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Guinea pig cytomegalovirus GP84 is a functional homolog of the human cytomegalovirus (HCMV) UL84 gene that can complement for the loss of UL84 in a chimeric HCMV

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Abstract

The guinea pig cytomegalovirus (GPCMV) co-linear gene and potential functional homolog of HCMV UL84 (GP84) was investigated. The GP84 gene had delayed early transcription kinetics and transient expression studies of GP84 protein (pGP84) demonstrated that it targeted the nucleus and co-localized with the viral DNA polymerase accessory protein as described for HCMV pUL84. Additionally, pGP84 exhibited a transdominant inhibitory effect on viral growth as described for HCMV. The inhibitory domain could be localized to a minimal peptide sequence of 99 aa. Knockout of GP84 generated virus with greatly impaired growth kinetics. Lastly, the GP84 ORF was capable of complementing for the loss of the UL84 coding sequence in a chimeric HCMV. Based on this research and previous studies we conclude that GPCMV is similar to HCMV by encoding single copy co-linear functional homologs of HCMV UL82 (pp71), UL83 (pp65) and UL84 genes.

Introduction

Human cytomegalovirus (HCMV) is a ubiquitous pathogen that causes asymptomatic infection in normal healthy individuals. However, it has emerged as a serious infection in immune suppressed individuals, including organ transplant and AIDS patients as well as the fetus during pregnancy (Pass, 2001). Indeed, congenital infection of the fetus by HCMV (1–2% of live births in the US) is a major cause of mental retardation and hearing loss in surviving newborns in the developed world (Ross & Boppana, 2004; Griffiths & Walter, 2005). Although there are candidate vaccines in clinical trials there is currently no effective vaccine to HCMV (Schleiss & Heineman, 2006). Antivirals are available but these act at late stages of the virus life cycle and result in the development of resistant strains emerging during prolonged antiviral therapy (Biron, 2006; Chou, 2008; McGregor 2010). Consequently, there is a need for the development of new vaccine strategies as well as the development of novel antiviral strategies that act at an earlier stage of the virus life cycle and less likely to lead to the development of resistant strains of virus.

In human cytomegalovirus (HCMV) the UL82, UL83 and UL84 genes are thought to have evolved from a common ancestor by gene duplication, with subsequent differentiation to

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fulfill unique functions associated with the virus life cycle (Davison & Stow, 2005). Both UL82 and UL83 encode tegument proteins whereas UL84 encodes a non-structural protein (He et al., 1992; Hensel *et al.*, 1992; Schmolke *et al.*, 1995). Both tegument proteins, pp71 (UL82) and pp65 (UL83), have roles in initiating viral life cycle in the infected cell as well as avoiding the cellular innate immune response (Abate *et al.*, 2004; Baldick *et al.*, 1997; Cantrel *et al.*, 2006). The function of UL84 protein in the HCMV viral life cycle is not entirely understood but it has an essential role insofar as knockout HCMV UL84 mutants are non viable (Dunn *et al.*, 2003; Xu *et al.*, 2004; Yu *et al.*, 2003). The UL84 proteins are unique to betaherpesviruses and in HCMV it is required in an auxiliary role for HCMV DNA replication (Sarisky & Hayward, 1996; Xu *et al.*, 2004). Specific interaction of pUL84 with the DNA polymerase accessory protein (pUL44) has been demonstrated (Strang *et al.*, 2009). However, Reid *et al.*, (2003) using HSV-1 DNA replication fork proteins showed that replication from the HCMV origin site is independent of UL84 protein. Initially identified as a nuclear protein, pUL84 can shuttle between the nucleus and the cytoplasm and multimerize with itself (Colletti *et al.*, 2004; He *et al.*, 1992; Lischka *et al.*, 2003; Lischka *et al.*, 2006; Xu *et al.*, 2002). The purpose of pUL84 shuttling has yet to be defined but involves the alpha beta importin pathway (Lischka *et al.*, 2003). Potentially, shuttling may be linked to pUL84 homology with DExD/H box family of helicases, which shuttle between the nucleus and cytoplasm (Colletti *et al.*, 2005; Lischka *et al.*, 2006). The viral protein is also suggested to have UTPase activity (Colletti *et al.*, 2005) and other pUL84 homologs, including GPCMV, carry UTPase motifs (Davison & Stow, 2005). Additionally, pUL84 exhibits a transdominant inhibitory growth effect on HCMV and interaction studies determined that the inhibitory effect is linked to specific interaction with IE2-p86 that impairs transactivation (Gebert *et al.*, 1997; Colletti *et al.*, 2004). Since IE2-p86 is important for the initiation of HCMV viral replication, this transdominant inhibitory effect could potentially be developed as a novel antiviral strategy, which would impact on virus replication at an earlier stage than current antivirals. Therefore in addition to identifying GPCMV GP84 as a functional homolog we sought to identify a minimal peptide domain with a transdominant inhibitory effect on GPCMV growth for potential development as an antiviral in the guinea pig model.

The strict species specificity of HCMV precludes studying this virus in a non-human animal model. Consequently, animal CMVs are used in their respective hosts for viral pathogenicity or candidate vaccine studies. Various animal models have been explored in the study of CMV (Schleiss, 2006; Barry *et al.*, 2006). The guinea pig model of CMV is uniquely useful for the study of congenital infection and the development of intervention strategies that prevent infection of the fetus (Kumar & Nankervis, 1978; Kern, 2006; Schleiss, 2002). One focus of vaccines against HCMV has been directed towards the pp65 viral tegument protein (UL83), the major CTL target (Gonczol & Plotkin, 2001). The homolog protein, GP83, in GPCMV has been successfully used as protective vaccine in the guinea pig model (McGregor *et al.*, 2004; Schleiss *et al.*, 2007). In contrast, the M84 gene in murine cytomegalovirus (MCMV), while co-linear to UL84, encodes a functional homolog to pp65 (UL83) and is a major CTL target (Ye *et al.*, 2004). Accordingly, M84 vaccines provide protection against MCMV challenge in mice (Holtappels *et al.*, 2001; Morello *et al.*, 2000). Hence, from the standpoint of vaccine development studies in the guinea pig model it was important to more properly define the function of the co-linear homolog to UL84, GP84, in the GPCMV life cycle. We previously identified proteins encoded by the GPCMV genes GP82 and GP83 as tegument proteins and direct functional homologs of HCMV encoded proteins pp71 (UL82) and pp65 (UL83) (McGregor *et al.*, 2004; Schleiss *et al.*, 1999). Preliminary studies suggest that the predicted pGP84 is potentially a functional homolog of pUL84 based on the presence of conserved UTPase motifs not found in the MCMV homolog (Davison & Stow, 2005). Therefore in this paper we attempted to demonstrate that pGP84 is a functional homolog pUL84.

Results

GP84 expression and cellular location of pGP84 protein

The GP84 ORF encodes a protein of 483 aa. in length that exhibits the highest homology to HCMV UL84 protein (22% identity by BLAST analysis). Analysis of the kinetics of gene expression in wild type virus infected cells reveals the GP84 gene expression to be delayed early kinetics (Figure 1), which is similar to HCMV UL84 (He *et al.*, 1992). This conclusion is based on comparative RT-PCR assays also performed for different classes of GPCMV genes previously characterized (Figure 1): immediate early, IE2, unique exon GP122 (Yamada *et al.*, 2009); early, GP54, the viral polymerase (Schleiss, 1995); late, GP83, encoding the homolog to pp65 (Schleiss *et al.*, 1999). Additionally, the class of kinetics was also confirmed by the inclusion of protein inhibitor cycloheximide (for IE transcripts) or DNA replication inhibitor phosphonoacetic acid, PAA (for detection of IE and E transcripts) in virus infected cultures. GP84 transcripts were detected in the presence of PAA but not in the presence of cycloheximide which confirmed GP84 as an E transcript, see supplementary Figure S1.

The full length GP84 ORF was cloned in frame into the expression vector pCMV2A (Promega), which tagged the N-terminal domain of the ORF with an epitope FLAG tag. Transient expression studies of pGP84 were carried out on GPL cells. The cellular distribution of pGP84 was determined at various times between 3 and 48 hr post transfection via immunofluorescence assay. Figure 1(ii), image A, indicates that pGP84 is a nuclear protein and this result is consistent with the cellular location reported for HCMV pUL84 (Xu *et al.*, 2002). Using convenient restriction enzyme sites a series of collapses were made to the full length GP84 ORF to generate N- and C-terminal deletions as described in materials and methods (and shown in Supplementary Figure S2). In transient cellular expression assays, the N-terminal collapsed pGP84 (lacking the first 211 aa of full length pGP84) encoded on plasmid pFLAGGP84(B) appeared only in the cytoplasm and not in the nucleus (Figure 1(ii), image C). In contrast, the C-terminal and combined N- and C-terminal truncated versions of pGP84 (encoded on plasmids pFLAGGP84(S) and pFLAGGP84(B) respectively) were found in both nuclear and cytoplasmic compartments (Supplementary Figure S3). This indicated that pGP84 carries a N-terminal NLS as described for HCMV pUL84 (Xu *et al.*, 2002). However, further study would be required to determine if pGP84 is dependent upon the alpha and beta importin pathway for nuclear targeting as described for HCMV (Lischka *et al.*, 2006).

pGP84 co-localizes with the viral DNA polymerase accessory protein (pGP44)

HCMV pUL84 plays an accessory role in viral DNA replication and has recently been shown to interact with the viral DNA polymerase accessory protein, pUL44, (Strang *et al.*, 2009). The UL44 homolog gene (GP44) in GPCMV (Schleiss *et al.*, 2008) encodes a protein of 407 amino acids that exhibits 57% identity with HCMV pUL44 by BLAST analysis (McGregor, unpublished data). In an effort to demonstrate similar interactions between GPCMV homolog proteins (pGP84 and pGP44) the GP44 coding sequence was cloned in frame into a FLAG tag expression plasmid to epitope tag the GP44 ORF. Transient expression studies were carried out in the presence of GFP tagged pGP84 expression plasmid or control GFP plasmids (expressing GFP only or a HCMV pp65 GFP fusion). Interactions were followed by cellular co-localization studies via immunofluorescence assay. Figure 2, panels A–F, show the co-localization of pGP84 and pGP44 in the nucleus of cells expressing both proteins. Identical patterns of nuclear aggregates were observed under GFP filter for pGP84 (panels A and D) or Texas red filter for pGP44 (panels B and E). Co-expression of pGP44 and GFP protein did not indicate any specific co-localization (Figure 2, panels J and K). The GFP protein was seen evenly distributed in both nuclear and

cytoplasmic compartments, whereas pGP44 was only located in the nucleus and was uniform in the nucleus without aggregates seen in the presence of pGP84. In another control experiment, co-expression of pGP44 with HCMV pp65-GFP fusion protein did not give rise to pGP44 nuclear aggregates (Figure 2, panels G and H). The pp65 protein produced characteristic punctate nuclear fluorescence but the pGP44 protein was evenly distributed in the nucleus. The expression pattern of pGP44 only (Figure 2, panel M) is the same pattern seen in GFP and GFP-pp65 co-transfection cells (K and H respectively). Overall the transient GP84 expression studies suggest that the pGP84 has similar cell localization properties to pUL84 and potentially interacts with the viral DNA replication machinery as demonstrated for HCMV pUL84 (Strang *et al.*, 2009; Sarisky & Hayward, 1996).

Transdominant inhibitory effect of overexpression of GP84 on GPCMV growth

Gerbert *et al.*, (1997) demonstrated that over expression of pUL84 in a human cell line resulted in subsequent impairment of HCMV growth, indicating that pUL84 has a transdominant inhibitory effect on HCMV when over expressed under HCMV IE promoter control. The specific interaction of pUL84 with the immediate early transactivation protein IE2 is the basis for the inhibitory effect (Gerbert *et al.*, 1997). A GPCMV homolog to IE2 has recently been identified with the encoded protein sequence in the unique GPCMV IE2 exon (GP122) showing 62% identity to HCMV pUL122 (McGregor, unpublished data; Schleiss *et al.*, 2008; Yamada *et al.*, 2009). Therefore it seemed probable that a similar transdominant inhibitory effect for GPCMV could be demonstrated. Two transdominant inhibitory GPCMV growth assays were developed. In the first assay, infectious GPCMV BAC DNA (McGregor & Schleiss, 2001) was co-transfected onto confluent monolayers of GPL cells in the presence or absence of GP84 expression plasmids or control plasmids. Virus development was allowed to proceed for a similar length of time and final viral titers from individual wells determined at the end of the experiment (day 18). During the experiment GFP positive transfected cells, GPCMV replication and viral spread could easily be followed in real time by virtue of the GFP gene incorporated into the GPCMV genome. Transfection of the GPCMV BAC DNA by itself or with the empty expression vector resulted in rapid generation of viral plaques and GFP viral spread across the monolayer (Figure 3, panels A and C). The presence of the empty expression vector (pCMV2A) did not affect the development of viral spread and only slightly impaired viral yield, 1.5×10^6 pfu/ml in the absence of pCMV2A (empty vector) compared to 1.4×10^6 pfu/ml in the presence of pCMV2A. In contrast, co-transfection of GPCMV BAC DNA with the GP84 expression vector (pFLAGGP84) severely impaired development of virus and viral spread across the monolayer (Figure 3, panel B) with an average virus yield of 1×10^2 pfu/ml. N-terminal or C-terminal truncated versions of GP84 were equally as effective at inhibiting GPCMV virus growth (average virus yield 1×10^2 pfu/ml). Surprisingly, the double truncated N- and C-terminal GP84 collapse that encodes 99 aa (codons 212–311) of pGP84 sequence (pFLAGGP84BS) was equally capable of inhibiting GPCMV (average virus yield 1×10^2 pfu/ml) as full length pGP84 (pFLAGGP84), Figure 3 (image B and D). The use of VP16 (HSV-1) or GP83 (GPCMV pp65 homolog) expression plasmids (McGregor *et al.*, 2004) produced similar results to GPCMV BAC co-transfection with empty vector (1.2×10^6 pfu/ml and 1.3×10^6 pfu/ml respectively) and had no effect on virus growth. These results indicate that pGP84 has a trans-dominant inhibitory activity against GPCMV similar to that described for pUL84 against HCMV (Gerbert *et al.*, 1997). Overall the pGP84 transdominant inhibitory assay impaired virus yield by approximately 10,000 fold. However, only a small 99 aa domain (codons 212–311) would appear to be sufficient for inhibitory growth effect. A similar minimal inhibitory domain has not been identified for pUL84.

The GPCMV BAC based transdominant inhibitory assay did not demonstrate a direct inhibitory effect on live virus infection as virus was derived from GPCMV BAC transfected

cells. Consequently, a second inhibitory growth assay was developed that tested the ability of pGP84 to impair live GPCMV infection of GPL cells. Initially GPL cell lines were established that expressed the pGP84 minimal inhibitory domain fused to the C-terminal domain of GFP (pEGFP-C1) as described in materials and methods. As a control, GPL cell lines were established that only expressed GFP. Clonal cell lines were designated GPL-GFPGP84ID and GPL-GFP respectively and were tested for their ability to inhibit virus growth at different input virus moi. The pGP84 inhibitory effect on virus growth was most pronounced at the lowest moi (0.001–0.01 pfu/cell) with a 3 log drop in virus yield in comparison to the equivalent infection on control GPL–GFP cells (Table 1). The results demonstrate that the minimal pGP84 inhibitory domain when expressed in trans was effective at inhibiting live virus infection and verifies the results seen in the initial inhibitory assay. The pGP84 inhibitory effect was specific to GPCMV as no viral inhibition was seen with HSV-1 (data not shown). In HCMV the transdominant effect is thought to occur by pUL84 interacting with IE2-p86 to inhibit transactivation of early genes by IE2 protein (Gerbert *et al.*, 1997). Although a homolog to IE2 protein has recently been identified in GPCMV (Schleiss *et al.*, 2008; Yamada *et al.*, 2009) further studies are required to characterize the IE2 transactivation function as well as IE2 protein-protein interactions.

Targeted knockout of GP84 in GPCMV

In HCMV the UL84 gene is essential for virus replication (Dunn *et al.*, 2003; Xu *et al.*, 2004; Yu *et al.*, 2003) we sought to determine the essential nature of GP84 by specific gene knockout in an infectious GPCMV BAC (McGregor *et al.*, 2001). An insertion deletion was introduced into the GP84 ORF by the insertion of a kanamycin cassette between two *Bam*H I sites close to the start of the GP84 ORF in a GP84 shuttle vector for targeted knockout of GP84. Figure 4(i), shows the location of the GP84 gene knockout on the GPCMV genome. Full length mutant GPCMV BACs were screened by *Hind*III restriction profile analysis with the correct insertion of the Km cassette resulting in the disruption of the GPCMV *Hind* III 'A' band, co-ordinates 103,313–147,381 bp, (Schleiss *et al.*, 2008; Gao and Isom *et al.*, 1984). Figure 4(ii) shows the modified restriction pattern of the GP84 GPCMV BAC mutant under *Hind*III restriction enzyme profile analysis. The modified GP84 locus was further verified by PCR analysis using common PCR primers flanking the modified GP84 locus. PCR analysis of wild type and GP84 mutant GPCMV BAC DNA verified the GP84 locus was correctly modified by insertion of a Km cassette and small deletion to the GP84 coding sequence, see Figure 4(iii). Transfection of the GP84 mutant GPCMV BAC onto GPL cells resulted in the generation of infectious virus, designated vGP84d. PCR analysis of viral DNA confirmed that the mutant GP84 locus was retained in the virus unless rescued back to wild type, see Figure 4(iv). A multi-step growth curve of vGP84d, Figure 4(iv), demonstrated that vGP84d had impaired growth kinetics in comparison to wild type virus, or rescue virus (GP84R). Additionally, the mutant produced greatly enlarged cells in comparison to wild type virus (data not shown). The ability to generate a viable GP84 knockout virus contrasts with an absolute requirement for UL84 to generate viable HCMV (Dunn *et al.*, 2003; Yu *et al.*, 2003; Xu *et al.*, 2002). However, there is a greater level of impairment associated with a GP84 knockout in comparison to knockout of the co-linear UL84 gene homolog in MCMV (M84), which does not exhibit any impaired growth kinetics in tissue culture (Morello *et al.*, 1999).

Substitution of GP84 in place of UL84 in a chimeric HCMV

In an ultimate test of similarity of function between UL84 and GP84 we sought to determine if the GP84 coding sequence could functionally replace the UL84 coding sequence in a chimeric HCMV. Mutant HCMV were generated using a Towne strain HCMV BAC (Hahn *et al.*, 2003). Two types of UL84 HCMV mutants were generated: a UL84 deletion mutant (HCMV::UL84d); a GP84 chimeric HCMV, encoding GP84 in place of UL84

(HCMV::GP84). A shuttle vector was generated for the UL84 locus for targeted modifications as described in materials and methods and is summarized in Figure 5(i). For generation of the chimeric HCMV, the FLAG tagged GP84 ORF was cloned into the pUL84d shuttle vector to generate pUL84d/GP84, which placed the GP84 ORF under UL84 promoter control. In separate recombination reactions the linearised plasmid DNA (pUL84d or pUL84d/GP84) was electroporated into recombination induced DH10B bacteria carrying the HCMV (Towne strain) BAC. Full length HCMV mutants (HCMV::UL84d or HCMV::GP84 BACs) were isolated and screened by *Hind*III restriction digestion to verify modification of the 7.8 kb subgenomic *Hind*III fragment (HCMV Towne co-ordinate nucleotides 120,817 – 128,615) predicted to encode UL84. Figure 5(ii)A shows the modifications to the UL84 locus, which were a result of the introduction of a novel *Hind*III site present in the Km cassette. The deletion mutant removes the majority of the UL84 coding sequence (codons 59-401) but introduces a Km cassette. The chimera removes the majority of the UL84 coding sequence and introduces the GP84 ORF and a Km cassette. The modifications to the UL84 locus were further verified by diagnostic PCR using common primers flanking the modified UL84 locus which confirmed the specific modifications to the UL84 locus, see Figure 5(ii)B.

Two independent HCMV BAC clones for each mutant were transfected separately onto HFF cells to generate virus. Despite repeated transfections of the UL84 deletion knockout HCMV BAC DNA (HCMV::UL84d) no virus was generated. This confirmed the essential nature of the UL84 gene. The lethal UL84 knockout could be rescued by co-transfection of the HCMV::UL84d BAC mutant with a rescue PCR product (3.7 kb fragment) encoding the wild type UL83-UL85 locus to generate virus (see material and methods). The chimeric HCMV BAC encoding GP84 (HCMV::GP84) generated viable virus, designated HCMV::GP84. A one step growth curve of HCMV::GP84 indicated that the chimera grew with similar kinetics to wild type HCMV (Towne) on HFF cells, see Figure 5(iii). Expression of FLAG tagged GP84 protein in chimeric HCMV infected HFF cells was confirmed by Western blot of infected cell lysate which detected a band of approximately 70 kDa, the predicted size of the GP84 protein, see Figure 5(iv). This data suggests that GP84 can functionally replace the loss of UL84 in a chimeric HCMV and provides the strongest evidence that GP84 is a functional homolog of UL84.

Discussion

In conclusion our studies on GP84 protein demonstrate that it is most likely the functional homolog of pUL84 in GPCMV as it shares a number of common features with pUL84. Furthermore, the GP84 ORF can substitute for the loss of the essential UL84 coding sequence in a chimeric HCMV. Further, studies are required to characterize the pGP84 NLS and the interactions of pGP84 with the GPCMV IE2 homolog. Preliminary studies indicate that the pGP84 NLS cannot be easily transplanted to another protein by the simple grafting of only the N-terminal domain to a reporter gene (McGregor, unpublished data). This suggests that the pGP84 carries a more complex bipartite NLS as described for HCMV pUL84 (Lischka et al., 2006). Analysis of the GPCMV IE2 unique exon GP122 indicates that it has high homology with HCMV UL122 exon, which suggests that GPCMV IE2 may function in a similar manner to HCMV IE2. However, no transactivation studies have been performed with the full length IE2 protein. Previous success has been achieved for analysis of transactivation activity associated with the GPCMV pp71 homolog, encoded in GP82 (McGregor *et al.*, 2004). Additionally, the GPCMV genome has recently been fully sequenced (Schleiss *et al.*, 2008; Yamada *et al.*, 2009), which should allow the development of suitable IE2 transactivation/pGP84 interference assays. Since pUL84 is linked to virus replication it is curious that a MCMV M84 mutant is not impaired in tissue culture. This result certainly confuses the issue of MCMV being the primary animal model for

pathogenicity, vaccine strategies against CMV. However, the GPCMV model is not a perfect substitute. The GP84 knockout mutant was capable of generating viable virus despite it being impaired. Possibly rodent CMV have evolved a DNA replication strategy that is more dependent upon a cellular protein rather than the viral pUL84 homolog. Reid *et al.*, (2003) have demonstrated HCMV origin dependent DNA replication in the absence of pUL84, which supports an alternative DNA replication strategy not dependent on pUL84. This area awaits further investigation. The transient expression protein localization studies were performed with FLAG epitope and GFP tagged ORFs. This is a common strategy to study protein cellular localization in the absence of specific antibodies to the respective protein(s). Similar results were obtained for the same protein (pGP84 and pGP44) under both GFP or FLAG tagging and it is therefore unlikely that these tags contributed to cellular localization patterns. However, ideally future pGP84 studies should additionally employ protein specific antibodies as well as the continued use of FLAG or GFP tagged constructs.

In HCMV, the UL83 protein, pp65, is the protective T cells target and not pUL84 (Wills *et al.*, 1996). In MCMV the co-linear homolog gene to UL84, M84, encodes the protective T cell target antigen but not the UL83 homolog, M83 (Morello *et al.*, 1999; Ye *et al.*, 2004). It was important to define GP84 as a functional homolog of UL84 as this has direct implications for the development of CMV candidate vaccines in the guinea model. Our results demonstrate the potential problem associated with the study of species specific animal CMV in vaccine studies and necessitates the functional definition of homolog proteins in addition to their use as candidate vaccine targets. In earlier studies we had identified pGP83 as encoding a tegument protein with properties similar to HCMV pp65 that acted as a protective T cell target antigen (Schleiss *et al.*, 1999; McGregor *et al.*, 2004; Schleiss *et al.*, 2007). The immune response to pGP84 is unknown but does not seem to be as dominant as the T cell response to pGP83 in wild type virus infected animals (Schleiss *et al.*, 2007). Therefore pGP84 is not likely to be a good vaccine target antigen as is the case for HCMV pUL84. Presumably, identification of pGP84 as a functional homolog to pUL84 will allow the future study of the role of pUL84 homolog in an animal model or the development of novel pUL84 based antiviral strategies in the guinea pig model.

Antivirals against HCMV are an important concern due to the development of resistant strains under current antiviral therapies (Biron *et al.*, 2006). The finding that pGP84 has a transdominant inhibitory effect on viral growth, as does pUL84 on HCMV, is of potential interest from the standpoint of development of a novel antiviral drug strategy that could be tested in the guinea pig animal model. The ability to minimize the pGP84 inhibitory domain to 99 aa, or perhaps even smaller, could allow the future development of novel compounds that mimic this domain and which are also inhibitory towards the virus (Robinson *et al.*, 2008). Potentially, an antiviral that inhibits IE2 protein could be more successful in combating CMV infection rather than current antivirals that inhibit late stages of the virus life cycle (Biron, 2006; McGregor, 2010) and allow emergence of resistant strains. Antiviral peptide strategies have been demonstrated to be effective against herpesvirus infections (Loregian *et al.*, 2005). However, the further development an antiviral strategy in GPCMV awaits characterization of pGP84/IE2 homolog protein interactions. Stamminger and colleagues recently tested the ability of a peptide aptamer antiviral that targets the NLS of pUL84 (Kaiser *et al.*, 2009). This could also be investigated in GPCMV but would first require further characterization of the pGP84 NLS.

Previously, in our attempts to increase the relevance of the GPCMV antiviral model we successfully generated a chimeric GPCMV encoding HCMV UL97 in place of the homolog gene GP97. This chimeric virus grew with wild type kinetics and was viable in vivo (McGregor *et al.*, 2008; 2010). The generation of a viable HCMV chimera encoding GP84 in place of the essential UL84 is a reverse approach that also demonstrates similarity of gene

function between GPCMV and HCMV. In conclusion, our findings on GPCMV GP84 and our previous studies on GP83 indicate a divergence of functions between co-linear homologs to UL83 and UL84 in MCMV and GPCMV. In GPCMV the co-linear homologs appear to strictly retain function similar to HCMV but in MCMV these functions are less conserved and appear to be shared between both proteins. Overall, these results in conjunction with our previous studies on GP83 and GP82, encoding homologs to pp65 and pp71 respectively, further increases the significance of the GPCMV animal model for pathogenicity, vaccine and antiviral studies.

Materials and Methods

Cells, viruses and oligonucleotides

GPCMV (strain 22122, ATCC VR682), GPCMV BAC derived mutant virus, and vAM403, a green fluorescent protein (GFP) tagged wild-type GPCMV (McGregor and Schleiss, 2001) were propagated on guinea pig fibroblast lung cells (GPL; ATCC CCL 158) as previously described (McGregor et al., 2004). HCMV was propagated on human foreskin fibroblast cells in DMEM (Gibco-BRL) with 10% FCS supplemented with $1 \times$ anti-anti (Invitrogen). Virus titrations were carried out on six-well plates. Plaques were stained with 10% Giemsa stain or visualized by fluorescence microscopy. All oligonucleotides were synthesized by Sigma-Genosys (The Woodlands, TX).

Cloning of GPCMV GP84 and generation of GP84 expression vectors and GP84 knockout shuttle vector

The full length GP84 ORF was PCR cloned from GPCMV viral DNA as a *EcoR* I/*Hind* III fragment into plasmid pNEB193 (New England Biolabs) using the PCR primers FGP84Ec (5' GAATTCGATGATCGAAGAGACGGCCGAAC) and RGP84Hd (5' AAGCTTACACGGATTTGGGCGCGGGATCGC) to generate pNEBGP84 and sequenced to verify the GP84 coding sequence. The GP84 ORF was cloned in frame as a *EcoR* I/*Hind* III fragment into the expression vector pCMV2A (Promega) to generate the plasmid pFLAGGP84, expressing the full length GP84 ORF (483 aa) tagged at the N-terminus with a FLAG epitope tag. Using convenient restriction enzyme sites (*Bam*H I and *Sal* I) a series of collapses were made to the full length GP84 ORF to generate N- and C-terminal deletions (see supplementary Figure S1). A *Sal* I collapse at the 3' end of the ORF generated a C-terminal deletion mutant encoding codons 1–311, designated pFLAGGP84(S). A 5' *Bam*H I collapse generated an inframe deletion at the N-terminus of the GP84 coding sequence between the FLAG tag and codon 212, designated pFLAGGP84(B). Collapse of both termini generated pFLAGGP84(BS). The complete GP84 ORF was also PCR cloned in-frame into the C-terminal polycloning site of pEGFP-C1 (Clontech) as an *EcoR* I fragment using the primers FGP84Ec and RGP84Ec (GAATTCACACGGATTTGGGCGCGGGATCGC). Additionally, a truncated version of the GP84 coding sequence (codons 170–309) was PCR cloned in frame into pEGFP-C1 (Clontech) as a *Bgl* II/*Sal* I fragment to generate pGFPGP84ID using the primers FGP84Bg (5' AGATCTAAGAACGGCGACGAGCGACACAGCATC) and RGP84Sal (5' GTCGACTTAGGGCGCCGTCACGATCAAGTG). For generation of a GP84 knockout shuttle vector the plasmid pNEBGP84 was cut with *Bam*H I to create an internal deletion in the GP84 coding sequence (codons 140–213). A kanamycin (Km) cassette was PCR amplified from pACYC177 (NEB) using the primers using primers previously described (McGregor et al., 2004) and cloned into the pNEBGP84 *Bam*H I collapse to generate the GP84 knockout vector pNEBGP84km(Bm). The GPCMV DNA polymerase accessory protein, GP44, coding sequence (Schleiss *et al.*, 2008) was PCR cloned as a *Bam*H I fragment in-frame into the expression vector pCMV2C to FLAG epitope the N terminus of the GP44 protein. GP44 PCR was performed using the primers F44BmXh (5'

GGATCCTCTCGAGCTTCTGCGACGCCGGAGGGTATCG) and R44BmXh (5' GGATCCTCGAGTCAGGCGGAACATTTCTGCTTCTTGACGTTAGAC).

Generation of HCMV UL84 knockout shuttle vector and GP84/UL84 chimera shuttle vector

A shuttle vector was generated for the HCMV UL84 locus (UL84 CDS co-ordinates 157,514–155780c) based on HCMV Towne strain sequence (Genbank # AY3151972) for targeted modifications of the HCMV (Towne) BAC. Left and right arms of flanking homology around the UL84 locus were generated by PCR. Left arm (HCMV Towne coordinates nucleotides 157,671–157,390) and right arm (HCMV Towne co-ordinates 156,341–155,871) were cloned into the same pUC19 vector sequentially as *EcoR* I, *Bam*H I and *Hind* III, *Bam*H I fragments respectively (see Figure 5). The primers used for the right arm were FBBRFLKUL84 (5' GGATCCATAAGATCTCGACCCTTCTTTTCGGACGC) and RHPRFLKUL84 (5' AAGCTTGCATTAATTAACAGTCTTGCGGTTCCGTCTCCG). The left arm primers were FELFLKUL84 (5' GAATTCGCGACAGCTGGATGACCTCATCCGCGAAC) and RBLFLKUL84 (5' GGATCCGGTCGTGGTGGTAGTGGTGGTGGCGAG). This construct deletes the majority of the UL84 coding sequence (codons 59 to 401) but retained the upstream UL84 promoter. Unique *Bam*H I and *Bgl* II sites between the left and right flanking arms allowed cloning of a Km selection marker (McGregor et al., 2004) and the GP84 ORF. Insertion of the *Bam*H I Km cassette generated plasmid pUL84d for deletion of the UL84 coding sequence. For generation of the chimeric GP84-HCMV, the FLAG tagged GP84 ORF was PCR amplified from pFLAGGP84 as a *Bgl* II fragment using primers FFLAGGP84Bg (5' AGATCTGCCACCATGGATTACAAGGATGACGACGATAAG) and RGP84BgEcV (5' AGATCTGATATCTTACACGGATTTGGGCGCGGGATCGCTTC) and cloned into a unique *Bgl* II site in pUL84d to generate pUL84d/GP84, which placed the GP84 ORF under UL84 promoter control. The GP84 insertion also introduced a unique *EcoR* V site 3' to the GP84 coding sequence. This allowed the insertion of a kanamycin cassette flanked by *EcoR* V restriction sites (McGregor et al., 2004) to generate pUL84d/GP84Km (Figure 4). This construct would be used to introduce the GP84 ORF into the HCMV Towne BAC.

Generation of recombinant BACmids and analysis of BAC structure

An inducible recombination system (ET system) was introduced into DH10B bacterial cells containing either the GPCMV BAC plasmid (McGregor and Schleiss, 2001) or the HCMV (Towne) BAC plasmid (Hahn et al., 2003) using a protocol previously described (McGregor et al., 2008). For knockout of the UL84 gene the plasmid pUL84d was linearized by a unique restriction enzyme (*Pac* I) outside the flanking homologous region. For generation of the chimeric GP84 HCMV BAC construct the chimeric shuttle vector pUL84d/GP84km was linearised by *Pac* I digestion. Linearized shuttle vector DNA was band isolated and resuspended at a concentration of 1 µg/ul, and electroporated into recombination induced HCMV BAC-containing cells in separate reactions for each mutant. Recombinant bacterial colonies of GPCMV BAC or HCMV mutants were isolated by chloramphenicol (12.5 µg/ml) and kanamycin (20 µg/ml) antibiotic selection in LB agar bacterial Petri dishes. Bacterial plates were initially incubated at 39° C to remove the ts ET recombination plasmid (Genebridges). Mutant GPCMV BAC DNA was analyzed by *EcoR* I and *Hind* III restriction digestion to verify the accuracy of the predicted genome configuration after mutation (McGregor and Schleiss, 2001). HCMV BAC mutants were analysed by *Hind* III restriction profile analysis. Insertion of the Km drug resistance cassette into the viral genome introduced a novel *Hind* III restriction. Full length HCMV mutants (HCMV::UL84d or HCMV::GP84 BACs) were further analyzed at the UL84 locus by diagnostic PCR using common primers (F84 5'CACTTTCTCGCCACCACTAC; R84 5'GTAAAAGCGGCGGTAGATACG) flanking the modified UL84 locus. Diagnostic PCR

reactions were carried out using conditions described in McGregor et al. (2008) except the extension time at 72° C was modified based on the size of each gene modification (based on an extension time of 30 sec. per 500 bases).

For knockout of the GP84 gene on the GPCMV BAC, the GP84 knockout shuttle vector pNEBGP84km(Bm) was linearized by *EcoR* I digestion which cut the plasmid in the polylinker sequence outside the GP84 coding sequence. DNA was band purified and 1 ug was electroporated into an aliquot of ET induced electrocompetent DH10B cells containing the GPCMV BAC as previously described (McGregor et al., 2004). GP84 knockout mutant GPCMV BAC colonies were selected under Km selection and screened by restriction profile analysis to identify full length mutants. The GP84 gene knockout was confirmed by comparative PCR analysis between wild type and mutant GPCMV BACs using common flanking primers FGP84g (5' GAGATGATCGAAGAGACGGCCGAAC) and RGP84g (5' GTCTACGCCCATAGCGTCGTAGTTCG) to amplify either the complete GP84 gene (1.4 kb) or the modified GP84 gene locus (2.1 kb).

Generation of mutant GPCMV, chimeric HCMV and rescue of lethal HCMV knockout mutant

For generation of recombinant viruses, large-scale BAC DNA was purified from *E. coli* using a Qiagen maxi plasmid kit. GPCMV BAC DNA was used to transfect GPL cells in six well dishes using Lipofectamine 2000 (Invitrogen) transfection reagent as previously described (McGregor et al., 2004). HCMV BAC DNA was transfected onto HFF cells in six well dishes using supertransfect reagent (Qiagen) following manufacturer's protocol. Transfections were followed for at least 3–4 weeks for the production of viral plaques. GPCMV GFP positive viral plaques were detected via microscopy using an inverted microscope equipped for GFP fluorescent imaging (McGregor and Schleiss, 2001). The HCMV BAC did not carry a GFP tag and formation of viral plaques was followed under bright field microscopy. Mutants were rescued back to wild type phenotype by mutant BAC co-transfection with the appropriate rescue plasmid (GPCMV GP84d knockout mutant) or PCR product (HCMV UL84 knockout mutant). HCMV rescue PCR product (UL83-UL85 PCR product) was generated by PCR amplification of Towne genomic DNA. PCR primers were generated from AD169 genomic sequence (UL83 forward primer 5' GTTCATCAACAGGTTACCTGAGATGCT, UL85 reverse primer 5' GCTGCGCAATATGACGCTGAGGTTGATG). Amplifying a predicted 3.7 kb PCR product (121,003–124,762 AD169 co-ordinates). PCR product was band purified and used to rescue lethal HCMV UL84 knockout by co-transfection of PCR product (10 µg) with BAC DNA (1 µg) per transfection well. Rescued viral plaques were picked and subject to further rounds of plaque purification by limiting dilution. Viral DNA was extracted from virus-infected cells as previously described (McGregor and Schleiss, 2001). Rescue was confirmed by PCR analysis using common flanking gene primers for GP84 (GPCMV) or UL84 (HCMV) as described previously.

GPCMV multi-step growth curve and HCMV one step growth curve

GPCMV infection was performed on six well dishes as previously described (McGregor and Schleiss, 2001) except that the input virus (moi) was 0.1 pfu/cell per well. Virus time samples were harvested immediately after 1hr virus adsorption (day 0) and every 24 hrs (days 1–7). Samples were harvested, stored at –80° C and titrated after the final time point had been harvested as previously described. For HCMV a one step growth curve was performed on confluent monolayers of HFF cells in 6 well dishes with an input virus moi of 3 pfu/cell. One step growth curve was performed as previously described (McGregor and Schleiss, 2001). Virus time samples were taken every 12 hours from 0–96 hrs post infection. Time samples were stored at –80° C and titrated after the final time point had been harvested.

Immunofluorescence and Western blot analysis

Immunofluorescence assays were performed on full length and truncated GP84 proteins transiently expressed in GPL cells by transfection of GP84 FLAG tagged expression plasmids pFLAGGP84, pFLAGGP84(B), pFLAGGP84(S) and pFLAGGP84(BS). Assay was performed as previously described on GPL cover slips in six well dishes (McGregor et al., 2004) except that samples were fixed in -20° C methanol at various time points post transfection (3, 5, 6, 8, 12, 18, 24, 36 and 48 hr post transfection). The mouse anti-FLAG antibody (Sigma) was used at 1/1000 dilution and the secondary antibody anti-mouse IgG (Sigma) conjugated to FITC was used at 1/1000 dilution. For co-localization study of plasmid based transient expression of GFP tagged GP84 (pGFPGP84) and FLAG tagged GP44 (pFLAGGP44) plasmid transfections were performed as described above but cells were fixed with 4% paraformaldehyde for 5 min to maintain GFP fluorescence and post fixation treated with 0.2% tritonX100 to increase cell membrane permeability. Immunofluorescence assay was performed as described above except the secondary antibody was anti-mouse IgG conjugated to Texas red (Santa Cruz Biotech.) at 1/200 dilution. Cells were also counterstained with DAPI (Vector labs) to identify the cell nucleus. Co-localization experiments were harvested at 12 hr. post transfection to enable optimal detection of Texas red (conjugated to secondary antibody) tagged GP44 protein. Western blot was carried out as previously described (Schleiss *et al.*, 1999) except that the primary antibody was mouse anti-FLAG (1/1,000 dilution) and the secondary was anti-mouse IgG conjugated to horseradish peroxidase (Santa Cruz Biotech.) used at a 1:10,000 dilution followed by detection by chemiluminescence using ECL substrate (Amersham) following the manufacturer's protocol.

Generation of GPL cell lines expressing GFP or GFP-GP84ID

A PCR cassette was generated that contained the minimal transdominant inhibitory domain for GP84 identified in the expression vector pFLAGGP84(BS) plus additional flanking sequence (codons 170–309). The PCR product was cloned as a Bgl II fragment inframe into the C-terminal domain of GFP expression plasmid pEGFPC-1 (Clontech) cut with *Bgl*II to generate pGFPGP84ID as described in plasmid constructs section. This plasmid expressed GFP under HCMV IE enhancer control but also encoded a neomycin drug resistance cassette under SV40 promoter control to allow selection of G418 (Invitrogen) antibiotic resistant mammalian cells. Plasmids pEGFP-C1 and pGFPGP84ID were separately transfected onto GPL cells in six well dishes (4 μ g per well) as previously described (McGregor *et al.*, 2004). At 36–48 hr post transfection the F-12 media was replaced with media containing G418 at a concentration of 600 μ g/ml and the media was changed every two days for the first 7–10 days. At that stage the G418 concentration was dropped to 500 μ g/ml and the media changed every three days. Resistant colonies of cells were detected by about 18–24 days post transfection. GFP positive colonies were detected using an inverted microscope equipped for GFP fluorescent imaging. Between days 30–40, well separated, GFP positive cell colonies were individually isolated using cloning rings and transferred to 25cm² flasks and expanded as cell lines. 10 cell lines were generated for each plasmid. 6 out of the 10 GFP cell lines maintained a strong level of GFP through multiple cell passages but only 3 out of 10 GFPGP84ID cell lines maintained consistent levels of GFP under multiple cell passages. Cells were maintained under G418 selection under passage in tissue culture (300 μ g/ml). One GFP cell line per plasmid was selected for use in transdominant inhibitory growth assays.

Transdominant Inhibitory Growth Assays

(A) GPCMV BAC and GP84 expression plasmid transient inhibitory growth assay. Six well dishes of confluent monolayers of GPL cells were transfected with different combinations of GPCMV BAC DNA (1 μ g) and GP84 FLAG tag expression vectors (4 μ g). The different

combinations were GPCMV BAC alone or plus: (1) pFLAGGP84; (2) pFLAGGP84(B); (3) pFLAGGP84(S); (4) pFLAGGP84(BS); (5) pTAG2A (empty vector). Transfected plates were transfected as previously described (McGregor et al., 2004). Assays were followed by GFP reporter gene expression for identification of GFP positive GPCMV BAC transfected cells and GFP viral spread across the cell monolayer. Assays were extended to day 18 where virus stocks were harvested and titrated out on GPL cells. Individual assays were repeated multiple times.

(B) GPCMV transdominant inhibitory growth assay on GFP and GFP84ID GPL cells. Clonal transformed GPL cells expressing GFP or GFP84ID (the minimal inhibitory domain from GP84 fused to the C-terminal domain of GFP) were used to seed six well dishes. Confluent monolayers were tested for their ability to support virus replication at different moi of GPCMV (vAM403), where moi was 0.1, 0.01 or 0.001 pfu/cell. Virus infections were carried out in duplicate wells and allowed to progress for 5 days at which time the wells were harvested and the viral titers determined by titration on regular GPL cells. Herpes simplex virus (type 1) at a moi 0.001 pfu/cell was used to infect both GFP and GFP84ID cells as an additional control to demonstrate specificity of the GP84 transdominant inhibitory growth effect to GPCMV.

RT-PCR GP84 expression

RT-PCR was performed as previously described (McGregor et al., 2008). The GP84 gene expression in ATCC wild type GPCMV infected GPL cells (input moi 1 pfu/cell) was determined using primers FGP84RT (5' CTACGGCTTCATCAAGATG) and RGP84RT (5' CATCGCGTCTGGATGAAGTAC). Control cellular gene expression was for GAPDH as previously described (McGregor et al., 2008). IE2 (GP122) gene expression was determined using RT-PCR primers FGP122RT 5'GACATGGAGCGCCGGTAGCG and RGP122RT 5'CATGGCAGACCTGATGCGAC. The viral DNA polymerase gene (GP54) was assayed by primer set FGP54RT 5'GAGACGGAGGACGTTACTAAG and RGP54RT 5'CTCACAATCTATCTCGATGTC. For specific selection of IE transcripts only, cells were treated with cycloheximide (100 µg/ml) 1 hr. prior to virus infection and for 4 hr. post infection at which point the cells were harvested and RNA extracted for analysis. For exclusion of late gene transcripts, phosphonoacetic acid (PAA) was present (200 µg/ml) as previously described Schleiss et al. (1999) and cell monolayers were harvested at 12 hr. post infection for RNA extraction and analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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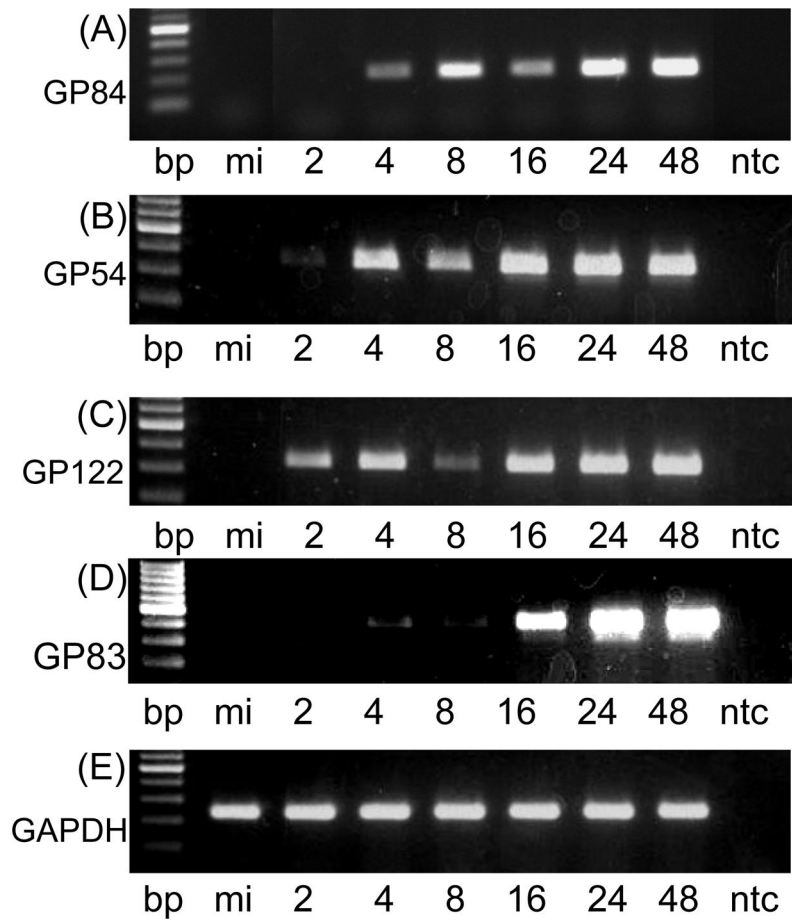
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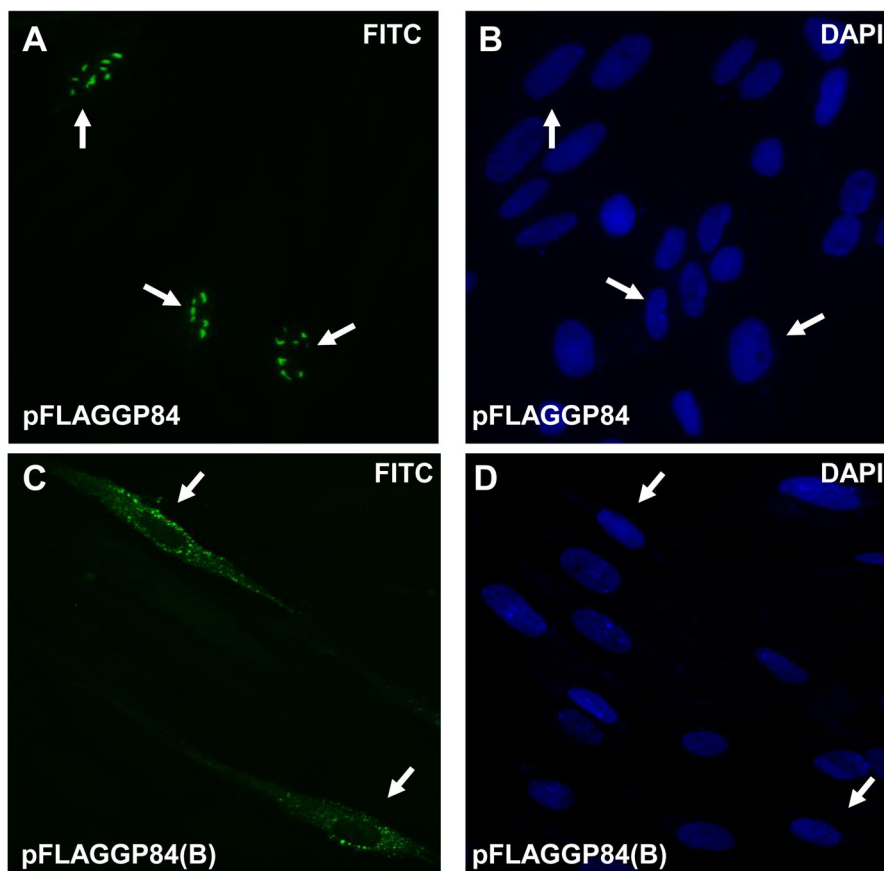


Figure 1. Expression studies of GP84

1(i) RT-PCR assay of GP84 mRNA expression in virus infected cells. Wild type virus infected GPL cells (moi=3 pfu/cell) were harvested at different time points for RNA extraction and mRNA analysis by RT-PCR using GP84, GP54 (viral polymerase), GP122 (IE2 unique exon) and cellular GAPDH specific primers as described in materials and methods. Viral gene classes: immediate early, IE2, unique exon GP122 (Yamada et al., 2009); early, viral polymerase GP54 (Schleiss, 1995); and late, GP83, encoding pp65 homolog (Schleiss et al., 1999). RT-PCR products were analyzed by agarose gel electrophoresis: GP84 (gel A); GP54 (gel B); GP122 (gel C); and GAPDH control (gel D). In all gels lanes are as the same. 100 bp ladder (NEB) followed by mock infected cells (mi), time samples 2–48 hr post infection (hpi) and no template control (ntc). Time samples 2, 4, 8, 16, 24 and 48 hr post infection.

1(ii) Transient expression studies of full length and N-terminal truncated GP84.

Immunofluorescence assay of FLAG-tagged GP84 protein transiently expressed in GPL cells transfected with pFLAGGP84 or pFLAGGP84(B). Assay performed at 10 hr post transfection. Primary antibody anti-FLAG and secondary antibody anti-mouse IgG conjugated to FITC (images A and C). Cells counterstained with DAPI (images B and D). Arrow indicates same cell under FITC and DAPI filters. Images A and B, full length pGP84 (pFLAGGP84 plasmid). Images C and D, N-terminal truncated pGP84 (pFLAGGP84(B) plasmid).

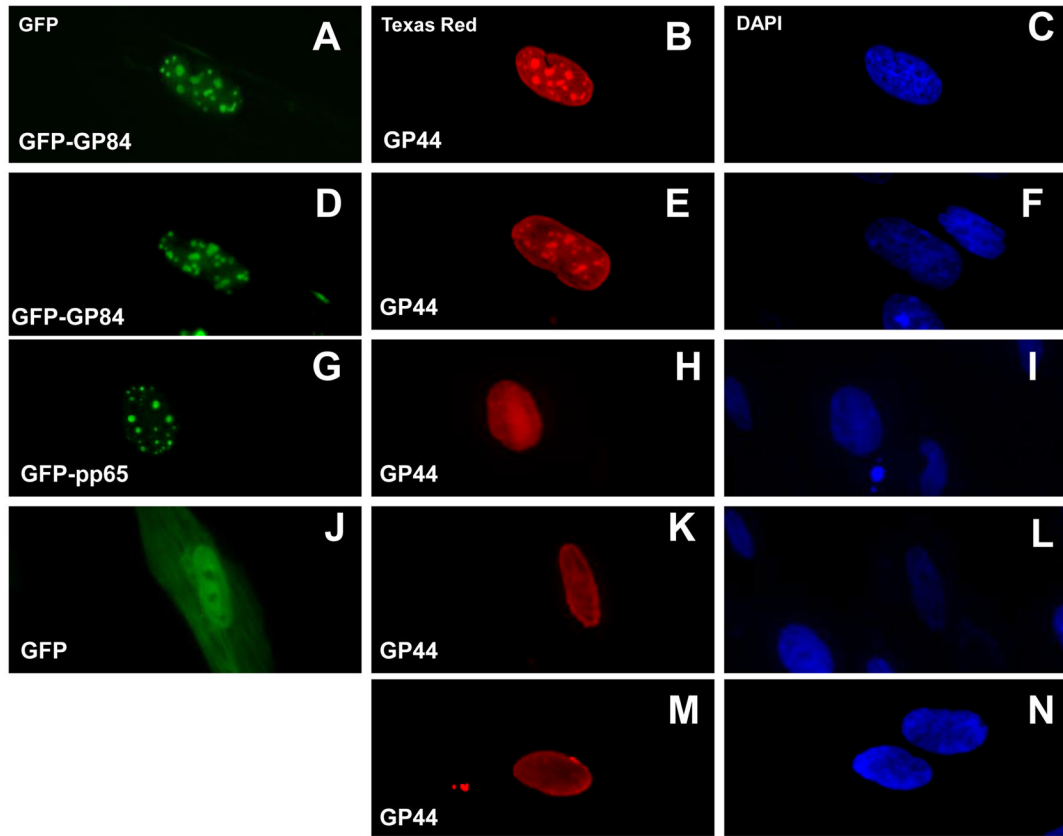


Figure 2. Co-localization studies of transiently expressed pGP84 and pGP44

Transient plasmid expression assay of FLAG tagged pGP44 (pFLAGGP44 plasmid) in the presence of GFP tagged vectors: GFP tagged pGP84 (images A–F); GFP tagged pp65 (G–I); GFP control (J–L). Images M and N correspond to transient expression of pGP44 only cells. pGP44 detected by anti-FLAG primary antibody and secondary antibody anti-mouse IgG conjugated to TRITC. pGP44 images B, E, H and M. Cells counterstained with DAPI to identify cell nucleus (right column). Image A and D, pGP84 expressed as N-terminal tagged GFP protein (pGFPPGP84). Image G, pGFPPp65 (HCMV pp65 N-terminal GFP tagged). Image J, control GFP plasmid pGFP-C1 (Clontech). Protein cellular localization determined at 12 hr post transfection as described in materials and methods.

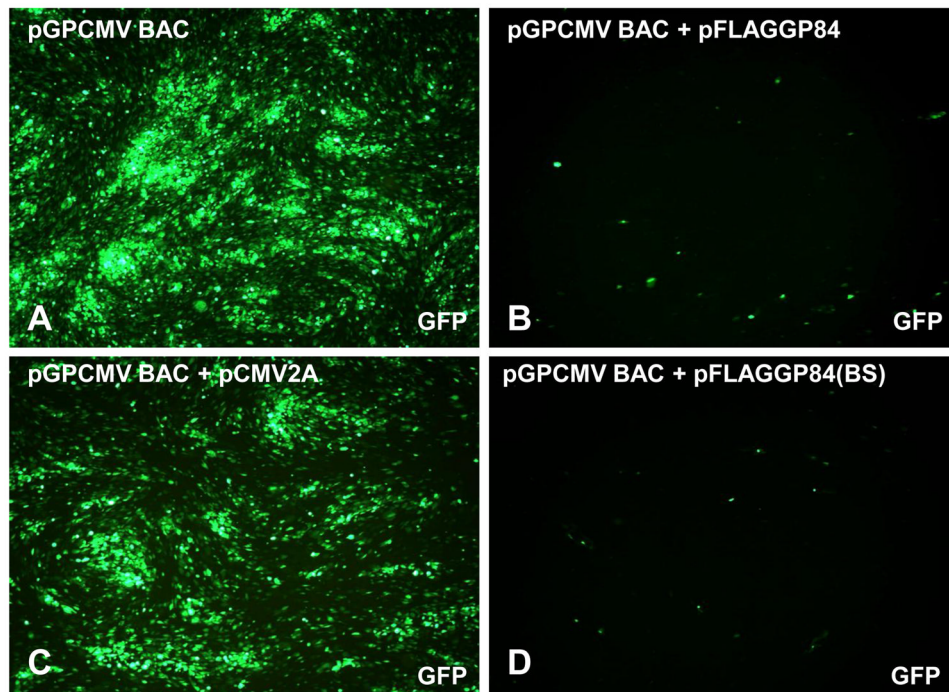


Figure 3. GPCMV pGP84 transdominant growth inhibition assay

GFP tagged GPCMV BAC DNA was transfected onto GPL cells alone or co-transfected with GP84 expression vectors (pFLAGGP84 or pFLAGGP84BS) or empty expression vector (pCMV2A). Development of viral plaques could be detected by GFP reporter gene expression in infected cells. Viral spread was monitored between 7 and 18 days post transfection. At day 18 all wells were harvested and viral titer determined. Data shown is representative GFP virus spread in wells of a six well dish at 16 days post transfection. Images: GPCMV BAC DNA only well, panel A; GPCMV BAC DNA plus empty vector (pCMV2A), panel C; B. GPCMV BAC DNA plus pFLAGGP84 (full length pGP84), panel B; GPCMV BAC DNA plus pFLAGGP84BS (truncated pGP84), panel D. Virus inhibition was obtained in the presence of full length or truncated pGP84 (images B and D).

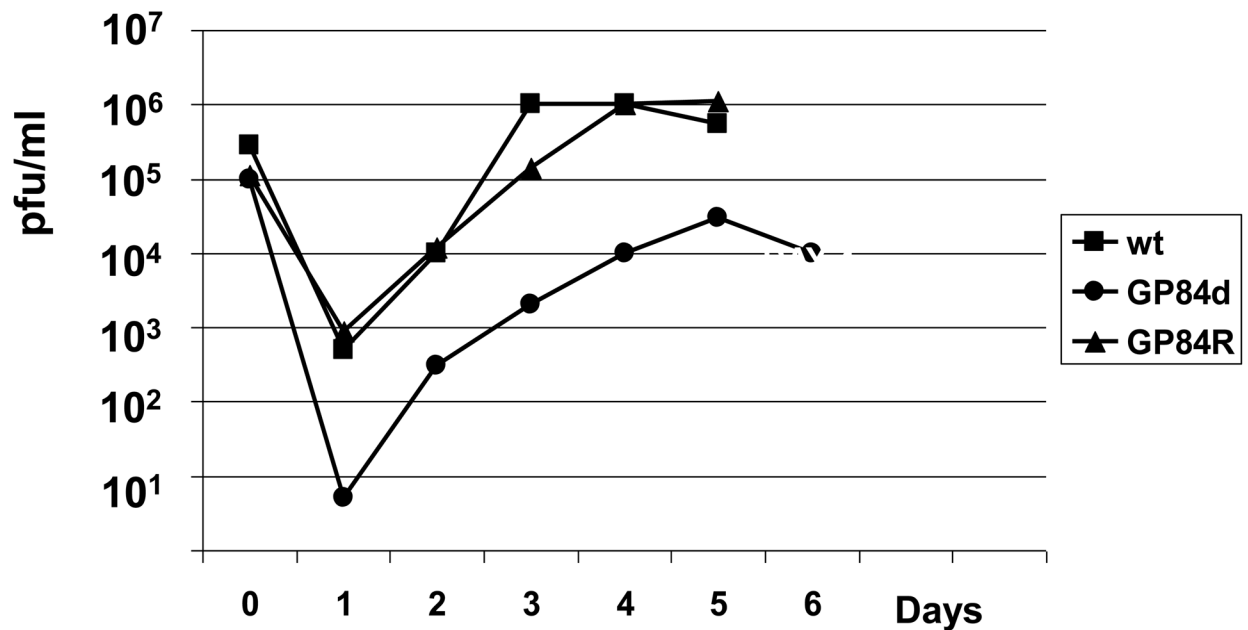
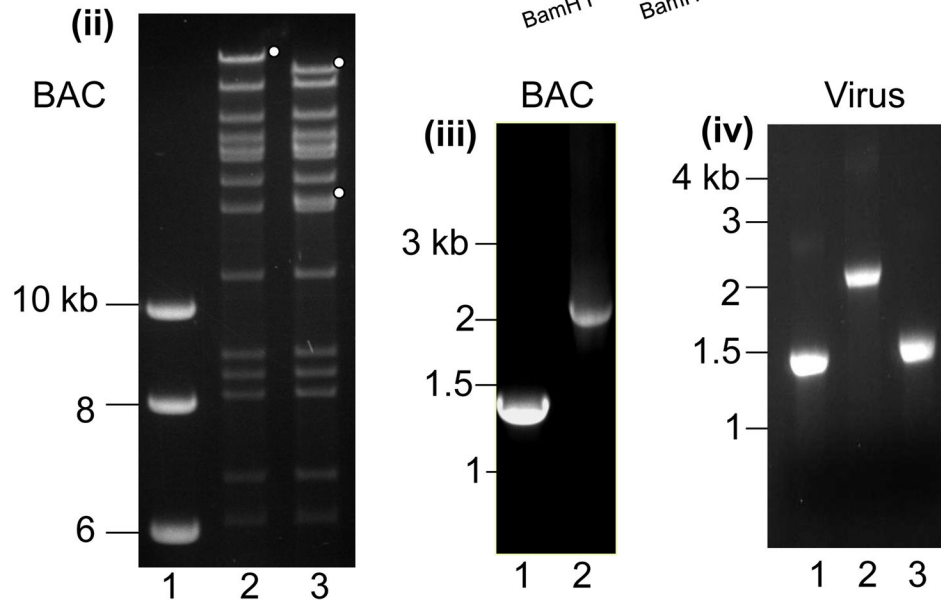
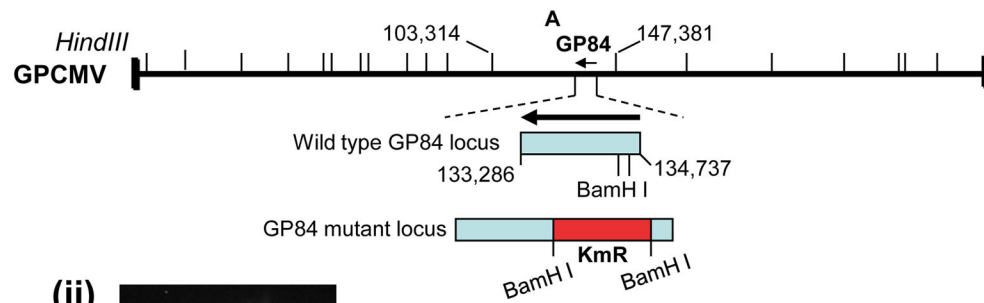


Figure 4. Characterization of GP84 knockout GPCMV mutant (GP84d)
 (i) GPCMV genome map of *Hind* III sites and the location of the knockout GP84 gene. Shown is the GPCMV *Hind* III ‘A’ locus (co-ordinates 103,314–147,381 bases) encoding GP84 CDS (complement 133,286–134,737 bases). Insertion of a kanamycin resistance

cassette into the GP84 coding sequence between two *Bam*H I sites (134,097 and 134,314 bases) results in an insertion deletion disruption of the GP84 coding sequence.

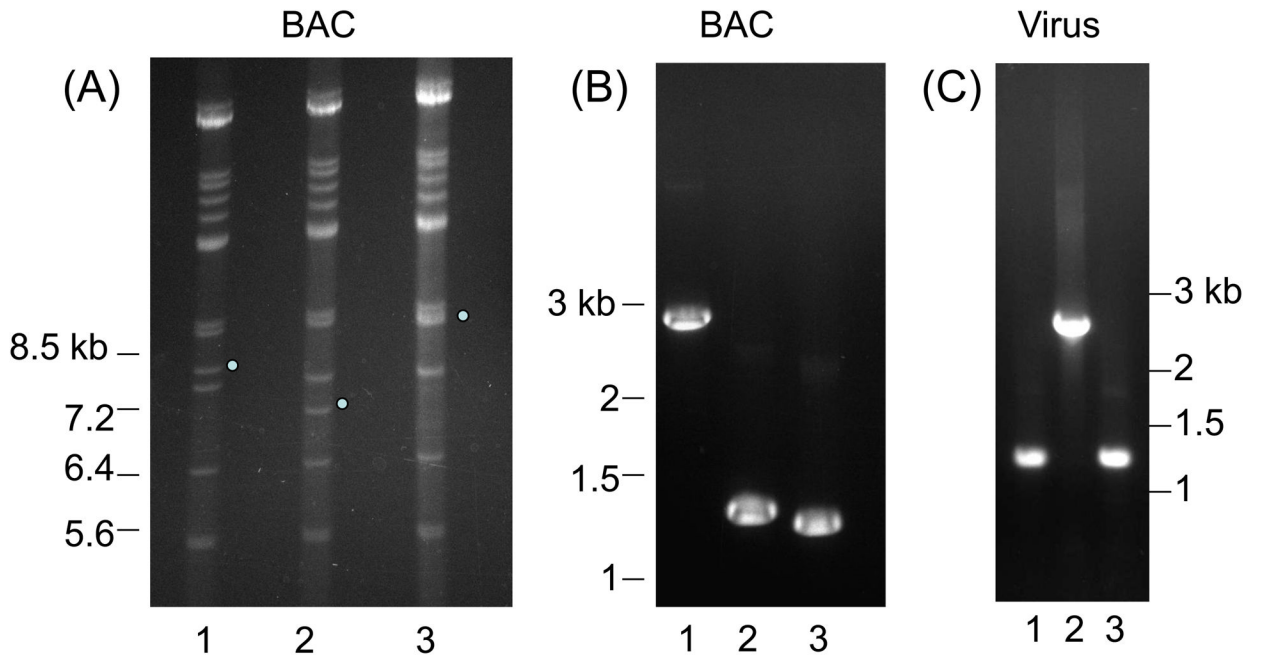
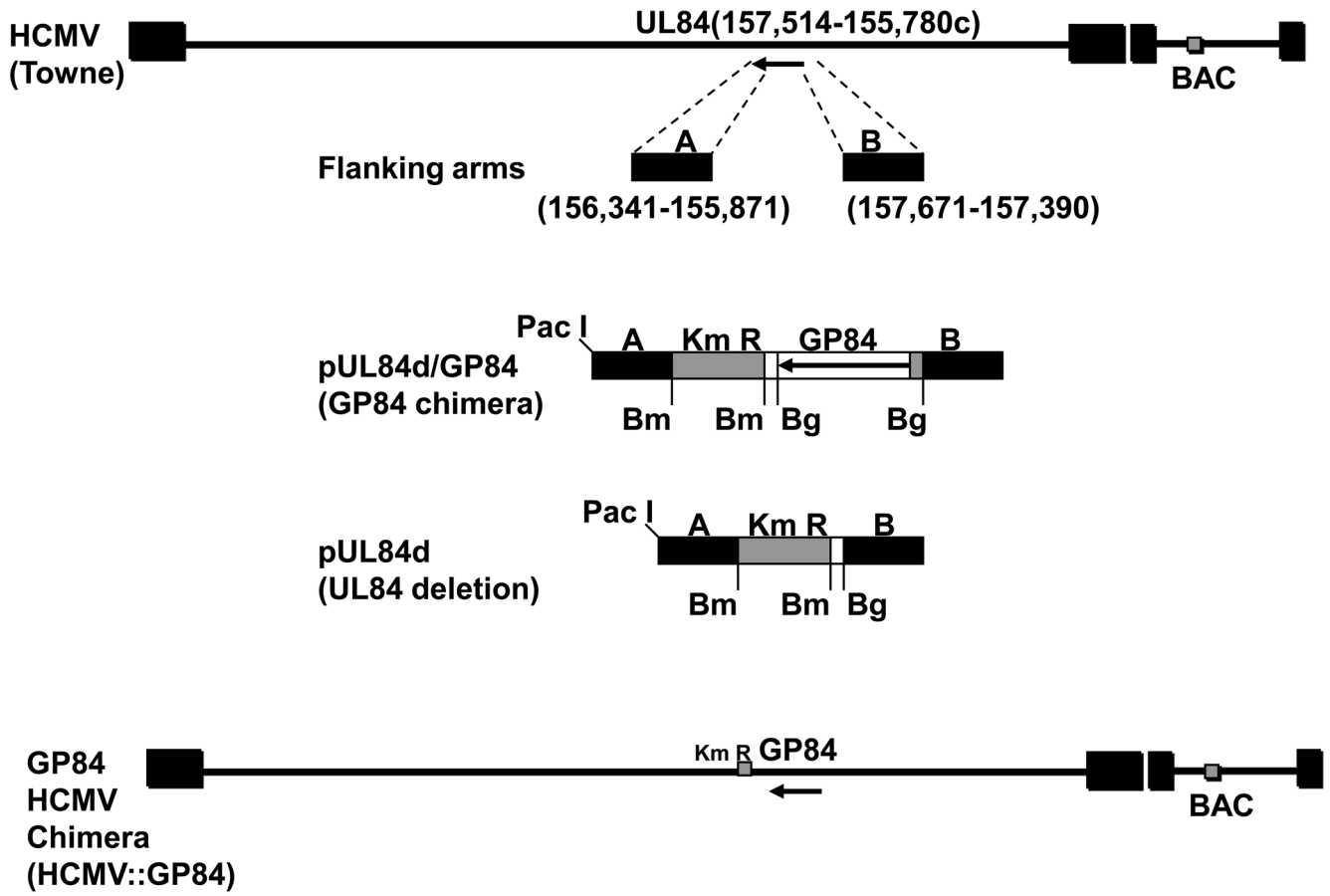
(ii) Wild type and GP84 mutant GPCMV BAC *Hind* III restriction profile

analysis. Agarose gel of the DNA restriction profile analysis of mutant and wild type GPCMV BACs. Lanes: 1. kb ladder (NEB). 2. wt GPCMV BAC; 3. GP84d GPCMV BAC. Insertion of a kanamycin cassette into the GP84 gene introduces a new *Hind* III site into the GPCMV genome and disrupts the *Hind* III 'A' band encoding the GP84 gene. Full length (lane 2) and modified bands encoding part of the *Hind* III 'A' band (lane 3) are indicated with circles.

(iii) GPCMV BAC Diagnostic PCR analysis of the GP84 locus. Common primers flanking the site of modification in the GP84 locus were used to verify the modification to the GP84 locus in comparison to the wild type locus. Shown is an agarose gel of GP84 PCR products from different BAC DNA templates. Lanes: 1. wt GPCMV BAC; 2. GP84d GPCMV BAC mutant. Shift in the size of the locus confirms correct insertion of the kanamycin cassette plus deletion of GP84 sequence between *Bam*H I sites.

(iv) Viral DNA Diagnostic PCR analysis of the GP84 locus. Common diagnostic GP84 locus primers were used to confirm the status of the GP84 locus in viral genomes: 1. wt GPCMV; 2. GP84d mutant; 3. GP84d rescue virus. The mutant virus retains the same modification generated in the GPCMV BAC mutant and the rescue virus has a wild type sized GP84 locus.

(v) Multi step growth curve of GPCMV GP84 mutant (GP84d) Growth curve performed as described in materials and methods. GPCMV GP84 knockout mutant (GP84d) was compared to wild type GPCMV (wt) and GP84d rescue virus (GP84R). Input virus moi was 0.1 pfu/cell with time points taken at 24 hr. intervals between 0–6 days post infection.



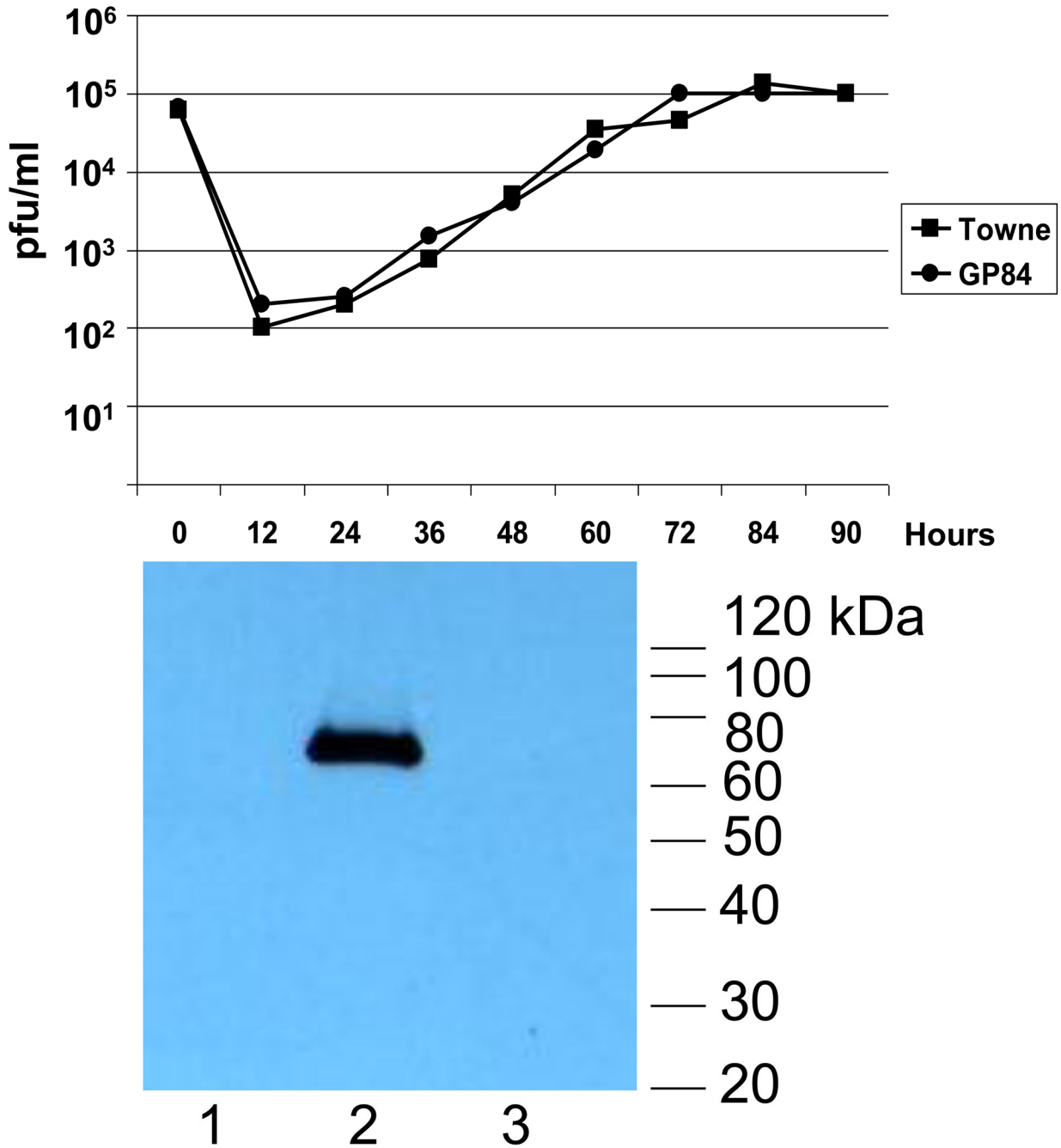


Figure 5. Generation and characterization of GP84 chimeric HCMV

(i) Overall strategy for the generation of UL84 knockout and GP84 chimera HCMV BAC mutants. Construction of UL84 knockout shuttle vectors. UL84 flanking sequence was generated by PCR (right arm left arm, A and B). For UL84 deletion HCMV BAC a *Bam*H I Km cassette was cloned into the shuttle vector to generate pUL84d. For generation of the *GP84* chimeric HCMV the FLAG tagged GP84 ORF from pFLAGGP84 was PCR cloned as a *Bgl*II cassette 5' to the Km cassette in plasmid pUL84d to generate construct pUL84d/GP84. Shuttle vectors were linearised by *Pac*I digestion prior to recombination in bacteria with HCMV Towne BAC following protocol described by McGregor *et al.* (2004). Structure of *GP84* HCMV chimeric virus is shown in the bottom of the figure.

(ii). Analysis of HCMV wild type and UL84 mutant structures. (A) HCMV BAC DNA *Hind* III restriction profiles. Lanes: 1. wt HCMV BAC DNA; 2. UL84 deletion mutant HCMV BAC DNA; 3. GP84 chimera HCMV BAC DNA. Dots mark wild type or modified 7.8 kb *Hind* III subgenomic fragment (co-ordinates 120,817–128,615 HCMV Towne) encoding the UL84 wild type locus (lane 1) and modified by the deletion of the majority of the UL84 coding sequence and insertion of a Km cassette (UL84 deletion mutant HCMV BAC, lane 2) plus the insertion of the GP84 coding sequence (GP84 HCMV chimera BAC, lane 3).

(B) PCR analysis of wild type and mutant UL84 loci from HCMV BAC clones.

Common PCR primers amplified the modified UL84 locus from the HCMV BAC constructs and PCR products were analysed by agarose gel electrophoresis. Lane: 1. GP84 chimeric HCMV BAC; 2. wild type HCMV BAC; 3. UL84 deletion HCMV BAC.

(C) PCR analysis of wild type and mutant UL84 loci from viral DNA. Common PCR primers amplified the modified UL84 locus from wild type and GP84 chimeric HCMV viral DNA and PCR products were analysed by agarose gel electrophoresis. Note that the rescue virus was generated to the lethal UL84 knockout. Lane: 1. wild type HCMV; 2. GP84 HCMV chimera; 3. UL84 deletion rescue virus. Diagnostic PCR confirms that the chimeric virus encodes a modified UL84 locus similar to the GP84 HCMV chimeric BAC (B, lane 1).

(iii) One step growth curve of wild type HCMV (Towne) and GP84 chimeric HCMV.

Growth curve carried out as described in materials and methods with an initial input of virus of 3 pfu/cell (moi) with time point samples taken every 12 hr (0–90 hr post infection) as described in materials and methods.

(iv) Western blot of FLAG-tagged GP84 protein expressed in chimeric virus infected cells. Lanes: (1) Mock infected HFF cells; (2) *GP84* chimeric virus infected cells; (3) HCMV (Towne) infected cells. Protein ladder (Invitrogen). GP84 protein detected with primary antibody mouse anti-FLAG and secondary anti-mouse IgG conjugated to HRP as described in materials and methods.

Table 1
Transdominant inhibitory effect on GPCMV growth by the pGP84 minimal inhibitory domain expressed by permanently transduced cell

Input virus infection is indicated and final virus yield determined by titration of infected cells at a set time point post infection as described in materials and methods. GPL-GFP, control cells expressing GFP. GPL GFP-GP84ID, cells expressing the pGP84 99 aa inhibitory domain fused to the C-terminal of GFP.

Input GPCMV (pfu/cell)	Viral titer GFP cells	Viral titer GFP84ID cells
0.1	2.5×10^6 pfu/ml	3×10^4 pfu/ml
0.01	1×10^6	4×10^3
0.001	2×10^6	5×10^3