing target and essential for infection of all cells (26, 27, 44–46). However, an HCMV gB subunit vaccine, despite evoking high antibody titers, provided only approximately 50% efficacy in phase II clinical trials (47, 48). GPCMV gB subunit vaccine strategies in the guinea pig model fail to fully protect against cCMV and provide efficacy similar to that found in human gB vaccine trials (46, 49, 50). In both HCMV and GPCMV, gB antibodies are less effective at neutralizing virus infection on nonfibroblast cells than on fibroblasts, which might be a basis for reduced vaccine efficacy (30, 51–53). Logically, other target antigens are being explored as vaccine candidates. In addition to neutralizing antibodies, individuals in the convalescent phase of HCMV infection have a robust T cell response to various viral proteins (including pp65, IE1, and pp150), which likely contributes to a protective T cell-mediated immune response (54). GPCMV encodes potential homologs to these viral proteins (18, 26, 55, 56). The HCMV tegument protein pp65 is considered the major T cell target (57) and has been explored as a vaccine strategy in both animal models and clinical trials (58).

A complication in animal CMV studies is that each virus has closely evolved with its respective animal host, and animal CMV colinear genes to the HCMV genome do not necessarily equate with encoding fully functional homolog proteins, which potentially weakens the translational aspect of animal CMV studies. The homolog *UL82-UL84* gene locus in various animal CMVs presents a potential problem in CMV translational

studies. In HCMV, *UL83* encodes structural tegument protein pp65, which is nonessential for virus replication on fibroblasts but has an important role in innate immune evasion by targeting interferon gamma inducible protein 16 (IFI16) and cyclic GMP–AMP synthase (cGAS) and blocking activation of the cGAS/stimulator of interferon genes (STING) antiviral pathway (59–63). IFI16 acts as a DNA sensor, present in both cytoplasm and nucleus, and cGAS is a cytosolic DNA sensor critical for the innate immune response (59, 62–65). HCMV *UL82* encodes tegument protein pp71 (UL82) with specific innate immune evasion functions to enhance virus infectivity (66, 67). *UL84* encodes a nonstructural protein (UL84) that is associated with DNA replication (68, 69). Although murine cytomegalovirus (MCMV) encodes a pp71 homolog (M82), this protein curiously lacks the ability to complement a herpes simplex virus (HSV) VP16 mutant, unlike HCMV pp71, suggesting divergence of function (67, 70). MCMV has positional duplicate gene copies of *UL83* (*M83* and *M84* encoding pp65 homologs) but no functional *UL84* (69, 71). Although rhesus cytomegalovirus (RhCMV) encodes homolog UL84 and UL82 proteins (69, 72), it also encodes two UL83 homolog proteins, pp65a and pp65b (*Rh111* and *Rh112*), where pp65b is considered to be an important T cell target (73). In contrast, GPCMV appears to encode single positional homologs to HCMV UL82 (GP82), UL83 (GP83), and UL84 (GP84) proteins (55, 69, 74, 75). Although GP83 has been demonstrated to be a tegument protein present in both GPCMV virions and extracellular dense bodies (74), a function associated with GP83 has not been identified. Similar to pp65, GP83 is nonessential for GPCMV replication on fibroblasts (55, 76). The original *GP83* knockout mutant virus (vAM409) (55) was generated in the backdrop of a PC-negative virus, which would impact the ability to infect nonfibroblast cells and disseminate in the animal model, further clouding the importance of GP83 for GPCMV tropism and pathogenicity. Additionally, the original mutant was generated via conventional homologous recombination and insertion of a guanine phosphoribosyltransferase-green fluorescent protein (GPT-GFP) cassette, containing two flanking simian virus 40 (SV40) poly (A) sequences, that potentially modified both *GP83* and *GP82* expression (55).

In the present study, a new GP83 deletion mutant GPCMV was generated in the backdrop of a PC-positive (PC+) virus (GP83dPC+) to enable evaluation of the impact of the loss of GP83 on a fully tropic virus. GP83 mutant virus studies were carried out on fibroblast, renal epithelial, and trophoblast cell lines. These studies demonstrated that although GP83 was nonessential for infection of fibroblasts, it was important for infection of nonfibroblast cell types, with additive impact to the requirement of PC for infection of nonfibroblast cells. Similar to HCMV pp65, GP83 specifically interacted with guinea pig homologs to cellular DNA sensors IFI16 and cGAS in transient-expression immunoprecipitation assays. Transient-expression studies of GP82 protein demonstrated that it was a functional homolog of HCMV pp71 and directly interacted with nuclear domain 10 (ND10) body component DAXX protein. A *GP83* null GPCMV was highly sensitive to type I interferon (IFN-I), demonstrating a role for GP83 in innate immune evasion linked to the IFI16/cGAS/STING pathway. Degradation of IRF3 by bovine viral diarrhea virus (BVDV) N^{pro} protein expressed ectopically by renal epithelial cells prevented activation in mutant virus-infected cells and restored GP83 mutant virus growth. Additionally, ectopic expression of the CMV fibroblast cell receptor PDGFRA enabled PC-independent virus cell entry of epithelial cells and restored growth similar to that of fibroblasts. GPCMV major immediate early locus IE protein expression in *GP83* knockout virus was enhanced by overexpression of IFI16 and GP83 prior to virus infection. A recombinant defective adenovirus (Ad) vector encoding GP83 was evaluated as a T cell target antigen vaccine and induced a specific response to GP83. However, AdGP83 vaccine efficacy was limited to prototype strain GPCMV 22122 and was ineffective against a novel strain isolate (TAMYC). Both strains shared 100% identity in GP83 protein sequence, but the novel strain was highly cell associated and tropic to epithelial cells. Overall, results demonstrate that GP83 is a functional homolog of HCMV pp65, important for innate immune evasion, and requires the PC-dependent pathway of infection. However, as a vaccine target antigen for adaptive immune

response, it provides limited protection in wild-type (WT) virus challenge, especially in the context of cross-strain protection.

RESULTS

GP83 deletion mutant virus is not impaired on fibroblasts but highly sensitive to IFN-I. A GP83 knockout mutant virus (vAM409) was previously generated via a conventional homologous recombination strategy (55) in the backdrop of a lab-adapted virus which did not encode the viral glycoprotein pentameric complex (27, 55). Retrospectively, it was unclear if the restricted virus dissemination *in vivo* (55) was associated with the lack of viral PC unique components (GP129, GP131, and GP133) (27), which would result in failure to infect nonfibroblast cells (27, 29, 33, 43), and/or because the mutant virus lacked GP83 (potential pp65 homolog but no demonstrated function), which further attenuated the virus. Additionally, the original GP83 knockout mutant virus was generated by insertion of a GPT-GFP double gene cassette into the *GP83* sequence with dual flanking SV40 poly(A) sequences (55, 77). Potentially, the original strategy may have modified *GP82* (pp71 homolog) expression in addition to knocking out the *GP83* gene, since *GP82* was expressed as a bicistronic transcript with *GP83* (74). Consequently, a second *GP83* mutant virus was generated to remove the majority of the GP83 coding sequence (codons 7 to 485), which was replaced with a kanamycin marker cassette. GPCMV bacterial artificial chromosome (BAC) mutagenesis was carried out in the backdrop of a viral genome retrofitted to encode a pentameric complex by ectopic expression of a missing full-length UL128 homolog (GP129) in the *GP25-GP26* intergenic locus, which restored virus tropism to nonfibroblast cells and pathogenicity *in vivo* (27, 29, 43) (Fig. 1A). The *GP83* gene is encoded in the antisense strand (nucleotides 131319 to 133016) in the HindIII "A" locus subgenomic fragment, and the new *GP83* mutant carried a deletion in the GP83 coding sequence from codons 7 to 485 of the 566-codon open reading frame (ORF) by insertion of a kanamycin cassette in place of the majority of the GP83 coding sequence between the BglII and KpnI sites within the ORF (nucleotides 131551 to 132990), as indicated in Fig. 1B, via targeted mutagenesis with shuttle vector pGP83dKm (Fig. 1C). Some of the 3' coding sequence of *GP83* was retained to avoid any impact on *GP82* expression. Construction of the *GP83* knockout mutant GPCMV BAC is more fully described in Materials and Methods. A comparative genomic HindIII restriction profile analysis between mutant and wild-type GPCMV BACs is shown in Fig. 1D and demonstrated specific modifications in the GP83 mutant BAC profile associated with modification of the HindIII "A" subgenomic fragment encoding GP83 and also a modification in the *GP25-GP26* intergenic locus encoding an ectopic copy of GP129 cDNA (see Materials and Methods).

Two independent *GP83* null mutant GPCMV BAC clones were separately transfected onto guinea pig lung fibroblast (GPL) cells to generate virus and simultaneously excise the BAC plasmid from the viral genome via Cre-LoxP recombinase (78). A virus stock was generated based on one clone, and the knockout mutant virus was designated GP83dPC+. A rescue virus (GP83R) was also generated to restore the GP83 coding sequence as described in Materials and Methods. Western blot analysis of GP83dPC+ virus-infected cell lysate (Fig. 1E) demonstrated ectopic expression of GP129myc-tagged protein similar to that of parental GPCMV BAC restored for GP129 ectopic expression (GP129FRT), which enabled expression of the full complement of unique PC components (27). An additional unique PC component, GP133, was detected in GP83dPC+ virus-infected renal epithelial (REPI) (Fig. 1G) and GPL fibroblast (Fig. 1H) cell monolayers by immunohistochemical (IHC) staining with mouse monoclonal anti-GP133 (GenScript) (27). Next, a comparative one-step growth curve (multiplicity of infection [MOI], 1 PFU/cell) on GPL fibroblasts for wild-type GPCMV GP83dPC+ and GP83R on separate six-well plates in triplicate demonstrated that the GP83 mutant virus had near normal growth on fibroblasts, compared to the wild-type virus (Fig. 2A), with a phenotype similar to that of a UL83 knockout HCMV virus (76).

HCMV has developed multiple strategies to evade cellular innate immunity and IFN-I (79). In GPCMV, we evaluated whether the loss of GP83 modified the ability of the

FIG 1 Generation of *GP83* null PC+ GPCMV mutant virus (GP83dPC+). **(A)** Linear diagram of the GPCMV genome showing the *Hind*III genomic map and location of excisable BAC plasmid (GFP-BAC) and ectopic GP129 in the *GP25-GP26* intergenic locus. **(B)** and **(C)** *GP83* locus encoded in *Hind*III "A" GPCMV subgenomic fragment and location of *Bgl*II and *Kpn*I restriction enzyme sites within the *GP83* coding sequence (**B**) used to delete the majority of the ORF in shuttle vector pGP83dKm (**C**). The GPCMV subgenomic plasmid clone carrying the complete *GP83* gene and partial *GP82* and *GP84* flanking coding sequence (55) was digested with unique sites within *GP83*, *Kpn*I (132990) and *Bgl*II (131551), to remove the majority of the *GP83* coding sequence (codons 7 to 485) in the shuttle vector, and in place, a kanamycin (Km) PCR cassette with flanking *Kpn*I (5') and *Bam*HI (3') restriction sites was inserted to generate the *GP83* knockout shuttle vector pGP83dKm (**C**). The GPCMV subgenomic plasmid clone carrying the complete *GP83* gene and partial *GP82* and *GP84* flanking coding sequence (55) was digested with unique sites within *GP83*, *Kpn*I (132990) and *Bgl*II (131551), to remove the majority of the *GP83* coding sequence (codons 7 to 485) in the shuttle vector, and in place, a kanamycin (Km) PCR cassette with flanking *Kpn*I (5') and *Bam*HI (3') restriction sites was inserted to generate the *GP83* knockout shuttle vector pGP83dKm (**C**). **(D)** Comparative GPCMV BAC genomic DNA restriction profile analysis (*Hind*III profile) of *GP83* mutant BAC (lane 1), parental GPCMV BAC encoding ectopic GP129 (*GP25-GP26*) locus (lane 2), and original second-generation GPCMV BAC without GP129 (lane 3). Mutagenesis of the GPCMV BAC (**A**) was performed using the shuttle vector (**C**) as previously described (108). Insertion of the Km drug resistance cassette into *GP83* introduced a novel *Hind*III restriction enzyme site, creating two novel subgenomic fragments to enable verification of the modified *Hind*III "A" locus in *GP83* mutant BAC (lane 1, red dots) in comparison to the original *Hind*III "A" fragment (lane 2, yellow dot) of parental GPCMV BAC encoding ectopic GP129 in the modified *GP25-GP26* locus (lane 2, blue and red dots) and the parental GPCMV BAC with the wild-type *GP25-GP26* locus (lane 3, yellow dot). *GP83* mutant virus (GP83dPC+) was generated by transfection of BAC DNA onto GPL fibroblast cells and excision of BAC plasmid. **(E)** Expression of ectopic GP129 verified by Western blotting for myc epitope-tagged protein. Western blot analysis of virus-infected GPL cell lysates: parental virus GP129FRT (lane 1), GP83dPC+ (lane 2), *GP83* rescue (GP83R) virus (lane 3), mock-infected GPL (lane 4). **(F)** β -Actin as a loading control for Western blot analysis in panel E. **(G)** and **(H)** IHC staining of GP133 protein expression in GP83dPC+ virus-infected REPI (**G**) and fibroblast (**H**) cell monolayers. Monolayers in 6-well plates infected with GP83dPC+ (MOI, 1 PFU/cell) (**a** and **c**) and mock-infected (MI) monolayers (**b** and **d**) were immunostained with monoclonal anti-GP133 (GPCMV) primary antibody (GenScript) plus anti-mouse IgG-HRP secondary antibody (Vectastain) (**a** and **b**) or with secondary antibody only (**c** and **d**). Individual bright-field images are representative of multiple fields. Magnification, $\times 20$.

FIG 2 GPL one-step growth curve for GP83PC+, wild-type (WT) GPCMV, and GP83 (GP83R) rescue virus and virus IFN-I sensitivity. (A) Comparative one-step growth curve on GPL fibroblasts for GP83dPC+ (blue triangles), wild-type GPCMV (orange circles), and GP83 rescue virus (green squares) on 6-well plates. MOI, 1 PFU/cell. Time point samples were harvested at 1 to 6 days postinfection (dpi) and titrated in duplicate. (B) IFN-I susceptibility of GP83 mutant (blue bars) in comparison to wild-type (WT) virus (black bars) and GP83 rescue virus (red bars). Cells in 6-well plates were incubated 6 h in the absence or presence of 100 IU/ml or 1,000 IU/ml IFN-I (R&D Systems) prior to GPCMV infection (MOI, 0.1 PFU/cell). After 72 h, the supernatant and monolayer were harvested and titrated in duplicate on GPL cells. Results are plotted as percentage of virus growth in IFN-I-treated groups compared to untreated groups (% PFU). Experiments were carried out in triplicate for each condition and virus. **, $P < 0.01$; ns, not significant (one-way analysis of variance [ANOVA] with Tukey's post hoc test).

virus to replicate in cells pretreated with various concentrations of universal IFN-I (alpha). An IFN-I susceptibility assay was developed in earlier GPCMV IE1 mutant studies (56), and a similar comparative IFN-I susceptibility assay was carried out for wild-type, GP83dPC+, and GP83R viruses. On GPL fibroblasts, the GP83 null virus had increased susceptibility to IFN-I in comparison to wild-type GPCMV on cells pretreated for 6 h with IFN-I prior to infection (MOI, 0.1 PFU/cell). GP83dPC+ mutant virus was highly impaired for growth (80% knockdown compared to no-IFN control) in cells pretreated with 100 IU/ml of IFN-I (R&D Systems) and was further impaired (95% knockdown) when the IFN-I pretreatment concentration was increased to 1,000 IU/ml prior to infection (Fig. 2B). In contrast, wild-type GPCMV was only slightly impaired for growth at 100 IU/ml (approximately 10% knockdown compared to no-treatment control) but more severely impaired with 1,000 IU/ml IFN-I pretreatment (80% knockdown). A GP83 rescue virus had sensitivity to IFN-I similar to that of the wild-type virus (Fig. 2B). This demonstrated a specific phenotype associated with the GP83dPC+ mutant virus on fibroblast cells and indicated that GP83 has a potential role in innate immune evasion related to the JAK/STAT IFN-I pathway.

GPCMV GP83 targets guinea pig cellular DNA sensors IFI16 and cGAS. In HCMV, pp65 interacts with the constitutively expressed cellular DNA sensor IFI16 to block the innate immune response but also potentially enhances IE protein expression (61). The guinea pig encodes an IFI16 homolog (XP_023419508.1) which has 53% identity with human IFI16 protein based on BLAST analysis and has two conserved HIN domains, indicating that it is a member of the PYHIN family (80). Guinea pig IFI16 (gplFI16) has not been previously investigated, and a lack of cross-reacting antibodies prevented studies with constitutively expressed protein. A synthetic gene encoding a C-terminal FLAG-tagged gplFI16 was generated (GenScript) for expression studies, and subsequently, a recombinant defective Ad5 vector was created that expressed gplFI16 (AdIFI16Flag), as described in Materials and Methods. Transient expression of gplFI16 by AdIFI16Flag-transduced cells resulted in expression of a protein of approximately 55 kDa, similar to the predicted protein size (Fig. 3B). Protein-protein interactions between GP83 and IFI16 were evaluated by transient expression of GP83 and IFI16 by transduction of cells with recombinant defective adenovirus (Ad) vectors encoding GP83 (AdGP83) and gplFI16 (AdIFI16) (Fig. 3A and B). The GP83 ORF encoded a C-terminal in-frame fusion of green fluorescent protein (GFP) that was generated by PCR cloning of a full-length GP83 ORF into pAcGFP-N1 (Clontech) to generate a GP83-GFP fusion construct (100 kDa) used to generate a recombinant GP83 Ad5 expression vector

FIG 3 GP83 (pp65 homolog) interacts with cellular guinea pig IFI16 and cGAS. (A) Schematic of gene cassettes used in transient-expression assays shown in panels B to D. Recombinant defective Ad5 vectors were used to express a GP83-GFP fusion construct (AdGP83-GFP), a C-terminal FLAG-tagged (striped box) guinea pig IFI16 (AdIFI16-FLAG), or GFP alone (AdGFP). A transient-expression plasmid encoded GFP-tagged HCMV pp65 (pp65-GFP). All expression vectors are under the control of the HCMV immediate early promoter (CMVIE; gray box) and contain a 3' terminal SV40 polyadenylation sequence (red box). (B to D) Interaction of GP83 with guinea pig IFI16 by transient-expression immunoprecipitation (IP) assay using GFP-Trap (Chromotek). GPL fibroblasts were transfected to express IFI16-FLAG and either GP83-GFP or GFP only or were transfected with a pp65-GFP expression plasmid. WB, Western blotting. (B) IP of GP83-GFP and IFI16-FLAG. (C) Control IP of GFP and IFI16-FLAG. (D) IP of HCMV pp65-GFP and guinea pig IFI16-FLAG. GP83-GFP, GFP, and pp65-GFP were detected by Western blotting using anti-GFP (rabbit) (Novus); IFI16-FLAG was detected using anti-FLAG (mouse) (Sigma). Proteins were detected with appropriate secondary antibody-HRP conjugates (Sigma) as previously described (26). Lanes 1, 5, 9, 13, 17, and 21 indicate total cell lysate; lanes 2, 6, 10, 14, 18, and 22 indicate flowthrough; lanes 3, 7, 11, 15, 19, and 23 indicate IP reactions. Lanes 4, 8, 12, 16, 20, 24 are GPL mock lysates. (E) Schematic of gene cassettes used in transient-expression assays in panels F and G using C-terminal FLAG-tagged guinea pig cGAS plasmid (pcGAS-FLAG), AdGP83-GFP, and AdGFP. CMV immediate early promoter (CMVIE; gray box), FLAG epitope tag (striped box), and terminal SV40 polyadenylation sequence (red box) are indicated. (F and G) Interaction of GP83 with guinea pig cGAS by transient-expression IP assay using GFP-Trap. (F) IP of GP83-GFP and cGAS-FLAG. (G) Control IP assay of GFP and cGAS-FLAG. GP83-GFP and GFP were detected by Western blotting using anti-GFP, and cGAS-FLAG was detected using anti-FLAG and appropriate secondary antibody-HRP conjugates. Lanes 25, 29, 33, and 37 indicate total cell lysate, lanes 26, 30, 34, and 38 indicate flowthrough, and lanes 27, 31, 35, and 39 demonstrate IP reactions. Lanes 28, 32, 36, and 40 are GPL mock lysates. All IPs were carried out with GFP-Trap (Chromotek) in accordance with the manufacturer's protocol (26). Broken red box indicates expected location of target IP protein IFI16 (lanes 7, 15, and 23) or cGAS (lanes 31 and 39).

(AdGP83). Interactions were determined by immunoprecipitation (IP) assays via GFP-Trap (ChromoTek) as previously described (26). AdGP83- and AdgpIFI16Flag-cotransduced cell monolayers in six-well plates were harvested at 36 h posttransduction for the IP assay. Results (Fig. 3B) demonstrated a specific interaction between GP83 and gpIFI16. A control Ad vector encoding GFP (AdGFP) (26) was used to demonstrate the lack of interaction between GFP and gpIFI16 in additional coexpression IP studies (Fig. 3C). We concluded that GP83 specifically interacted with gpIFI16 in a manner similar to that observed for HCMV pp65 and huIFI16 (62). In an additional experiment, transiently expressed GFP-tagged HCMV pp65 (55) was also capable of interacting with gpIFI16 in an IP assay (Fig. 3D) and potentially indicated that HCMV pp65 could in part overcome the species specificity barrier.

In addition to IFI16 protein, HCMV pp65 specifically targets cGAS to interfere with the cGAS/STING pathway and prevent activation of constitutively expressed IRF3 (81).

The cGAS/STING pathway is a strongly conserved innate immune pathway between various species. It has not been investigated previously in the guinea pig, but this animal model encodes orthologs of cGAS, STING, and IRF3 proteins based on the genome sequence. Since an inability to target cGAS might contribute to the GP83 mutant virus sensitivity to IFN-I, we evaluated whether GP83 could bind to cGAS in transient-expression studies. A synthetic gpcGAS gene was generated based on the predicted cGAS sequence (GenBank accession no. [XM_003478261](#)) and incorporated a C-terminal FLAG epitope tag (GenScript). The ORF was cloned into the mammalian expression vector pcDNA3.1 to enable transient plasmid (pgpcGAS) expression studies in GPL cells, where Western blotting of total cell lysate identified a protein of approximately 72 kDa (Fig. 3F), which was similar to the predicted protein size. An additional lower-molecular-weight protein which was likely an N-terminally processed version of cGAS was also detected, as another synthetic cGAS gene with an N-terminal myc epitope tag was not detected in transient-expression assays (data not shown), as similarly noted for N-terminally tagged GPCMV glycoproteins with signal peptides (26). Next, the ability of transiently expressed cGAS to interact with GFP-tagged GP83 was evaluated in a GFP-Trap IP assay (Fig. 3E to G). GP83 protein was able to immunoprecipitate cGAS protein (Fig. 3F), unlike control GFP (Fig. 3G). We concluded that GP83 is a functional HCMV pp65 homolog with conserved interactions with both IFI16 and cGAS.

GP83 enhances transcription of GPCMV immediate early locus and IE protein expression. IFI16 can be inhibitory to herpesviruses when oligomerized (82) but also enhances HCMV infection through pp65/IFI16 interactions with a major immediate early locus (83). In order to evaluate the impact of GP83 on GPCMV major immediate early locus expression, ectopic expression of GP83 was carried out in the context of two specific assays (Fig. 4): (i) a GPL cell luminescence assay of firefly luciferase reporter gene protein expression under GPCMV major immediate early promoter control with and without ectopic GP83 and with and without overexpression of IFI16; (ii) GPL cell Western blot analysis of GPCMV IE2 protein expression in GP83dPC+ virus-infected cells with and without ectopic GP83. In the first assay, a GPL cell line permanently transfected with a Sleeping Beauty plasmid encoding GPCMV immediate early promoter driving a firefly luciferase cassette (designated GPL GPCMVIE Luc) (Fig. 4A) (18) was used to evaluate luciferase luminescence of cells in 96-well plates nontransduced or transduced with recombinant Ad vectors AdGP83, AdGP83 plus AdIFI16, or control AdgL (26). An additional control was GPL cells not expressing luciferase. Cell luminescence assays of individual wells were evaluated at 48 h after Ad vector transduction or in nontransduced cells as described in Materials and Methods with triplicate sets of monolayers for each condition. Results (Fig. 4B) demonstrated that unlike GPL cells, GPCMVIE Luc cells produced luminescence, which indicated that luciferase was constitutively expressed. Further, luminescence was increased in GPCMVIE Luc cells transduced with AdGP83, and expression further increased in cells transduced with both AdGP83 and AdIFI16, which indicated enhanced reporter protein expression associated with GP83 and gpIFI16. Under conditions of IFI16 only, overexpression was not statistically significant but did induce a slight increase in luminescence (Fig. 4B). Results demonstrated that specificity of GPCMV MIE promoter-driven expression was associated with GP83 and gpIFI16. However, the enhancement of expression was more limited than the reported 5-fold increase for HCMV MIE enhancer by IFI16/pp65 (61). Potentially, this might be attributed to an absence of predicted SP1 transcription factor binding sites in the GPCMV MIE promoter and the presence of multiple SP1 sites in the HCMV MIE enhancer (84) coupled with the ability of IFI16 to sequester available SP1 and further influence transcription (85). However, further evaluation would require in-depth mutagenesis of the GPCMV MIE promoter sequence and was considered outside the scope of the present study.

In the second assay, GPCMV IE2 protein expression (56) was investigated in fibroblast cells in 6-well plates pretreated with AdGP83 or empty vector and subsequently infected with GP83dPC+ mutant GPCMV (MOI, 0.1 PFU/cell). IE2 immediate early

FIG 4 Ectopic GP83 enhances GPCVMIE Luc reporter expression and IE2 protein expression at a low MOI for GP83 mutant virus-infected GPL cells. (A) Schematic of plasmid luciferase reporter cassette used for generation of permanently transfected GPCMV major immediate early luciferase (GPCVMIE Luc) reporter GPL cell line (18), as described in Materials and Methods. The GPCVMIE promoter region is 5' to a firefly luciferase reporter gene with a 3' SV40 poly(A) sequence. (B) Luciferase luminescence assay for evaluation of MIE gene expression after 48 h. GPCVMIE Luc reporter fibroblasts were transduced to express AdIF16, AdGP83, AdGP83+AdIF16, or AdgL (vector control). GPL and GPCVMIE Luc monolayers served as untreated controls. Luminescence was measured in relative luminescence units (RLU). (C) Western blot analysis for detection of GPCMV IE2 protein expression following GP83dPC+ virus infection, with or without ectopic GP83 transient expression. GPL fibroblasts were transduced with either recombinant adenovirus encoding GP83 (+GP83) or control empty Ad vector (-GP83) 24 h prior to infection with GP83dPC+ mutant virus (MOI, 0.1 PFU/cell). Lysates were harvested at 24, 48, and 72 hpi with GP83dPC+. MI, mock-infected control GPL cell lysate. (D) β -Actin control for lane loading in panel C. (E) Quantification of Western blot bands in panels C and D. Protein expression was normalized to β -actin, and fold change was calculated for each time point. Experiments were carried out in triplicate. *, $P < 0.05$; **, $P < 0.01$; ns, not significant (one-way ANOVA with Tukey's *post hoc* test).

protein expression was evaluated by Western blotting (56) of total cell lysate at 24, 48, and 72 h postinfection (hpi) with mutant viruses. IE2 was a marker for IE protein expression (56) (Fig. 4C). β -Actin was evaluated as a control for cell lysate lane loading (Fig. 4D). Signal intensity for the IE2 Western blot at different time points and conditions normalized against β -actin are shown in Fig. 4E. Results indicated that the presence of GP83 enhanced IE protein expression. Overall, results from both assays suggest that GP83 in conjunction with IFI16 enhances GPCMV IE protein expression, which is similar to the pp65/IFI16 effect observed for HCMV IE protein (61). Since GPCMV IE1 and IE2 are expressed by transcripts with shared exons (1 to 3) and a unique 4th 3' exon (*GP123* and *GP122*, respectively) (56), the assumption is that IE1 is expressed similarly to IE2, but a lack of specific antibody to IE1 prevents verification by Western blotting. Results suggest that the importance of GP83 for infection of fibroblasts potentially increases as the MOI decreases and that the GP83dPC+ virus may have a more impaired viral phenotype at an MOI of 0.1 PFU/cell or lower.

GP82 targets DAXX and is a functional pp71 homolog. Although the focus of the study was directed at pp65, we considered it important to undertake preliminary studies with GPCMV GP82 because of gene duplication events in the *UL82-UL84* homolog

FIG 5 GP82 interacts with guinea pig DAXX as part of PML bodies. GP82 and gpDAXX interactions were evaluated by cellular colocalization (A to D) or by GFP-Trap immunoprecipitation (E and F). (A to D) Immunofluorescence detection of transiently plasmid expressed gpDAXX (FLAG tagged) and GP82 (GFP tagged) colocalization in the nucleus of GPL. Shown separately in the same cell are gpDAXX (anti-FLAG TRITC [tetramethylrhodamine isothiocyanate]) (A), GP82 (GFP fluorescence) (FITC, fluorescein isothiocyanate) (B), a merged image of panels A and B (C), and an overlay of panel C with DAPI (4',6-diamidino-2-phenylindole) nuclear staining (D). (E) GFP-Trap (Chromotek) IP of GP82-GFP and gpDAXXFLAG plasmid coexpression in GPL cells as previously described (26). GP82 was detected in lanes 1 and 3 (anti-GFP). DAXX was detected in lanes 4 and 6 (anti-FLAG). (F) Control IP of GFP and gpDAXX in wells cotransfected with pEGFPC1 plasmid and gpDAXXFLAG. GFP was detected in lanes 7 and 9 (anti-GFP), and DAXX (anti-FLAG) was only detected in cell lysate (lane 10) and not in IP (lane 12). Western blot secondary antibodies were anti-mouse IgG-HRP conjugate (for FLAG) and anti-rabbit IgG-HRP conjugate (for GFP). Proteins were detected by Western blotting of total cell lysate (lanes 1, 4, 7, and 10) or IP track (lanes 3, 6, 9, and 12). Control mock nontransfected GPL cell lysate is shown in lanes 2, 5, 8, and 11. Red broken box (lanes 6 and 12) indicates expected location of DAXX IP.

locus of animal CMV. The HCMV *UL82* gene encodes tegument protein pp71 (UL82), which targets DAXX protein present in nuclear ND10 bodies (66). We recently characterized the major guinea pig ND10 components and demonstrated that they have a function similar to that in human cells (56). A GPCMV IE1 protein similar to HCMV IE1 is necessary for the interaction and dispersal of ND10 components by interaction with PML and SP100 (56). However, previous studies did not demonstrate a direct interaction of IE1 with guinea pig DAXX (gpDAXX). Transient plasmid expression of GP82 (pGP82GFP) (55) and gpDAXX (pgpDAXXFLAG) (56) was used to evaluate protein-protein interaction in GPL fibroblast cells. In immunofluorescence studies, nuclear expressed gpDAXX was found to colocalize with GFP-tagged GP82 (Fig. 5A to D). In IP assays of plasmid transiently expressed proteins, GFP-tagged GP82 protein was able to specifically IP gpDAXX via GFP-Trap (ChromoTek) (Fig. 5E). In contrast, a control GFP vector, pEGFPC1 (Clontech), was unable to interact with gpDAXX protein, and only GFP protein was detected by IP (Fig. 5F). It was concluded that GP82 is a functional homolog of HCMV pp71. Overall, GPCMV is similar to HCMV in encoding single functional homologs of pp71 and pp65, unlike MCMV and RhCMV, which encode duplicate pp65 homologs.

A GP83 mutant GPCMV is impaired for infection of nonfibroblast cells. Since the GP83 mutant virus was capable of PC expression, we evaluated the ability of GP83dPC+ virus to infect available guinea pig nonfibroblast cell lines, renal epithelial (REPI) and placental trophoblasts (TEPI), established in the laboratory and previously demonstrated to require GPCMV PC for infection (27, 43). GPCMV (PC+) growth on REPI and TEPI cells is shown in Fig. 6A. In comparison, the GP83 mutant virus (GP83dPC+) at a similar MOI (1 PFU/cell) was impaired for infection on REPI and TEPI cells, unlike GPL fibroblast cell infection (Fig. 2A and 6B). The GP83 rescue virus (GP83R) had kinetics similar to that of the wild-type virus on renal epithelial cells (Fig. 6B). Transduction of TEPI cells with recombinant AdGP83 prior to infection with GP83dPC+ virus restored the ability of virus to infect

FIG 6 Comparative growth of wild-type (WT) GPCMV, GP83dPC+, and GP83 rescue virus on REPI and TEPI cells and rescue of GP83dPC+ mutant growth by ectopic GP83 expression on TEPI cells. (A) One-step growth curve of wild-type GPCMV infection on REPI (blue squares) and TEPI (green circles) cell monolayers. (B) One-step growth curve of GP83dPC+ virus on REPI (orange squares) and TEPI (gray circles) cells, GP83 rescue (GP83R) virus on REPI (black diamonds) cells, and GP83dPC+ virus on TEPI cells transduced to express GP83 prior to infection (yellow triangles). All infections were performed at an MOI of 1 PFU/cell. Experiments were carried out on 6-well plates in triplicate, and titers were determined at time points in duplicate on GPL cells. Results are plotted as virus titer (PFU/ml) against days postinfection (dpi).

cells to levels roughly similar to those seen in fibroblast cells (Fig. 6B). This demonstrated the requirement of GP83 as a novel tropism factor for infection of nonfibroblast cells, as the route of GPCMV cellular entry differs between cells: direct cell membrane fusion for fibroblasts and endocytosis for epithelial cells (27, 43). This potentially indicated that GP83 is important for viral cellular infection via the endocytic pathway at a postentry stage.

We recently demonstrated that fibroblast cells have two routes of GPCMV cell entry (29, 33) and that CRISPR knockout of the gH/gL/gO triplex cell receptor PDGFRA forces virus to enter cells via a PC-dependent endocytic pathway (33, 34). Importantly, there is no difference in ability between wild-type PC+ GPCMV to replicate on PDGFRA-positive or PDGFRA-negative fibroblast cells (33) (Fig. 7). We evaluated the ability of GP83dPC+ virus to infect PDGFRA gene knockout (GPKO) fibroblast cells (33). GPCMV GP83dPC+ infection of GPKO cells (MOI, 1 PFU/cell) demonstrated that unlike infection of GPL (PDGFRA+) fibroblasts (Fig. 7A), the GP83 mutant virus was impaired for infection of GPKO (PDGFRA-) fibroblasts based on viral titers at 3 days postinfection (dpi) (Fig. 7B). GP83 mutant virus infection of GPKO cells could be improved by transduction of Ad vector encoding GP83 prior to infection by the GPCMV GP83 mutant (Fig. 7B). Results indicated that GP83 has an important undefined role associated with the endocytic pathway of infection of nonfibroblast cells as well as fibroblasts lacking PDGFRA cell receptor. A similar relationship of pp65 with HCMV on nonfibroblast cells has not been evaluated.

We previously demonstrated that ectopic expression of PDGFRA on epithelial cells enables GPCMV to enter the cell by direct fusion, independent of the PC-dependent

FIG 7 Ectopic expression of GP83 in GPKO (PDGFRA[−]) cells restores GP83dPC⁺ mutant virus growth. (A) Titers of GPL cells infected with wild-type GPCMV (green bar), GP83dPC⁺ mutant (yellow bar), or GP83R virus (blue bar) at 3 dpi (MOI, 1 PFU/cell). (B) Titers of GPKO cells infected with wild-type GPCMV (green bar), GP83dPC⁺ mutant virus (yellow bar), or GPKO cells transduced to express GP83 prior to infection with GP83dPC⁺ mutant virus (black bar). Experiments were carried out in triplicate, and virus titer was determined in duplicate on GPL cells. Mean titer (PFU/ml) $\pm$ standard deviation (SD) is shown; *, $P < 0.05$; ns, not significant (unpaired Student's *t* test).

endocytic pathway (29, 34). We hypothesized that PDGFRA expression in epithelial cells could restore infection similar to that in fibroblast cells, as it would enable infection by a direct fusion pathway (Fig. 8A to C). Renal epithelial cells were permanently transfected with a PDGFRA expression plasmid to generate a cell line (REPI PDGFRA) that constitutively expressed ectopic guinea pig PDGFRA (Fig. 8B). GP83dPC⁺ virus was compared to wild-type and GP83R viruses for infection on REPI (Fig. 8A) and REPI PDGFRA (Fig. 8C) epithelial cell lines in six-well plates. Cells were infected with virus (MOI, 1 PFU/cell), and wells were harvested at 3 dpi to determine virus titers. On REPI cells, the GP83 mutant virus was impaired for infection (Fig. 8A), but in contrast, on REPI PDGFRA cells, GP83dPC⁺ virus produced a virus yield similar to that of the wild-type and GP83 rescue virus (Fig. 8C). This demonstrated that the GP83 mutant phenotype on epithelial cells could be rescued by an alternative route of virus cell entry. Overall, our studies suggested that the GP83dPC⁺ virus endocytic entry pathway resulted in activation of an innate immune response likely associated with IFI16/cGAS/STING and downstream activation of constitutively expressed IRF3 and an antiviral innate immune response, given the GP83dPC⁺ virus sensitivity to IFN- λ .

The guinea pig encodes an IRF3 homolog with 68.2% identity to human IRF3 (<https://www.uniprot.org/uniprot/H0VXN2>). We hypothesized that if endogenously expressed IRF3 protein could be inactivated, then GP83 mutant virus growth could be restored on epithelial cells (Fig. 8D and E). BVDV N^{pro} protein targets IRF3 for proteasomal degradation and is functionally active across various species in tissue culture, and ectopic expression of N^{pro} has been shown to complement HCMV growth (86–88). An expression vector that encodes a synthetic FLAG-tagged BVDV N^{pro} gene was generated (GenScript) in pcDNA3.1 and used to permanently transfect renal epithelial cells to constitutively express a C-terminal FLAG epitope-tagged N^{pro} protein. Ectopic expression of N^{pro} in REPI cells (Fig. 8D) enabled complementation of GP83 mutant virus growth, resulting in virus titers similar to those of the wild-type and GP83R rescue virus at 3 dpi (Fig. 8E).

Direct evaluation of N^{pro} on endogenous gpIRF3 protein levels in guinea pig cells was prevented by the lack of a specific or cross-reacting gpIRF3 antibody. However, using an expression plasmid for human IRF3 and a control GFP expression plasmid, pAcGFP1 (Clontech), with both ORFs under HCMV MIE enhancer control, we were able to evaluate transiently expressed IRF3 stability in the presence or absence of N^{pro} in epithelial cells compared to transient GFP or endogenous β -actin (Fig. 9). Plasmid-derived IRF3 protein levels remained high on REPI cells (Fig. 9A), but IRF3 levels rapidly

FIG 8 Growth impairment of GP83dPC+ mutant virus in REPI cells can be overcome by the PDGFRA-dependent (direct fusion) entry pathway or by expression of IFN-I pathway suppressor BVDV N^{PRO}. (A) Comparative infection of wild-type GPCMV (green bar), GP83dPC+ mutant (yellow bar), and GP83R virus (blue bar) on REPI cells at 3 dpi (MOI, 1 PFU/cell). (B) Western blot analysis for the detection of PDGFRA in wild-type REPI cells and a REPI-derived cell line generated to stably express PDGFRA (REPI PDGFRA) with a β -actin Western blot as a lane loading control. (C) Comparative infection of wild-type GPCMV (green bar), GP83dPC+ mutant (yellow bar), and GP83R virus (blue bar) on REPI PDGFRA cells at 3 dpi (MOI, 1 PFU/cell). (D) Western blot analysis for the detection of BVDV N^{PRO} in wild-type REPI cells and a REPI-derived cell line generated to stably express N^{PRO} protein (REPI N^{PRO}) with a β -actin Western blot as a control. (E) Comparative infection of wild-type GPCMV (green bar), GP83dPC+ mutant (yellow bar), and GP83R virus (blue bar) on REPI N^{PRO} cells at 3 dpi (MOI, 1 PFU/cell). Mean titer (PFU/ml) $\pm$ SD; **, $P < 0.01$; ns, not significant (unpaired Student's t test).

degraded in REPI N^{PRO} cells, with a progressive drop in levels of detectable protein between 24 and 72 h posttransfection (Fig. 9A) and with less than 10% of the 24-h level detected by 72 h in REPI N^{PRO} cell lysate based on signal intensity (Fig. 9B). In contrast, plasmid-expressed GFP levels remained similar between REPI and REPI N^{PRO} cells (Fig. 9C and D). We concluded that N^{PRO} expression in REPI cells results in degradation of IRF3, and an inhibitory innate immune response to GPCMV GP83 mutant virus infection was initiated by the endocytic pathway of cell entry. It is likely that infection of trophoblast cells and PDGFRA KO fibroblasts could be similarly restored for GP83dPC+ growth by N^{PRO} expression but awaits future evaluation and generation of N^{PRO} expression cell lines.

A PC-positive GP83 mutant GPCMV is impaired for pathogenicity in the animal model. The pathogenicity of the GP83dPC+ virus in animals was evaluated in comparison to GP83+ parental BAC-derived virus (GP129FRT) restored for PC+ by ectopic GP129 expression in the *GP25-GP26* intergenic locus (27) and GP83 rescue virus (Fig. 10). Seronegative animals were randomly assigned to three groups ($n = 12$ per group) and were infected with virus (10^5 PFU subcutaneously [s.c.]) dependent on group, as follows: GP129FRT for group 1, GP83dPC+ for group 2, and GP83R for group 3. At various days postinfection, animals were euthanized ($n = 3$ /group) and the viral loads in target organs and blood were determined by real-time PCR of extracted tissue. Figure 10 shows the viral loads in infected animals at 4, 8, 12, and 27 dpi. Comparative viral loads between wild-type, rescue, and GP83 mutant viruses demonstrated a high level of attenuation associated with the GP83 mutant virus. Viremia was detected only at

FIG 9 REPI cells expressing BVDV N^{PRO} result in targeted degradation of IRF3. Transient plasmid expression in REPI (orange bars) and REPI N^{PRO} (blue bars) cells of IRF3-FLAG (A and B) or pEGFP-C1 (Clontech) (C and D) plasmids. (A and B) Western blot analysis of IRF3 (FLAG) expression in REPI and REPI N^{PRO} cells (A) and relative expression (B). (C and D) Western blot analysis of transient plasmid GFP expression REPI and REPI N^{PRO} cells (C) and relative expression (D). Total cell lysates were harvested at 24, 48, and 72 h following plasmid transfection; mock lanes indicate control nontransfected cell lysates. β -Actin was used as an internal Western blot lane loading control for cell lysates. Relative expression of signal intensity was quantified from separate blots using C-DiGit blot scanner (LI-COR) with Image Studio software and normalized to β -actin. Expression at 24 h served as the baseline. Experiments were performed in triplicate. *, $P < 0.05$; **, $P < 0.01$; ns, not significant (unpaired Student's *t* test).

8 dpi, compared to other groups with viremia at 4 to 12 dpi, with a significantly higher load at 8 dpi compared to that of GP83dPC+. The GP83 mutant virus was not detected in the salivary gland, unlike the wild-type or rescue virus, and had significantly lower levels or was not detectable in lung, liver, and spleen at each time point (Fig. 10). Overall, the results demonstrate that the GP83dPC+ virus was highly attenuated in the animal model despite encoding the PC. This indicated the importance of both PC and GP83 for virus pathogenicity in the animal and is likely linked to the requirement for infection of nonfibroblast cell types via the endocytic pathway. This would likely include both neutrophils and macrophage, important for virus dissemination, but requires future evaluation.

A recombinant defective Ad vector encoding GP83 induces a protective immune response. The pp65 protein is a major T cell target in HCMV convalescent patients. GPCMV GP83 protein evokes a cell-mediated response in virus-infected animals and in GPCMV disabled infectious single-cycle (DISC)-vaccinated animals, where the cell-mediated response is thought to contribute to vaccine protection together with the antibody response to viral glycoprotein complexes (28, 33). We previously demonstrated that full-length gB expressed in a defective Ad5 vector induces high titers of neutralizing antibodies (30). Therefore, we sought to determine if GP83 (22122 strain) expressed in a defective Ad5 vector vaccine strategy (AdGP83) can induce a protective cell-mediated immune response. Potentially, a combination of both an antibody response to gB and a cell-mediated response to GP83 might provide significant cumulative protection against GPCMV in subsequent Ad vector studies. An important aspect of CMV vaccine strategies is that the vaccine has the ability to provide cross-strain protection. This aspect of GPCMV vaccine protection has not been explored previously in the guinea pig model. Our laboratory recently isolated a novel strain of GPCMV (TAMYC) from the salivary glands of a commercial animal of a colony with GPCMV endemicity (89). Unlike the prototype GPCMV strain 22122, which was isolated in the 1950s and passaged extensively on fibroblast cells, the TAMYC strain has been passaged only on epithelial cells, exhibits high tropism to epithelial cells, and replicates poorly on fibroblasts unless adapted (89). Importantly, the

FIG 10 Pathogenicity studies of GP83PC+ mutant virus in animals. Three separate groups of animals ($n = 12$ per group) were infected with either parental GPCMV BAC-derived wild-type virus (GP83+) expressing ectopic GP129 (GP129FRT) (blue bars), GP83dPC+ mutant virus (orange bars), or GP83 rescue virus (GP83R) (input, 10^5 PFU s.c.). At 4, 8, 12, and 27 days postinfection (dpi), 3 animals per group were evaluated for viral load in target organs by real-time PCR of tissue-extracted DNA. Viral load is plotted as the number of viral genome copies/mg tissue. Salivary gland tissue was evaluated only at day 27 and is detectable in the GP129FRT and GP83R groups. (A) Lung; (B) liver; (C) spleen; (D) salivary gland. Blood viremia at 4, 8, 12, and 27 dpi is shown in panel E and plotted as the number of genome copies/ml blood. Statistical analysis was performed by one-way ANOVA with Tukey's *post hoc* test. *, $P < 0.05$; ns, not significant. #, viral load in GP83dPC+ is below level of detection.

TAMYC strain is highly cell associated, unlike 22122, and therefore is more similar to clinical HCMV isolates (89). The GP83 locus of the TAMYC strain genome was PCR cloned and sequenced to demonstrate that both GPCMV strains encode 100% identical predicted GP83 protein sequences (Fig. 11). Subsequently, AdGP83 vaccine efficacy against the two strains was evaluated to determine if there was a difference in protection between challenge wild-type strains. A specific concern regarding the 22122 strain of GPCMV is that the virus was extensively passaged on fibroblasts before being deposited with the ATCC. Consequently, the 22122 strain, despite being able to cause cCMV, might represent an impaired pathogenic virus in comparison to strains circulating in guinea pig colonies.

Seronegative animals were vaccinated with AdGP83 in accordance with a previously described protocol for AdgB vaccine, with three sequential inoculations with each separated by a 4-week interval (30). A guinea pig gamma interferon (IFN- γ) enzyme-linked immunosorbent spot (ELISPOT) assay of splenocytes isolated from AdGP83-vaccinated animal demonstrated a specific cell-mediated response to groups of 9mer peptides corresponding to a specific GP83 sequence previously found to be reactive in this assay (Fig. 12A; see Table S1 in the supplemental material) (28). In a challenge protection study (Fig. 12B to E), AdGP83-vaccinated animals ($n = 12$ /group) were challenged with wild-type GPCMV (10^5 PFU s.c.), as follows: group 1, 22122 (Fig. 12C); group 2, TAMYC (Fig. 12E). GPCMV-seronegative animals were similarly challenged with wild-type virus: group 3, 22122 (Fig. 12B); group 4, TAMYC (Fig. 12D). At 4, 8, 12, and 27 dpi, animals in the groups were randomly euthanized ($n = 3$ /group) and the viral loads in target organs (lung, liver, spleen) and blood were evaluated. Additionally, at 27 dpi, salivary gland tissue was also evaluated for viral load. Results (Fig. 12B and C)

FIG 11 Comparative alignment of the predicted GP83 protein amino acid sequences from GPCMV strains 22122 and TAMYC. ClustalW alignment of the predicted amino acid sequences of GP83 from GPCMV strain TAMYC and strain 22122 (GenBank accession no. [AB592928](#)) is shown. Sequence alignment was carried out using MacVector software. Gray background with consensus letter in the third row indicates amino acid sequence identity.

demonstrated that the AdGP83 vaccine was effective in reducing viral load in target organs in 22122 challenge animals in comparison to the no-vaccine control. Virus was absent in the liver by 12 dpi, and no virus was detected in any target organs by 27 dpi (Fig. 12C) in comparison to seronegative control animals infected with wild-type virus (Fig. 12B). Importantly, the vaccine prevented dissemination of the 22122 strain of GPCMV to salivary glands (Fig. 12C) and reduced detectable viremia up to 4 dpi (Fig. 12C), compared to viremia (4 to 12 dpi) in the no-vaccine control group of 22122-infected animals (Fig. 12B). In contrast, the AdGP83 vaccine had limited impact against the new strain of GPCMV (TAMYC), and vaccinated animals had viral dissemination patterns that were very similar to those of the no-vaccine control group, with virus in all target organs (Fig. 12D and E). Importantly, there was extensive viremia and a high viral load in the salivary gland in the vaccinated animals (Fig. 12E), which contrasted with the results for the 22122 challenge study (Fig. 12B and C). Overall, results indicated that the cell-mediated immune response to GP83 contributes to limiting 22122 strain virus dissemination but not against TAMYC strain, despite identical predicted GP83 protein sequences (Fig. 11). Since the TAMYC GPCMV strain might be considered to be more similar to clinical HCMV strains, our results suggest a limitation of pp65-based vaccine strategies against HCMV, especially in the context of cross-strain protection.

DISCUSSION

Cytomegalovirus tegument proteins are associated with enhancing early stages of virus infection and/or important for virus assembly and maturation (90). The HCMV pp65 protein is a major component of virions and dense bodies. Importantly, in dense bodies, pp65 is 95% of the protein content and has the ability to enter cells, similar to infectious virions. GPCMV is like HCMV in that the virus produces extracellular dense bodies, and GP83, the presumptive pp65 homolog, is present in both dense bodies

FIG 12 Immune protection of AdGP83 vaccine strategy against challenge by different WT GPCMV strains (22122 and TAMYC). (A) Cell-mediated immune response to AdGP83 vaccine measured by guinea pig IFN- γ ELISPOT. Two previously identified GP83-reactive peptide pools, X and XX (purple and red bars), reacted with primed splenocytes from AdGP83 vaccine animals. ConA-positive control, blue; DMSO control, orange; unstimulated control, gray; unresponsive negative peptide pool, green. Final counts were calculated based on the number of spot-forming cells (SFC) per 10⁶ cells after background subtraction. (B and D) Pathogenesis of wild-type GPCMV in seronegative animals ($n=12$ /group) infected s.c. with virus (10⁵ PFU) of strain 22122 (B) and strain TAMYC (D). At various time points postchallenge (4, 8, 12, and 27 dpi), target organs and blood were collected from 3 animals/time point/group. The numbers of viral copies were measured by real-time PCR from extracted DNA. Salivary gland was collected only on day 27. (C and E) Pathogenesis of wild-type GPCMV in AdGP83-vaccinated animals ($n=12$ /group) using strain 22122 (C) and strain TAMYC (E). At various time points (4, 8, 12, and 27 dpi), target organs and blood were collected from 3 animals/time point. The numbers of viral copies were measured by real-time PCR from extracted DNA. Salivary gland was collected only on day 27. Day 4 (D4), blue; D8, orange; D12, gray; D27, yellow.

and viral particles (74). In HCMV, the pp65 protein has identified roles in innate immune evasion by targeting the cellular DNA sensors IFI16 (62) and cGAS (81). Additionally, pp65 can stimulate HCMV major immediate early promoter expression through interaction with IFI16 (61, 83). However, this contrasts with the fact that pp65 protein appears nonessential for HCMV growth on fibroblast cells. HCMV has two routes of cell entry: direct membrane fusion (PC independent) or the endocytic pathway (PC dependent). GPCMV infection similarly has two pathways of cell entry (27, 29, 34). We hypothesized that the pp65 homolog in GPCMV (GP83) is more important for virus infection of nonfibroblast cells via the endocytic PC pathway and is a basis for GP83 as a pathogenicity factor. This aspect has not been evaluated for HCMV or in any animal CMV. A complication of animal CMV studies is that species-specific evolution of the animal CMV results in divergent functions and that colinear homolog genes do not necessarily equate with proteins with similar function to HCMV. An important example is that mouse CMV does not encode a PC but instead another gH/gL-based trimer

complex, which is more similar to that found in Epstein-Barr virus (91) and affects the ability to evaluate the PC as a vaccine target in the mouse model. The *UL82-UL84* homolog locus in animal CMV is another example where gene duplication events have resulted in animal CMV (e.g., RhCMV and MCMV) with additional proteins encoded in this locus or proteins with altered functions. This aspect has a potential to detract from the translational impact of animal CMV studies and development of preclinical intervention strategies against CMV. This also has ramifications for preclinical development of CMV as a novel vaccine vector platform against other diseases (92). RhCMV encodes two potential pp65 homologs (Rh111 and Rh112) with about 40% homology to each other (93). A RhCMV double knockout (pp65a and pp65b) mutant virus was only slightly impaired in culture and did not appear to be required for primary infection or to establish persistent infection in the non-human primate (NHP) model (73). However, these studies were carried out in the backdrop of a virus lacking a PC, which limits the research. The importance of RhCMV pp65 homologs in innate immune evasion has not been demonstrated, but RhCMV has the ability to block IRF3 activation (94). In MCMV, there is no homolog to UL84, and the virus carries two potential pp65 homologs (M83 and M84) that are both nonessential for infection in culture, but independent null mutants are impaired in the mouse model, indicating a potential innate immune evasion function (71, 95). However, MCMV does not encode a PC, and a specific functional role for a MCMV pp65 homologs awaits future characterization. An additional potential complication in the mouse model is that IFI16 is not encoded by the mouse but instead an ortholog p204 (96).

We previously demonstrated that GP84 is a functional UL84 homolog (75) and that GP82 shares some functions associated with pp71 with an ability to complement a VP16 HSV-1 mutant virus (55). Although GP83 has previously been demonstrated to be a tegument protein and nonessential for infection of fibroblasts, no function associated with GP83 has been assigned, which potentially jeopardizes translational studies of GP83 as a pp65 homolog, especially in the context of vaccine strategies. Importantly, previous GP83 mutant studies were carried out with a PC-negative virus, so virus studies on nonfibroblast cells were not possible and pathogenicity studies were clouded by the lack of PC. In the present study, we confirm that GPCMV encodes functional homologs of tegument protein pp65 (GP83) and pp71 (GP82). GP83 interacts with cellular protein gpIFI16 similar to HCMV pp65, and GP82 targets DAXX, as observed for pp71 (97). These observations are important, since a common criticism of the guinea pig model is that it lacks similarity to HCMV, which negatively impacts the perception of GPCMV-based research.

Although HCMV has developed multiple strategies to counteract the IFN-I pathway, the virus remains sensitive to IFN-I (98). In HCMV, pp65 and pp71 target cGAS and STING, respectively, to block the IFN/ISG response pathway (67, 81). IFN-I studies in GPCMV demonstrated that wild-type GPCMV encoding GP83 was less sensitive to low-level IFN-I treatment of cells (100 IU/ml) than GP83 knockout virus (MOI, 0.1 PFU/cell). The IFN-I response in guinea pigs is similar to that in humans and other mammals (99). IFI16 expression would be upregulated upon IFN-I induction as well as endogenously expressed in the cell, which increases the importance of the GP83/IFI16 interaction, since oligomerization of IFI16 can serve as a restrictome structure to herpesviruses and epigenetic silencing of the viral genome (82, 100). GP83 mutant virus growth studies were carried out at MOI of 1 PFU/cell, which identified an impairment of infection on nonfibroblast cells but not on fibroblasts. However, infection studies on fibroblasts at a lower MOI (0.1 PFU/cell) demonstrated a deficit in IE2 protein expression compared to wild-type virus, and it is likely that growth studies at the lower MOI might demonstrate an impaired fibroblast growth phenotype. Potentially, on fibroblast cells, where virus enters cells by direct fusion with the cell membrane, an MOI of 1 PFU/cell enables rapid IE protein expression and compensates for the loss of GP83 or the limited requirement for GP83 in fibroblast cells. This contrasts with the PC-dependent endocytic pathway of cell entry in nonfibroblast cells, where normal growth is more dependent upon GP83.

Our initial GPCMV epithelial tropism studies indicated that the PC was sufficient for virus cell entry and infection (27, 29, 43). However, studies with PC-positive wild-type virus and a GP83 knockout mutant virus (GP83dPC+) on nonfibroblast cells (REPI and TEPI cells) demonstrated that GP83 protein was also necessary (Fig. 6). Transient expression of GP83 on trophoblast cells prior to GP83dPC+ virus infection restored virus growth on TEPI cells to levels similar to those seen on fibroblast cells and indicated that GP83 has an important function related to the endocytic pathway at the postentry stage. This was also demonstrated by GP83dPC+ virus infection of fibroblast cells lacking PDGFRA (viral cell receptor for gH/gL/gO), which forces the virus to infect fibroblasts via the endocytic pathway (27, 33, 34) (Fig. 7). In both cases, transient expression of GP83 restores infection, and this is likely the case for other cell lines that are dependent on PC for GPCMV infection (27, 29, 42, 43). Increased interdependence of IFI16 and cGAS has been noted for effective innate immune response in nonfibroblast cells, including epithelial cells, macrophage, and monocytes (101, 102). In the case of monocytes, the virus can establish latency as a method of bypassing the innate immune response, but studies on permissive epithelial or macrophage cells are lacking in regard to a UL83 HCMV mutant. Overall, we concluded that the pp65 homolog is important for virus dissemination to target organs and that this is likely related to GP83 innate immune evasion function and the PC-dependent endocytic pathway of entry. The requirement of PC is likely dominant to the requirement for GP83, since the PC enables the virus to enter the cell. However, both pp65 and PC are highly important for CMV dissemination and disease.

Potentially, GP83 could be considered a novel GPCMV factor for cCMV, since a GP83 mutant virus is impaired for PC-dependent infection of guinea pig placental trophoblasts (Fig. 6B) (29, 43). Importantly, the lack of detectable viremia in GP83 mutant-infected animals possibly suggests that GP83 is necessary to enable PC-dependent infection of macrophages, monocytes, and neutrophils (42), but this awaits further evaluation. The salivary gland is normally a site of sustained high-titer virus production in guinea pigs, and GP83 would appear to be a significant tropism factor for infection of this tissue because of the lack of detectable mutant virus. Potentially, this could relate to higher levels of IFI16 constitutively expressed in this tissue, but the lack of a guinea pig salivary gland cell line limits further evaluation of this aspect. Earlier GPCMV studies indicate that the virus targets salivary gland ductal epithelial cells, which would doubtless require PC for the endocytic pathway of infection (103). Specific studies related to the importance of pp65 for the HCMV endocytic pathway infection of nonfibroblast cells awaits evaluation. Potentially, a PC+ GP83 knockout mutant virus could function as a candidate live attenuated vaccine strategy against cCMV.

A protective vaccine strategy against cCMV is a high priority, and both antibody and T cell responses are likely to contribute to protection, as suggested by DISC vaccine studies in this model (28, 33). In GPCMV, a defective alphavirus vaccine encoding GP83 resulted in partial protection against cCMV, which indicated the importance as a T cell target antigen in this model (104). We recently demonstrated that expression of a full-length GPCMV gB antigen in a recombinant defective adenovirus vector vaccine strategy (AdgB) induced a potent neutralizing antibody response (30). However, despite the essential requirement for gB on all cell types, the neutralizing antibodies were less effective on nonfibroblast cells, including renal epithelial cells, trophoblasts, and amniotic sac cells (29, 30). Consequently, the AdgB vaccine strategy reduced but did not fully prevent wild-type virus dissemination in vaccinated animals (30). The result indicated that additional target antigens are necessary to induce an effective protective response. Consequently, we tested the ability of a recombinant Ad vector encoding GP83 to induce a cell-mediated response in AdGP83-vaccinated animals.

In a vaccine protection study, we demonstrated that the AdGP83 vaccine induced a protective immune response against GPCMV wild-type challenge virus (22122 strain). This result sharply contrasted with the inability of the AdGP83 vaccine to be protective in animals against challenge by a novel strain of GPCMV (TAMYC). This new strain differs from the 22122 prototype strain, as it preferentially grows on epithelial cells and

poorly on fibroblasts unless adapted. Additionally, TAMYC is highly cell associated, unlike 22122 virus, and is therefore more similar to HCMV clinical strains. Overall, the AdGP83 vaccine results suggest that a pp65-based vaccine approach has limited efficacy in the GPCMV model. In RhCMV, a modified vaccinia Ankara (MVA) vaccine strategy against pp65b induced a T cell response but failed to prevent horizontal RhCMV infection (105), which indicates a general limitation of pp65 stand-alone vaccine. In GPCMV, a combination vaccine strategy of GP83 and gB has been evaluated in a recombinant lymphocytic choriomeningitis virus (LCMV) strategy, but this vaccine did not encode full-length gB antigen and the use of both gB and GP83 only marginally increased protection over that of gB only (106). In contrast, a recent GPCMV DISC vaccine strategy that evoked a neutralizing antibody response to PC as well as gB and a cell-mediated response to GP83 was highly effective in preventing virus dissemination of challenge wild-type virus, but only a DISC vaccine encoding the PC was fully protective against cCMV (33). Potentially, the DISC vaccine strategy encodes additional T cell target antigens, including the IE1 protein, which is a notable T cell target antigen in HCMV convalescent patients. Although the IE1 homolog has been characterized in GPCMV (56), the immune response to the protein has not been evaluated. The immune response to IE1 may be more protective than the response to GP83, especially in the context of cross-strain protection. However, this awaits future evaluation, either by a recombinant Ad vector strategy or another recombinant vaccine vector platform approach.

In summary, GPCMV GP83 is a functional homolog to HCMV pp65 involved in innate immune evasion. GP83 would appear to be highly important for tropism to cells and tissues that are dependent upon the PC for infection. It remains to be determined if this is also the case for HCMV pp65 and awaits additional studies. Although a recombinant defective Ad vector encoding GP83 induced a protective cell-mediated immune response, it potentially has limited efficacy against multiple GPCMV strains, especially if the challenge strain is more similar in phenotype to HCMV clinical strains. Overall, the results demonstrate that GPCMV encodes important functional homologs to HCMV, which strengthens the translational merit of the continued use of this preclinical animal model to gain a better understanding of cCMV disease and the development of intervention strategies. Furthermore, results demonstrate the importance of cross-strain protection studies for evaluation of vaccine candidates in this model to increase the translational impact of the research.

MATERIALS AND METHODS

Cells, synthetic genes, viruses, and oligonucleotides. GPCMV (strain 22122, ATCC VR682) and second-generation GPCMV BAC-derived viruses (77, 107) were propagated on guinea pig lung fibroblast cells (GPL; ATCC CCL 158) in F-12 medium supplemented with 10% fetal calf serum (FCS; Life Technologies), 10,000 IU of penicillin/liter, 10 mg of streptomycin/liter (Life Technologies), and 7.5% NaHCO₃ (Life Technologies) at 37°C and 5% CO₂. Additionally, GPCMV was grown on guinea pig renal epithelial cells (REPI cells) and placental trophoblasts (TEPI cells) for specific tropism studies (27, 43). REPI and TEPI cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 5% FCS (Life Technologies), 10,000 IU of penicillin/liter, and 10 mg of streptomycin/liter (Life Technologies), with growth at 37°C and 5% CO₂. A previously characterized novel GPCMV strain (TAMYC) was used in vaccine challenge studies (89). Synthetic genes for guinea pig IFI16 and cGAS were based on determined DNA sequences from the NCBI database, as follows: IFI16, GenBank accession no. [XP_023419508.1](#); cGAS, GenBank accession no. [XM_003478261.3](#). An additional synthetic gene was generated to bovine viral diarrhoea virus (BVDV) nonstructural protein N^{pro} (UniProtKB accession no. [Q6F647](#) [Q6F647_BVDV]). Synthetic genes were synthesized by GenScript, and each incorporated a C-terminal in-frame FLAG epitope tag. ORFs were cloned as BamHI fragments into pcDNA3.1 (Life Technologies) under HCMV MIE enhancer control. Protein expression was verified by plasmid transfection onto GPL fibroblast cells and anti-FLAG Western blotting of cell lysates as previously described (26). Recombinant defective adenovirus (Ad5) vectors generated as high-titer CsCl gradient purified recombinant defective adenovirus virus stocks (10¹² transduction units [TDU]/ml) were propagated by Welgen, Inc. (Worcester, MA), after preliminary characterization of primary virus stock by our laboratory. Recombinant defective Ad5 vectors included AdGP83-GFP (encoding full-length GP83 with a C-terminal GFP tag) and AdIFI16flag (guinea pig IFI16 with a C-terminal FLAG tag). All oligonucleotides were synthesized by Sigma-Genosys (The Woodlands, TX). DNA sequencing was carried out by Functional Biosciences and Eurofins Genomics.

IFN-I inhibitory growth assay. A trans-species type I alpha interferon (R&D Systems) was used to test the susceptibility of GPCMV to type I interferon (IFN-I). The IFN-I was initially tested on vesicular

stomatitis virus (VSV)-infected GPL cells to verify equivalent international unit (IU) strength on guinea pig fibroblast cells (56). For the GPCMV IFN-I susceptibility assay, GPCMV (wild type, GP83 mutant virus, or GP83 rescue virus) was used to infect monolayers in six-well dishes (MOI, 0.1 PFU/cell). Cells were either pretreated for 6 h with IFN-I (100 or 1,000 IU/ml) or left untreated (control) for normal virus growth (56). Virus infection was carried out as previously described, and infection progressed for 3 to 4 days, at which time viral titers were evaluated. Experiments were carried out in triplicate and repeated multiple times.

GP83 knockout mutant construction. The GPCMV sequence was based on the 22122 strain genome sequence (GenBank accession no. [AB592928.1](#)). A shuttle vector to remove the majority of the GP83 coding sequence was generated based on an existing GPCMV subgenomic plasmid clone carrying the complete GP83 gene and partial GP82 and GP84 flanking coding sequence as a BamHI-to-EcoRV fragment cloned into pUC18 devoid of a KpnI restriction site in the polylinker sequence (55). The GP83 locus plasmid was digested at unique sites in GP83 (KpnI [position 132990] and BglII [position 131551]) to remove the majority of the GP83 coding sequence (codons 7 to 485 of 566) in the shuttle vector, and in its place, a kanamycin (Km) PCR cassette with flanking KpnI (5') and BamHI (3') restriction sites was inserted into the shuttle vector as previously described (26). This generated the GP83 knockout shuttle vector pGP83dKm (Fig. 1C), which was used in targeted recombination of the GPCMV BAC to generate a GP83 knockout mutant (see next section).

Generation of gene mutant GPCMV BACs and analysis and generation of virus. An inducible ET recombination system (Gene Bridges) was introduced into DH10B bacterial cells containing a GPCMV BAC plasmid (77, 107). Mutagenesis of the GPCMV BAC was performed using linearized shuttle vectors encoding a kanamycin (Km) marker, as previously described (108). Isolated mutant GPCMV BAC colonies were characterized by separate EcoRI and HindIII restriction digestions of BAC DNA to verify the accuracy of the predicted genome profile configuration after mutation (77, 107). Insertion of the Km drug resistance cassette into the viral genome introduced a novel HindIII restriction enzyme site at the site of mutation to enable verification of locus modification. The GP83 mutation retained the 3' end of the GP83 gene to ensure normal expression of GP82, which is expressed as a bicistronic mRNA with GP83 similar to HCMV UL82 and UL83 (60, 74) (Fig. 1). The GP83 knockout was generated in the backdrop of a retrofitted second-generation GPCMV BAC which expressed a missing full-length GP129 (UL128 homolog) in an ectopic locus to enable expression of a complete homolog pentameric complex (gH/gL/GP129/GP131/GP133). Studies on the GPCMV homolog pentameric complex protein interactions and restored epithelial tropism in virus via ectopic expression of a missing full-length GP129 are described in another paper from our laboratory (27). The GP83 mutant GPCMV BAC was characterized by restriction profile analysis (Fig. 1) and comparative PCR of the GP83 locus (data not shown), which was additionally sequenced to verify correct modification of the GP83 gene. The insertion of the kanamycin cassette into the GP83 locus introduced a novel HindIII site in the genomic HindIII "A" fragment (positions 102380 to 146446). This generated two novel HindIII fragments of 29.9 kb and 13.7 kb (Fig. 1D). In Fig. 1D, novel fragments are indicated with a red dot (lane 1), and the original fragment is indicated by a yellow dot (lane 2). The second-generation BAC encoded a truncated GP129 mutant and was modified to encode a full-length GP129 cDNA under SV40 promoter control by insertion of a cassette into the GP25-GP26 intergenic locus of the GPCMV genome, as previously described (27). Modification of the GP25-GP26 locus introduced a novel HindIII site (within the SV40 promoter) in this locus, which altered the wild-type HindIII restriction fragment from 17.1 kb (lane 3, yellow dot) to 6.6 and 11.5 kb (lane 2, red and blue dots, respectively), as previously described (27). Specific gene modifications were confirmed by comparative PCR analysis between wild-type and mutant GPCMV BACs by using common flanking primers for each gene and DNA sequencing of the modified locus (27). The GP83 locus was amplified with primers F409 (5' CATCAAGATGGTCAACAGTGCACGAC) and R409 (5' TGTCGTAGAGCACTTCGAACCTGACTCTG).

Generation of recombinant virus. For generation of recombinant viruses, large-scale GPCMV BAC DNA was purified from *Escherichia coli* strain DH10B by using a maxi plasmid kit (Qiagen). BAC DNA was transfected onto GPL cells in six-well dishes using Lipofectamine 2000 (Invitrogen), as previously described (75). The BAC plasmid was also excised from the viral genome via the Cre-LoxP recombinase strategy (107). Two independent GP83 mutant GPCMV BAC clones were separately transfected onto GPL cells to generate virus and excise the BAC plasmid from the genome. A virus stock was generated based on one clone, and mutant virus was designated GP83dPC+. Unique components of the PC, GP129 and GP133, were verified by Western blotting for myc-tagged ectopic GP129 or immunohistochemical staining for GP133 with custom mouse monoclonal antibody anti-GP133 (GenScript) (27). GP133 was detected by immunohistochemical staining of virus-infected monolayers in accordance with a previously described protocol (29) with a mouse detection kit (Vector Laboratories). Western blot analysis of infected cell lysate with anti-myc (Novus) confirmed expression of GP129myc-tagged protein, which confirmed the ability of the virus to generate a pentameric complex (Fig. 1E) (27). Generation of GP83 rescue virus (GP83R) was by cotransfection onto renal epithelial cells of GP83dPC+ GPCMV BAC DNA with a wild-type GPCMV subgenomic DNA fragment encoding the GP82-GP84 locus and purification of rescue virus (GP83R) by limiting dilution. BAC plasmid insertion into the genome was removed by Cre-Lox recombination as previously described. Rescue virus was subsequently characterized by PCR sequencing of the GP83 locus to verify restoration of the GP83 coding sequence.

Ethics. Guinea pig (Hartley) animal studies were carried out under IACUC (Texas A&M University). All study procedures were carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. Animals were observed daily by trained animal care staff, and animals that required care were referred to the attending veterinarian for immediate care or euthanasia. Terminal euthanasia was carried out by lethal CO₂ overdose, followed by

cervical dislocation, in accordance with IACUC protocol and NIH guidelines. Animals purchased from Charles River Laboratories were verified as seronegative for GPCMV by toenail clip bleed and anti-GPCMV ELISA of sera, as previously described (26). Animal studies were carried out to determine (i) the pathogenicity of the GP83 knockout mutant in seronegative animals compared to wild-type virus, (ii) the immune response to the AdGP83 vaccine, and (iii) the level of protection provided by the AdGP83 vaccine against wild-type GPCMV challenge (22122 or TAMYC strain) compared to the no-vaccine control.

GPCMV pathogenicity and GPCMV vaccine protection studies. (i) Pathogenicity study. GP83dPC+ virus was evaluated for pathogenicity in the animal model in comparison to the wild-type virus. Pathogenicity studies were carried out as previously described (27). GPCMV-seronegative animals were randomly assigned to 3 groups of 12 per group. At day 0, animals were injected subcutaneously (s.c.) with 10^5 PFU of GP83dPC+, wild-type virus, or rescue virus. At 4, 8, 12, and 27 days postinfection (dpi), three animals per group were euthanized, and viral loads in target organs (liver, lung spleen) and blood were evaluated by real-time PCR assay. Additionally, at 27 dpi, salivary gland tissue was evaluated for viral load.

(ii) Vaccine protection study. Two groups of seronegative female guinea pigs ($n = 12/\text{group}$) were vaccinated subcutaneously with AdGP83 by following the same protocol recently described for AdgB (30). Animals received three sequential inoculations of AdGP83 strain, with each vaccination separated by 4-week intervals. At 4 weeks after the last vaccination, animals were challenged with wild-type GPCMV (10^5 PFU s.c.), challenge strain 22122, or TAMYC (dependent upon assigned group) and evaluated for wild-type virus spread. At 4, 8, 12, and 27 dpi, three animals per group were euthanized and viral loads in target organs (liver, lung, spleen, and blood) were evaluated by real-time PCR assay. Additionally, at 27 dpi, salivary gland tissue was evaluated for viral load. No-vaccine control groups (22122 and TAMYC) were similarly challenged with wild-type GPCMV and evaluated for viral load at the same time points postinfection. An additional 3 vaccinated animals not challenged with wild-type virus were used to evaluate the cell-mediated response in animals for GP83 as described in "Guinea pig IFN- γ ELISPOT assay."

Real-time PCR assay. Tissues were collected from euthanized guinea pigs to determine the viral load. For tissue DNA extraction, FastPrep 24 (MP Biomedical) was used to homogenize tissues as a 20% (wt/vol) homogenate in lysing matrix D (MP Biomedicals). DNA was extracted using a QIAxtractor (Qiagen) according to the manufacturer's tissue protocol instructions. Viral load was determined by real-time PCR on a LightCycler 480 instrument (Roche Applied Science). Primers and the hydrolysis probe were designed using the LightCycler Probe Design2 program to amplify a product from the GPCMV GP44 gene: forward primer, 5'TCTCCACGGTGAAGAGTTGT; reverse primer, 5'GTGCTGTCGGACCACGATA; hydrolysis probe, 5'FAM-TCTTGCTCTGCAGGTGGACGA-BHQ1. PCR master mix contained LightCycler probes master (Roche Life Science), 0.4 μM primers, 0.1 μM probe, and 0.4 U uracil N-glycosylase (UNG) in a 25- μl total reaction volume, including 10 μl of DNA per reaction volume. Standard controls and no-template controls (NTC) were run with each assay for quantification. LightCycler 480 amplification parameters were as follows: UNG step for 10 min at 40°C, followed by activation at 95°C for 10 min and then 45 cycles of denaturation at 95°C for 15 s, annealing at 56°C for 15 s, and elongation at 72°C for 10 s. Data were collected by "single" acquisition during the extension step. Data were analyzed with the LightCycler Data Analysis software (version 1.5.1; Roche). A standard curve was generated using serial dilutions of GPCMV GP44 plasmid (75) at known concentrations for quantification and assay sensitivity. The sensitivity of the assay was determined to be 5 copies/reaction. Viral load was expressed as copy number/mg tissue. Results calculated were a mean value of the results of triplicate PCR runs per sample.

ELISA, Western blot analysis, and immunoprecipitation assays. Anti-GPCMV ELISA was carried out as previously described (26). The net optical density (OD) (absorbance at 450 nm) of test sera was attained by subtracting the OD of uninfected cell preparations (Ag-) from that of GPCMV-positive cell preparations (Ag+). ELISA reactivity was considered positive if the net OD was greater than or equal to 0.2, as determined by GPCMV-negative serum. Western blot analyses were carried out on cell lysates separated by 10% SDS-PAGE and performed as previously described (26, 27). Immunoprecipitation (IP) assays were carried out using a GFP-Trap (ChromoTek) in accordance with the manufacturer's protocols, with inclusion of protease inhibitor cocktail (Pierce) in cell lysates, as previously described (27). For Western blot analyses, the following anti-epitope tag primary antibodies were used at 1/1,000: mouse anti-FLAG M2 (Sigma) and rabbit anti-GFP (Santa Cruz) (27, 33). Anti-mouse and anti-rabbit IgG horseradish peroxidase (HRP)-linked secondary antibodies for Western blotting (Cell Signaling) were used at 1/2,000 (33, 75). Rabbit anti-GPCMV IE2 was previously described (56).

Guinea pig IFN- γ ELISPOT assay. The guinea pig IFN- γ enzyme-linked immunospot (ELISPOT) assays were performed by a previously described protocol using freshly isolated splenocytes and GPCMV GP83 peptide pools (28). Anti-guinea pig IFN- γ monoclonal antibodies (V-E4 and N-G3) were previously characterized (109). Briefly, polyvinylidene difluoride (PVDF) membrane 96-well plates were coated with a guinea pig IFN- γ capture antibody, V-E4, incubated overnight at 4°C, and blocked, and then freshly isolated splenocytes were added before being exposed to GP83 GPCMV 9mer peptide pools and incubated for 18 h. A biotinylated detection antibody, N-G3, was added before streptavidin-AP conjugate (R&D Systems) and then detected with BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium) (Life Technologies). Membranes were dried before spots were counted on an ImmunoSpot S6 system (CTL). Final counts were calculated based on the number of spot-forming cells (SFC) per 10^6 cells after background spots (cells only without any stimulation) were subtracted. Concanavalin A (ConA; 10 $\mu\text{g}/\text{ml}$) was used as a positive control, and other controls included cells-only control, dimethyl

sulfoxide (DMSO) control (peptide background), and nonspecific peptide control and medium-only control. Reactive peptide pools are listed in Table S1 in the supplemental material.

GPCMV major immediate early promoter firefly luciferase reporter gene protein expression luminescence assay. A guinea pig GPCMVME luciferase reporter (Luc) fibroblast cell line was previously generated by permanent transfection of GPL fibroblast cells with the Sleeping Beauty transposon (pGPCMVME Luc) encoding a GPCMV major immediate early promoter driving a firefly luciferase reporter gene cassette with a 3' SV40 poly(A) sequence (18) to generate the GPCMVME Luc GPL cell line, which was maintained as described for GPL cells. Confluent monolayers of GPCMVME Luc GPL cells in triplicate wells in 96-well plates were transduced with recombinant defective Ad vectors AdGP83, AdIFI16, AdGP83+IFI16, or AdgL (control). Additional control wells were non-Ad-transduced wells of GPCMVME Luc GPL cells or normal GPL cells not expressing luciferase. Luciferase activities were evaluated by a luminescence assay at 48 h after Ad vector transduction by using a firefly luciferase luminescence assay kit (Biotium, Inc.) in accordance with the manufacturer's protocol, using a Cytation 5 imaging reader (BioTek instruments) to measure luminescence (in relative luminescence units [RLU]). Assays were carried out in triplicate and repeated 3 times.

Statistical analysis. All statistical analyses were conducted with GraphPad Prism (version 7) software. Replicate means were analyzed using one-way analysis of variance or Student's *t* test (unpaired), with significance taken as a *P* value of <0.05 or as specified in the figure legends.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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