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Characterization and immunological analysis of the rhesus cytomegalovirus homologue (Rh112) of the human cytomegalovirus UL83 lower matrix phosphoprotein (pp65)

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Rhesus cytomegalovirus (RhCMV) contains two open reading frames (Rh111 and Rh112) that encode proteins homologous to the phosphoprotein 65 (pp65) of the human cytomegalovirus (HCMV) UL83 gene. As HCMV pp65 elicits protective immune responses in infected humans and represents an important vaccination target, one RhCMV homologue of HCMV pp65, pp65-2 (Rh112), was characterized and analysed for its ability to induce host immune responses. Similar to its HCMV counterpart, RhCMV pp65-2 was expressed as a late gene, localized to the nucleus within pp65-2-expressing cells and was present within infectious virions. Longitudinal and cross-sectional studies of pp65-2 immunity in naturally infected rhesus macaques showed that humoral responses to pp65-2 were elicited early during infection, but were not always sustained over time. In contrast, pp65-2-specific T-cell responses, examined by gamma interferon ELISPOT, were broadly detectable in all of the animals studied during primary infection and persisted in the vast majority of RhCMV-seropositive monkeys. Moreover, there was considerable inter-animal variability in the pattern of the immune responses to pp65-2. Together, these results demonstrated that RhCMV pp65-2 exhibited biological and immunological homology to HCMV pp65. Thus, the rhesus macaque model of HCMV persistence and pathogenesis should be relevant for addressing pp65-based vaccine modalities.

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INTRODUCTION

Human cytomegalovirus (HCMV) infection is usually asymptomatic in immunocompetent individuals. This is true for both primary infection and non-primary exposure to virus following either reactivation of persistent viral genomes or horizontal transmission of novel immunological variants. However, HCMV is a significant source of morbidity and mortality in those without a fully functional immune system, such as immunosuppressed transplant recipients, congenitally infected fetuses and neonates, and AIDS patients (Alford & Britt, 1993). There is no licensed vaccine for HCMV, although there is a clear clinical need for one that could protect those most at risk for HCMV infection and disease. Natural history studies have identified immunological correlates of protection, particularly the role of both neutralizing (NT) antibodies and cytotoxic T lymphocytes (CTLs) in limiting transplacental transmission of HCMV (Boppana & Britt, 1995) and protecting from disease in bone marrow- and solid organ-transplant recipients (Li *et al.*, 1994; Reusser *et al.*, 1991, 1997, 1999; Walter *et al.*, 1995), respectively. Dominant viral targets for NT antibodies and CTL activity include the proteins encoded by the UL55 (glycoprotein B; gB) and UL83 (phosphoprotein 65; pp65) open reading frames (ORFs). As such, both HCMV proteins represent logical components of HCMV vaccine strategies. Likewise, other studies have indicated that the immediate-early 1 protein (IE1) should be included as a vaccine target (Bunde *et al.*, 2005), a conclusion bolstered by vaccine studies involving the IE1 protein of murine CMV (MCMV) (Del Val *et al.*, 1991; González Armas *et al.*, 1996; Jonjić *et al.*, 1988; Ye *et al.*, 2002).

Although the immune responses that control HCMV are not known precisely, the quality and magnitude of humoral responses (e.g. NT antibodies or high-avidity antibodies) appear to be critical for protection against primary infection and limiting virus dissemination (Boppana & Britt, 1995; Fowler *et al.*, 1992; Nigro *et al.*, 2005) and the severity of disease during recurrence (Campbell & Herold, 2004), whereas cell-mediated immune responses probably play the dominant role in protection against HCMV sequelae and

the recovery from fulminant HCMV infection (Li *et al.*, 1994; Reusser *et al.*, 1991, 1997, 1999; Walter *et al.*, 1995). Thus, induction of protective antibody and CTL responses should be considered for vaccine design. As an abundant structural protein, HCMV pp65 is the immunodominant target for CTL and lymphoproliferative responses (Beninga *et al.*, 1995; Boppana & Britt, 1996; Gyulai *et al.*, 2000; McLaughlin-Taylor *et al.*, 1994; Wills *et al.*, 1996) and, as such, has been studied as a vaccine candidate. A recombinant canarypox vector expressing HCMV pp65 has been shown to induce $CD8^+$ cytotoxic T cells that persist for at least 2 years following immunization in healthy, HCMV-seronegative humans (Berencsi *et al.*, 2001). Similarly, a modified vaccinia virus Ankara expressing pp65 elicits pp65-specific CTLs in mice (Wang *et al.*, 2004). However, there has not been an evaluation of their ability to protect against either infection or disease. DNA immunization against the MCMV homologue (M84) of HCMV pp65 stimulates cellular responses that reduce viral titres significantly following challenge (Morello *et al.*, 2000, 2005). Together, the natural history and vaccination studies point to the need to investigate pp65-based immunization strategies further.

Towards that end, we have characterized a rhesus CMV (RhCMV) homologue of HCMV pp65 as a prelude to evaluating its utility as a model system for testing pp65 strategies in a non-human primate host. RhCMV encodes two ORFs, Rh111 and Rh112, that are both positional and sequence homologues of HCMV pp65 (Hansen *et al.*, 2003). Of the two, the predicted protein encoded by Rh112 (pp65-2) has slightly higher amino acid identity (35 %) to HCMV pp65 than does the protein encoded by Rh111 (32 %). Immunologically, pp65-2 elicits antibody and cellular immune responses stronger than those generated by pp65-1 (Hansen *et al.*, 2003; unpublished data). This report characterizes the expression of pp65-2 in tissue-culture cells and host immune responses following natural exposure to RhCMV. Like its HCMV pp65 counterpart, RhCMV pp65-2 is expressed with late-gene kinetics, localizes to the nucleus and stimulates humoral and cellular immune responses in infected rhesus macaques (Fowler *et al.*, 1992). The results demonstrate that RhCMV pp65-2 is a target of host immune responses and, thus, represents a valid surrogate for investigating pp65 as a vaccine component.

METHODS

Cell culture and virus. Primary rhesus dermal fibroblasts (RhDFs) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 20 % (v/v) heat-inactivated fetal calf serum (FCS; Gemini Bio-Products), 2 mM glutamine, 100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1} (Invitrogen) (Pen/Strep). Telomerase-immortalized rhesus fibroblasts (Telo-RFs) (Chang *et al.*, 2002; Kirchoff *et al.*, 2002) were cultured in complete DMEM containing 10 % (v/v) FCS, 100 μg G418 ml^{-1} (Invitrogen) and Pen/Strep. 293T cells (DuBridge *et al.*, 1987) were grown in complete DMEM containing 10 % (v/v) FCS and Pen/Strep. All of the cell lines were tested and found to be free of mycoplasma contamination. Cell-free stocks of RhCMV strain 68-1 (Lockridge *et al.*, 1999) and total virus antigens for ELISA were prepared in RhDFs as described previously (Yue *et al.*, 2003) and stored at −80 °C. Telo-RFs were used for the kinetics of protein expression and localization of pp65-2 following RhCMV infection. 293T cells were used for the transfection of pND/pp65-2 and the preparation of antigens for pp65-2-specific ELISA.

Sequence analysis. Alignment of RhCMV pp65-1, pp65-2 and HCMV pp65 was performed with the Vector NTI 7 software package from Invitrogen. Identification of potential nuclear-localization signals (NLSs) was performed with PSORT (http://psort.nibb.ac.jp) (Nakai & Horton, 1999; Nakai & Kanehisa, 1992).

Expression plasmids for RhCMV pp65-2 gene. The RhCMV pp65-2 gene was amplified from the genome of RhCMV 68-1 by using primers that overlapped the start and stop codons of Rh112 (Hansen *et al.*, 2003) and contained *Xho*I and *Eco*RI restriction-endonuclease sites, respectively, for cloning into the *Sal*I and *Eco*RI sites of the pND expression vector (Loomis-Huff *et al.*, 2001) to create the vector pND/pp65-2. The fidelity of the amplification was confirmed by sequence analysis after cloning the amplicon into pND. The plasmid pECFP-pp65-2 was constructed by inserting the pp65-2 coding sequence downstream of and in frame with that of the enhanced cyan fluorescent protein (ECFP) by using the *Bgl*II and *Eco*RI sites of the pECFP-c1 expression vector (BD Biosciences). This resulted in the expression of a chimeric ECFP–pp65-2 fusion protein under the transcriptional control of the HCMV immediate-early promoter.

Transient-transfection assay. For the preparation of antigen for a pp65-2-specific ELISA, 293T cells were transiently transfected with pND/pp65-2 according to our previously published protocol (Yue *et al.*, 2003). Cells were harvested at 48 h post-transfection and resuspended in 0·1 M glycerin/saline buffer (Yue *et al.*, 2003). Untransfected 293T-cell extracts prepared similarly served as a source of negative-control antigen.

To investigate the subcellular localization of pp65-2, the plasmids pECFP-pp65-2 and pECFP-c1 vector were transfected individually into Telo-RFs with FuGENE 6, a lipid-based transfection reagent (Roche Diagnostics) at a ratio of 3 μl FuGENE : 0·5 μg DNA in the absence of serum (Chang *et al.*, 2002). Complete DMEM was replaced with 2 ml plain DMEM prior to transfection. After a 4 h incubation at 37 °C in 5 % CO_2, the medium containing the DNA–lipid complex was removed and fresh DMEM/serum was added. Cells were either fixed with 3 % paraformaldehyde at different times post-transfection (6, 12, 24, 48 and 78 h) or harvested into lysis buffer (Vogel *et al.*, 1994) at 48 h post-transfection for Western blot analysis. The fixed cells were observed under a fluorescence microscope for the subcellular distribution of the ECFP–pp65-2 fusion protein.

Immunofluorescence assay. Telo-RFs grown on chamber slides were infected with RhCMV strain 68-1 (m.o.i. of 2). At 24 h post-infection (p.i.), the cells were fixed in glacial acetic acid : ethanol (5 : 95) for 10 min and permeabilized with CSK buffer (50 mM NaCl, 300 mM sucrose, 10 mM PIPES, 3 mM MgCl2, 0·5 % Triton X-100) for 5 min. After rinsing in PBS, the cells were blocked with 20 % (v/v) normal goat serum in PBS for 30 min at 37 °C and then were incubated in either pp65-2-specific or non-immune mouse sera (both diluted 1 : 500 in 1 % goat serum/PBS) for 1 h at 37 °C. The cells were washed three times in PBS (5 min per wash) and incubated with Alexa Fluor 594-conjugated goat anti-mouse IgG (diluted 1 : 3000 in 1 % goat serum/PBS) (Molecular Probes Invitrogen Detection Techologies) for 1 h at 37 °C. After rinsing three times with PBS, the slides were mounted with SlowFade gold

anti-fade reagent with DAPI (4,6-diamidino-2-phenylindole; Molecular Probes Invitrogen Detection Techologies) and viewed on a Zeiss fluorescence microscope.

Protein-expression analysis. Telo-RFs were infected with RhCMV strain 68-1 at an m.o.i. of 1 and harvested at 6, 24, 48 and 72 h p.i. in the presence or absence of either cycloheximide (CHX, 50 μg ml^{-1}, 6 h) or ganciclovir (GCV, 20 μg ml^{-1}, 24, 48 and 72 h). Cell lysates were harvested at the different time points p.i. for Western blot analysis (Vogel *et al.*, 1994). Mock-infected Telo-RF cells were also prepared as a negative control. pp65-2 protein expression was detected by using serum from mice immunized with the pND/pp65-2 expression plasmid. The protocol for genetic immunization in mice has been described previously (Loomis-Huff *et al.*, 2001).

Animals and sample collection. To study the humoral and cellular responses to RhCMV pp65-2 after infection, blood samples were collected from three groups of healthy, immunocompetent rhesus macaques at the California National Primate Research Center (CNPRC) (University of California, Davis, CA, USA). Group I monkeys ($n=7$, <1 year of age) were part of an earlier study that evaluated genetic immunization against herpes B virus (BV) (Loomis-Huff *et al.*, 2001). These juveniles were housed indoors and were seronegative for RhCMV at the beginning of the BV immunization study. Their RhCMV serostatus changed from negative to positive during the course of the original study following an unknown exposure to RhCMV unrelated to the immunization procedure. Blood samples were collected every 2–4 weeks over a period of 8 months. Group II consisted of 14 RhCMV-seronegative juveniles ($n=14$, 1–2 years) that were relocated to a new housing arrangement into which a lone RhCMV-seropositive adult monkey was introduced. The seronegative juveniles seroconverted to RhCMV over the course of 36 weeks following initial horizontal transmission of RhCMV from the sentinel adult (L. Strelow, Y. Yue, A. Kaur, S. S. Zhou & P. A. Barry, unpublished data). Blood samples were collected every 1–3 months over a period of 1 year. Group III consisted of 79 randomly selected animals housed either in outdoor natal groups or indoors. Of these, 74 animals had pre-existing RhCMV antibodies at the start of the investigation and five animals seroconverted to RhCMV during the period of observation. Blood samples were collected at least twice from each animal over a period of 4–7 months. Blood samples were processed for either plasma/peripheral blood mononuclear cells (PBMCs) or, in some cases, just serum.

pp65-2 ELISA. pp65-2-specific antibodies were analysed by following previously described protocols (Yue *et al.*, 2003). For the detection of pp65-2-specific antibodies, lysates of 293T cells transiently transfected with pND/pp65-2 were used as coating antigen. Lysates of untransfected 293T cells served as a source of negative-control antigen. Microtitre plates (Immunlon 4 HBX; Fisher) were coated with 0·5 μg cell extract per well in 100 μl 0·05 M carbonate/bicarbonate buffer (Lockridge *et al.*, 1999) and incubated overnight at 4 °C. All subsequent steps were carried out at room temperature. The plates were blocked with 300 μl per well of PBS containing 1 % BSA for 2 h. After washing the plate six times in PBS/0·05 % Tween 20 (wash buffer), 100 μl plasma (diluted 1 : 100 in PBS/1 % BSA/0·05 % Tween 20 dilution buffer) was added to each well for 2 h. Each plasma sample was assayed in duplicate. After washing, 100 μl peroxidase-conjugated goat anti-monkey IgG (KPL Inc.) (1 : 150 000 in dilution buffer) was added to each well for 1 h. One hundred microlitres of tetramethylbenzidine liquid substrate (Sigma) was added to each well for 30 min following a six-time washing. The reaction was terminated by the addition of 50 μl 0·5 M H_2SO_4 per well. A_{450} was recorded spectrophotometrically. For each sample, the net A_{450} was determined by subtracting the mean A_{450} values obtained in the duplicate control antigen-coated wells from the mean A_{450} obtained in the pp65-2 antigen-coated wells. Samples were considered positive if the net absorbance values were $>0·1$.

Gamma interferon (IFN-γ) ELISPOT assay. pp65-2-specific T-cell responses were quantified by an IFN-γ ELISPOT assay (U-CyTech) using a previously published protocol (Kaur *et al.*, 2002). Briefly, cryopreserved PBMCs were stimulated for 16 h with either a recombinant vaccinia virus expressing pp65-2 (rVV-pp65-2) (Kaur *et al.*, 2003) or an overlapping peptide pool (15mers overlapping by 11 aa – 134 peptides in total) representing the entire amino acid sequence of pp65-2 (BioSource). Each peptide was resuspended in 100 % DMSO at a concentration of 100 mg ml^{-1} and equal volumes of each peptide were mixed to form the pool (final concentration of each peptide in the pool, 0·75 mg ml^{-1}). The peptide pool was diluted so that the final concentration of individual peptides used for stimulation was 1 μg ml^{-1} and the concentration of DMSO did not exceed 0·5 %. Spots were counted on a KS ELISPOT Automated Reader System (Carl Zeiss) using KS ELISPOT 4.2 software (performed by Zellnet Consulting). Frequencies of pp65-2-specific cells obtained after subtracting background spots in negative-control wells (control vaccinia NYCBH strain for rVV-pp65-2 and medium for pp65-2 peptides) were expressed as the number of spot-forming cells (SFCs) per million PBMCs. pp65-2-specific T-cell frequencies $\geqslant 50$ SFCs per 10^6 PBMCs were considered positive.

Statistical analysis. Statistical analysis was carried out by using Prism (GraphPad Software). The relationship between antibodies to RhCMV and antibodies to pp65-2 was analysed by a linear-regression test.

RESULTS

RhCMV pp65 sequence analysis

Previous analysis of the RhCMV genome (strain 68-1) demonstrated that RhCMV contains two positional and sequence homologues (Rh111 and Rh112) of HCMV UL83 (pp65) (Hansen *et al.*, 2003). The two pp65 proteins predicted from the nucleotide sequences are 539 (pp65-1) and 542 (pp65-2) aa in length. They have 32 % (pp65-1) and 35 % (pp65-2) identity to HCMV pp65 and 41 % identity to each other. Alignment of the predicted amino acid sequences derived from the Rh111, Rh112 and HCMV UL83 genes indicates that conserved amino acids amongst all three ORFs were dispersed throughout the proteins, with minimal stretches of contiguous conservation (Fig. 1). There were only three regions where five consecutive amino acids were absolutely conserved. In contrast, the pp65 proteins of HCMV and chimpanzee CMV (Dolan *et al.*, 2004) are 81 % identical (data not shown). Amino acids conserved between the two RhCMV ORFs were found throughout the proteins, including the regions with minimal identity to HCMV pp65.

Previous studies have demonstrated that HCMV pp65 localizes to the nucleus of tissue-culture cells following infection or transfection with a pp65 expression vector (Gallina *et al.*, 1996; Schmolke *et al.*, 1995). Nuclear localization is due in part to the presence of multiple NLSs, although there are other domains involved (Gallina *et al.*, 1996; Schmolke *et al.*, 1995). PSORT analysis of RhCMV pp65-1 and pp65-2 (Nakai

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   HCMV pp65     (1) MESRGRRCPEMISVLGPISGHVLKAVFSRGDTPVLPHETRLLQTGIHVRVSQPSLILVSQ
RhCMV pp65-1     (1) MDLSQRYRLDYVSELGPIAGKVIQSVFDEAREPVKPHETKIVKTGLRVQVHRPSIIFLTQ
RhCMV pp65-2     (1) MASRPHRFPDLVTDLGPTSGHLVKLIFNANKEHLNPHTTYIADTHLNVRVNQPSVILATQ
   Consensus     (1) M        * *  LGP  G      F        PH T *  T   V V  PS I   Q

   HCMV pp65    (61) YTPDSTPCHRGDNQ-LQVQHTYFTGSEVENVSVNVHNPTGRSICPSQEPMSIYVYALPLK
RhCMV pp65-1    (61) FARNLKPDPRYEHNTLQIKHMTFEDQELKDVYLHVHNPTDKPITPADEPMSFFLYALPLR
RhCMV pp65-2    (61) FTPESQPCQRYDTN-LQIKHTAFEPRSLEPLVVHVHNPTDRPIPTTAEPLSMYVYALPLD
   Consensus    (61) *     P  R*  * LQ* H  F*   *     *VHNPT* *I    EP S   YALPL

   HCMV pp65   (120) MLNIPSINVHHYPSAAERKHRKHLPVADAVIHASGKQMWQARLTVSGLAWTRQQNQWKEPD
RhCMV pp65-1   (121) HLVPADLTLHPG----GTYNPELPTYDAAVQAFAQG-YHTRFNAYHLQWTQRYNRWMPHG
RhCMV pp65-2   (120) PVTPPELILRQG----ESHKRRTTVADAVVQQIDDKKWHTRLTASRLVWSRNQSRYTANS
   Consensus   (121)    *  * *  *              DA  *       **R     L W     *

   HCMV pp65   (180) VYYTSAFVFPTKDVALRHVVCAHELVCSMENTRATKMQVIG--DQYVKVYLESFCEDVPS
RhCMV pp65-1   (176) VHHTASFYVNTSPMPLWSINVANELVCSLRSTHVRKIQLVDKVKGLVRIFLESFQEETPD
RhCMV pp65-2   (176) VYHTTSFIFNSQQMPLGSMNTASELVCSIPHTHVTNIRKIS--KDEVKVYLECLQEDTPE
   Consensus   (181) V *T *F  *   **L *   A ELVCS   T**  *      *  V   LE   E *P

   HCMV pp65   (238) GKLFMHVTLGSDVEEDLTMTRNPQPFMRPHERNGFTVLCPKNMIIKPGKISHIMLDVAFT
RhCMV pp65-1   (236) DKVFIHLAWE-QSSGIITMNRNPKPFLTPQQRNGYTILNPKRLHLKPREHANVMIDTYFE
RhCMV pp65-2   (234) TKCFVHLAWE-NGNHDIVMNRNPKPYLRAHDRNGFTILCPQTLHLKPGKTSHLMFDVCFE
   Consensus   (241)  K F H****      * M*RNP*P *    RNG T*L P  ***KP      M D  F*

   HCMV pp65   (298) SHEHFGLLCPKSIPGLSISGNLLMNGQQIFLEVQAIRETVELRQYDPVAALFFFDIDLLL
RhCMV pp65-1   (295) SDKYIGFICPKSVPGCSISCNPIMSTQCIFMEIRSLHDSVYIEPFQAIASLHFFDRNLFF
RhCMV pp65-2   (293) SDKYVAIICPRTIPGISMSCNPLLPLQNLFMEIKAVHESLYIEQYEELGWLYFFDRKMIL
   Consensus   (301) S***   *CP   PG S S*N*    Q  F*E*   * * ***       L FFD*

   HCMV pp65   (358) QRG-PQYSEHPTFTSQYRIQGKLEYRHTWDRHDEGAAQGDDDVWTSGSDSDEELVTTERK
RhCMV pp65-1   (355) TYKKTDHSIFR---DQYRITTSFEYHQGKG-VPIVQDSSEDDSSSSSESEDMDVFEVAS-
RhCMV pp65-2   (353) TKGTATEPAQARLVDQHRLMAKLEYHH------VYGDQADDESGSSASDSDMEVFRHPPS
   Consensus   (361) *             *Q R     EY*          *   D * *S    D* *

   HCMV pp65   (417) TPRVTGGGAMAGASTSAGRKRKSASSATACTSGVMTRGRLKAESTVAPEEDTDEDSDNEI
RhCMV pp65-1   (410) ------TTPHAGTSSSASKKRE------KLYKPLVQRKRFTEDDAAKKSSS-DDENDDFF
RhCMV pp65-2   (407) -----GSSARRPSQPPASSSRKPSS--SAASSTMTTRSKHRVTKAETSDESEEDDDDRET
   Consensus   (401)                  A*  R              R       *     *  *  D

   HCMV pp65   (477) HNPAVFTWPPWQAGILARNLVPMVATVQGQNLKYQEFFWDANDIYRIFAELEGVWQPAAQ
RhCMV pp65-1   (457) P---TLYWGIWQFGVRATELTPMIASVCGDQLPHQEFSWEGDDDLRIFSGLSGSWHMYQA
RhCMV pp65-2   (460) L---VFSWPNWQCGIKSTSLVPIVASAHGDRLPYQDFPWGEGDDYRTFSGIEIDLQPYQT
   Consensus   (451)        W  WQ G   * L P  A   G* L* Q F W   D* R F**       **

   HCMV pp65   (537) PKRRRHR-QDALPGPCIASTPKKHRG
RhCMV pp65-1   (514) PKRRHYSPSEQLPSTSGESVSKRPCE
RhCMV pp65-2   (517) QRRRRHTRLEPIPESCTVSVPKKHRS
   Consensus   (501)   RR      *  P     S* K
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Fig. 1. Alignment of RhCMV strain 68-1 pp65-1 and pp65-2 and HCMV pp65. Identical amino acids conserved in all three proteins are marked (consensus) and identical amino acids conserved only in RhCMV pp65-1 and pp65-2 are marked by asterisks. The critical lysine residue for protein kinase activity of HCMV pp65 (Yao *et al.*, 2001) is marked by an arrow. The NLSs predicted by PSORT are boxed.

& Horton, 1999; Nakai & Kanehisa, 1992) revealed that, of four potential NLSs, two have been conserved in RhCMV pp65-1 and three in pp65-2 (Fig. 1). Only one NLS was common to both RhCMV pp65 homologues.

RhCMV pp65-2 expression kinetics and virion association

The kinetics of pp65-2 protein expression following RhCMV infection of Telo-RFs were analysed by Western blot (Fig. 2a). There was a large increase in the intensity of pp65-2 between 24 and 48 h p.i., with abundant steady-state levels present also at 72 h p.i. The approximate size of pp65-2 detected by Western blot (63 kDa) was consistent with the predicted size of 61·7 kDa (Hansen *et al.*, 2003). Expression of pp65-2 was abrogated almost completely in the presence of the replication inhibitor GCV, consistent with true late-expression kinetics. There was faint staining observed at 6 h p.i. in both the presence and absence of the protein-synthesis inhibitor CHX. This was probably due to the uptake of the protein from the infecting virion following viral attachment and entry. This interpretation was substantiated by Western blot analysis demonstrating the presence of an immunoreactive protein of the expected size within sucrose gradient-purified RhCMV virions (Fig. 2b).

Nuclear localization of pp65-2

To determine whether pp65-2 localized to the nucleus, a plasmid expressing an ECFP–pp65 fusion protein

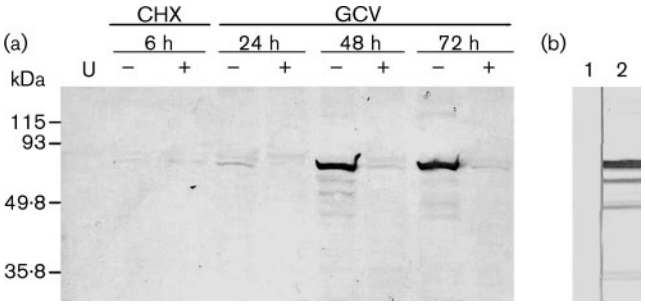

Fig. 2. (a) Kinetics of RhCMV pp65-2 expression. Telo-RF cells were infected with 1·0 m.o.i. RhCMV strain 68-1 in the presence or absence of cycloheximide (CHX) or ganciclovir (GCV). Cell extracts were prepared at 6, 24, 48 and 72 h p.i. Expression of RhCMV pp65-2 was detected by Western blot analysis using mouse anti-pp65-2 serum. U, Uninfected Telo-RF extracts. (b) Virion association of RhCMV pp65-2. The presence of pp65-2 in virions was analysed on the immunoblot of sucrose gradient-purified RhCMV virions with normal mouse serum (lane 1) and mouse anti-pp65-2 serum (lane 2).

(pECFP-pp65-2) was constructed and transfected into Telo-RFs. The parental plasmid (pECFP-C1) was transfected in parallel as a control. Western blot analysis confirmed the expression of the appropriately sized fusion protein (data not shown). At 6 h post-transfection, the ECFP–pp65-2 fusion protein was either diffused throughout the cell or concentrated almost exclusively within the nucleus (Fig. 3a). The latter pattern was characterized by relatively uniform nuclear staining together with a few punctate nodes of intense staining. At 78 h post-transfection, staining was confined to numerous intense foci of variable size within the nucleus (Fig. 3c). In contrast, the control ECFP protein remained distributed uniformly throughout the cell post-transfection (Fig. 3b, d).

The nuclear localization of pp65-2 was also observed in RhCMV-infected Telo-RFs. Immunofluorescent staining of RhCMV-infected Telo-RFs with pp65-2-specific mouse serum showed that pp65-2 was detected in both the cell nucleus and the cytoplasm at 24 h p.i. The pattern of nuclear accumulation was similar to that of ECFP–pp65-2 in transfected Telo-RFs (Fig. 3e–g).

Humoral immune responses to pp65-2

RhCMV is ubiquitous in captive rhesus macaques, with an infection rate of almost 100 % by the first year of age (Vogel *et al.*, 1994). To determine whether pp65-2 was immunogenic in RhCMV-seropositive macaques, a pp65-2-specific ELISA was developed. Antibody (IgG) responses to pp65-2 were characterized in two groups of seronegative juvenile macaques (groups I and II, $n=7$ and 14, respectively) that underwent a primary infection following natural exposure to RhCMV and a third group of animals of mixed ages and RhCMV serostatus (group III, $n=79$). The difference between groups I and II was that the source(s) of infectious virus for group I was not known, whereas the source of infectious virus for group II was a single seropositive adult macaque that was co-housed with the seronegative juveniles.

A total of 20 monkeys of the 21 animals comprising groups I and II (95 %) developed IgG to pp65-2 during primary infection (Fig. 4a; data from only four animals are presented). Generally, binding antibodies to pp65-2 developed contemporaneously with antibodies to a total viral-antigen preparation consisting of structural and non-structural viral proteins. Antibody responses to pp65-2 reached maximal levels prior to the time of peak responses to total viral antigens. In addition, pp65-2 antibody responses declined over time and became undetectable in approximately 50 % of the animals over a period of 2–6·5 months since the first time point of detectable pp65-2-binding antibodies. The levels of pp65-2-binding antibodies were quite variable

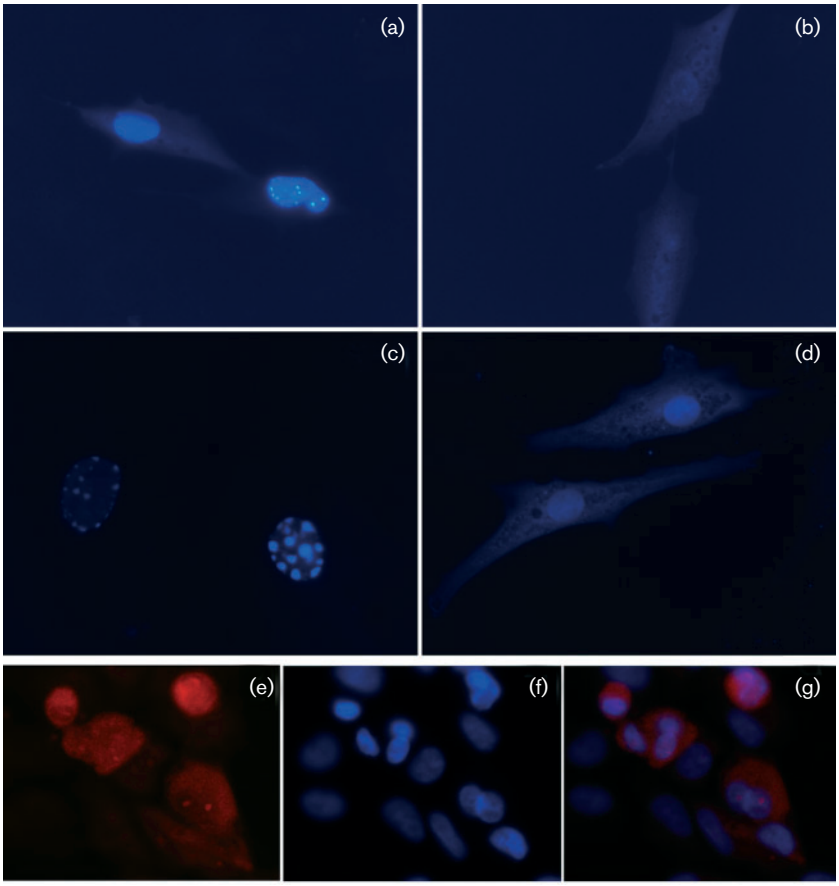

Fig. 3. Localization of RhCMV pp65-2 in expression plasmid-transfected and RhCMV-infected Telo-RFs. Telo-RFs were either transfected with pECFP-pp65-2 plasmid or pECFP vector, or infected with RhCMV strain 68-1 (m.o.i. of 2). The localization of pp65-2 in transfected or infected Telo-RFs was viewed under a microscope (Zeiss, 40×). (a) ECFP-tagged pp65-2 at 6 h post-transfection. (b) ECFP at 6 h post-transfection. (c) ECFP-tagged pp65-2 at 78 h post-transfection. (d) ECFP at 78 h post-transfection. (e) RhCMV-infected Telo-RFs were fixed at 24 h p.i. and stained with mouse anti-pp65-2 antibody. (f) The nuclei of RhCMV-infected Telo-RFs were stained with DAPI. (g) Colocalization of pp65-2 and nuclei.

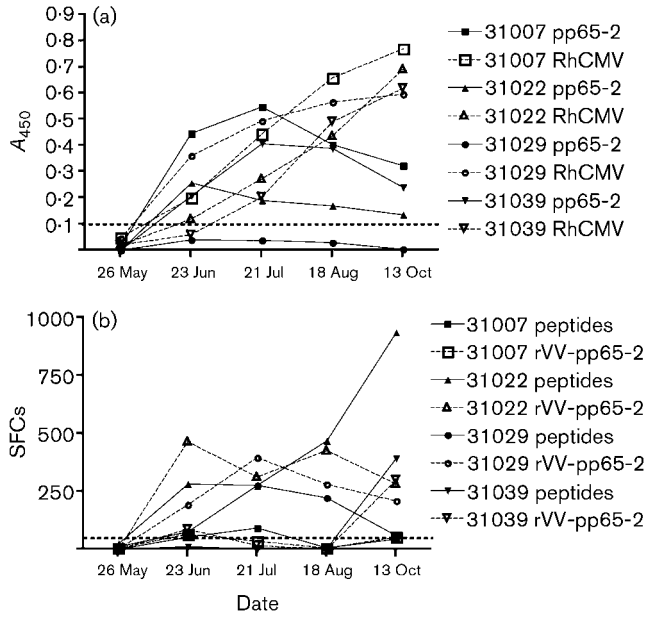

Fig. 4. Ontogeny of pp65-2-specific immune responses after primary infection. Longitudinal serum samples were collected from 21 juvenile rhesus macaques that seroconverted following natural exposure to RhCMV. Antibodies to RhCMV and pp65-2 were analysed by antigen-specific ELISA (a). 31022 and 31007 represented 17 of 20 animals in which antibodies to pp65-2 and total RhCMV antigens developed concurrently. 31039 represents three of 20 animals in which antibodies to pp65-2 were detected earlier than to a total RhCMV antigen preparation. 31029 was the animal that lacked detectable antibodies to pp65-2. pp65-2-specific T-cell responses were evaluated in these four monkeys by IFN-γ ELISPOT assay after restimulating PBMCs with rVV-pp65-2 or pp65-2 overlapping peptide pool (b). SFCs represents the number of spot-forming cells per million PBMCs. A_{450} represents absorbance at wavelength 450 nm. Dashed lines represent the cutoff values for ELISA and ELISPOT.

between the infected monkeys. Taken together, these data suggested that the humoral responses to RhCMV pp65-2 were elicited early, varied considerably among individuals and were not always sustained after production.

The pattern of pp65-2-specific antibodies was analysed during chronic infection in a pool of randomly selected, RhCMV-seropositive monkeys ($n=79$). A total of 46 monkeys (58 % of RhCMV-seropositive animals screened) had detectable IgG to pp65-2 at least once during a 4–7 month period (Table 1). Of these, 27 (59 % of those with pp65-2 antibodies) either had declining or fluctuating responses to pp65-2 over a period of time when contemporaneous responses to total RhCMV antigens remained relatively stable. Only five of the pp65-2-positive animals (11 %) exhibited stable antibody responses over time. As RhCMV DNA was detected periodically in the saliva and genital secretion, the fluctuating pp65-2-specific antibody responses may indicate the reactivation of RhCMV or reinfection (Huff *et al.*, 2003). Statistical analysis showed that the antibody responses to pp65 and those directed against total RhCMV antigens were related ($P<0{\cdot}001$; $r=0{\cdot}256$). This result was consistent with the interpretation that antibodies to pp65-2 were not dominant during chronic infection.

Cellular immune responses to pp65-2

Multiple studies have demonstrated the importance of cellular immune responses in limiting the pathogenic potential of HCMV (Li *et al.*, 1994; Reusser *et al.*, 1991, 1997, 1999; Walter *et al.*, 1995). The kinetics of cellular responses to pp65-2 during primary infection were analysed in four animals. Three of the four animals developed detectable pp65-2 IgG during the period of observation, whereas all four were positive for T cells secreting IFN-γ following stimulation with pp65-2 antigen (Fig. 4). The cellular responses to pp65-2 varied between animals. The peak responses ranged from 66 to 466 SFCs per million PBMCs following stimulation with an overlapping peptide pool of pp65-2 and from 94 to 938 SFCs per million PBMCs following stimulation with rVV-pp65-2. The kinetics of the pp65-2-specific T cells were generally concordant with seroconversion to RhCMV. It was noted that one animal (MMU31039; Fig. 4b) that was negative for antibody against pp65-2 exhibited robust T-cell responses (280–390 SFCs per 10^6 PBMCs) following stimulation with either rVV-pp65-2 or an overlapping peptide pool comprising the entire protein. It was also noted that the cellular responses declined over time in this animal.

To determine whether cellular responses persisted over time, a cross-sectional study was performed by using PBMCs from 20 long-term RhCMV-seropositive monkeys, ten of which were positive for IgG to pp65-2 and ten that were negative. Unlike long-term antibody responses to pp65-2, cellular responses were mostly retained in chronically infected animals (80 %, 16/20). Eight monkeys in each of the pp65-2 antibody-negative and -positive groups had detectable T-cell responses to either or both sources of pp65-2 antigen (Table 2). Conversely, two monkeys were IFN-γ ELISPOT-negative/IgG-positive and two were IFN-γ ELISPOT-negative/IgG-negative with respect to pp65-2. Although the frequencies of IFN-γ-positive T cells in some of the pp65-2 antibody-positive animals were higher than the highest frequencies observed in pp65-2 antibody-negative animals, there was no statistical difference between the two groups. The ages of the animals at which seroconversion occurred for this group were unknown. However, the dates of birth and times of the PBMC isolation were consistent with an interval of up to 2 years between primary infection and the time of sampling, based on previous seroepidemiological studies of colony-reared macaques (Vogel *et al.*, 1994). Thus, T-cell responses to pp65-2 appeared to persist for years.

Table 1. Summary of longitudinal antibody responses to RhCMV (total antigen preparation) and pp65-2

RhCMV*	pp65-2*	*n*	MMU†	DOB‡	Sample date	RhCMV§	pp65-2§
Stable	Stable	5	33012	25 Mar 2001	6 Mar 2002	1·26	1·02
					8 May 2002	1·37	1·01
					6 Jun 2002	1·38	0·97
Stable	Decreasing	24	33035	29 Mar 2001	11 Dec 2001	1·30	0·52
					23 Apr 2002	1·34	0·24
					8 Jul 2002	1·32	0·07
			32879	6 Mar 2001	11 Dec 2001	1·42	0·62
					23 Apr 2002	1·52	0·39
					8 Jul 2002	1·59	0·31
Stable ($n=8$) or increasing ($n=6$)	Increasing	14	33441	8 Jun 2001	11 Dec 2001	0·21	0·33
					23 Apr 2002	1·77	0·57
Stable	Fluctuating	3	32250	27 Mar 2000	11 Dec 2001	1·37	0·10
					28 Jan 2002	1·31	0·02
					17 Apr 2002	1·44	0·25
					3 Jun 2002	1·43	0·23
					18 Jul 2002	1·42	0·25

*Antibody responses to a total RhCMV antigen preparation (RhCMV) and pp65-2 were characterized as stable, increasing/decreasing or fluctuating if the absorbance values varied by $<20\,\%$ (stable), $\pm\ \geqslant 20\,\%$ (increasing or decreasing) or exhibited changes up and down over time of $\geqslant 20\,\%$ (fluctuating), respectively.
†*Macaca mulatta*.
‡Date of birth.
§Anti-RhCMV and -pp65-2 antibody responses in five representative monkeys were demonstrated by ELISA (A_{450}).

DISCUSSION

The protein encoded by the Rh112 ORF of RhCMV (Hansen *et al.*, 2003), pp65-2, is expressed late in the replication cycle, localizes to the nucleus after expression, is present within the mature virion and elicits both humoral and cellular immune responses. As such, RhCMV pp65-2 bears strong similarity to the pp65 protein (UL83) of HCMV in terms of genomic collinearity, sequence homology, expression kinetics, localization within the cell and virion, and immunogenic profile (Boppana & Britt, 1996; Chee *et al.*, 1990; Depto & Stenberg, 1989; Gallina *et al.*, 1996; Geballe *et al.*, 1986; Gyulai *et al.*, 2000; Jin *et al.*, 2000; McLaughlin-Taylor *et al.*, 1994; Ohlin *et al.*, 1995; Sanchez *et al.*, 1998; Schmolke *et al.*, 1995; van Zanten *et al.*, 1995; Wills *et al.*, 1996).

The localization of RhCMV pp65-2 in discrete areas within the nucleus is consistent with the patterns identified for the pp65 proteins of HCMV and guinea pig CMV (GPCMV) (Gallina *et al.*, 1996; McGregor *et al.*, 2004; Schleiss *et al.*, 1999; Schmolke *et al.*, 1995). The punctate staining pattern in our study is consistent with co-localization in the nucleoli, although this remains to be proved formally. If substantiated, this would be similar to that of HCMV pp65, which has been shown to traffic rapidly to nucleoli after infection (Arcangeletti *et al.*, 2003). The early distribution of virion-associated pp65 to the nucleus, recapitulated by pp65 expressed from a plasmid, suggests a possible regulatory role in the growth of CMV-infected cells, virus replication and transcription, and subviral particle assembly (Arcangeletti *et al.*, 2003; Sanchez *et al.*, 1998; Sindre *et al.*, 2000). Whatever functions pp65 performs, the distribution of the protein within the cell nucleus further suggests that it may be conserved amongst HCMV, RhCMV and GPCMV.

HCMV pp65 represents a critical vaccine candidate because it is an immunodominant target of host cell-mediated immunity, and HCMV-specific CTLs are vital for protection from HCMV pathogenesis associated with transplantation (Riddell *et al.*, 1992; Walter *et al.*, 1995). In the present study, pp65-2-specific IFN-γ-secreting T lymphocytes were detected in the peripheral blood of all animals with primary infection and roughly two-thirds of RhCMV-seropositive macaques with long-standing RhCMV infection following stimulation of PBMCs with either rVV-pp65-2 or an overlapping pp65-2 peptide pool. It has been reported that recombinant vaccinia virus-delivered antigen mainly stimulates specific $CD8^+$ T cells and that 15mer overlapping peptide pool stimulates both specific $CD4^+$ and $CD8^+$ T cells. The disparate results between rVV-pp65-2 and pp65-2 peptide stimulation indicate that pp65-2 is likely to elicit both $CD4^+$ and $CD8^+$ T-cell responses. As the macaque colony at the CNPRC is an outbred population with multiple MHC haplotypes (Penedo *et al.*, 2005), the results

Table 2. Cellular and humoral immune responses to pp65-2 in RhCMV-seropositive macaques

Animal (RhCMV$^+$)	pp65-2 IgG	SFCs (per million PBMCs)*		Summary of pp65-2 immune responses
		rVV-pp65-2	pp65-2 peptide pool	
32076	+	2960	2616	Ab$^+$/T cell$^+$ ($n=8$)
32250	+	1560	1352	
31846	+	396	362	
32969	+	390	538	
33437	+	126	72	
32101	+	124	(0)	
33405	+	56	50	
32879	+	(40)	70	
32858	+	(28)	(44)	Ab$^+$/T cell$^-$ ($n=2$)
33431	+	(0)	(22)	
33317	−	466	510	Ab$^-$/T cell$^+$ ($n=8$)
32175	−	452	364	
33349	−	78	64	
33014	−	78	226	
33428	−	78	(0)	
32156	−	66	(0)	
33100	−	(0)	74	
33035	−	(0)	96	
33368	−	(36)	(36)	Ab$^-$/T cell$^-$ ($n=2$)
33007	−	(0)	(30)	

* A response of $\geqslant 50$ SFCs per 10^6 PBMCs was considered a positive, antigen-specific response. Numbers in parentheses were considered a negative response to pp65-2.

indicate that pp65-2 contains epitopes recognized in the context of diverse HLA backgrounds. The results of this study, however, do not attest to either the relative cellular immunodominance of pp65-2 or the protective efficacy of these responses in limiting the extent of RhCMV replication. The ontogeny of RhCMV pp65-2-specific cell-mediated immunity observed in this study was coincident with the clearance of viral DNA from the blood following either experimental inoculation with or natural exposure to RhCMV observed in previous studies (Lockridge *et al.*, 1999; Sequar *et al.*, 2002; L. Strelow, Y. Yue, A. Kaur, S. S. Zhou & P. A. Barry, unpublished data). CD8$^+$ CTLs against MHC-matched target cells either pulsed with an overlapping pp65-2 peptide pool or infected with rVV-pp65-2 have been observed in the peripheral blood of naturally infected macaques by using pp65-2 antigen-stimulated PBMCs (A. Kaur, unpublished data). However, a previous report has noted that an early RhCMV protein(s) was primarily responsible for stimulating cytolytic activity of bulk peripheral CTLs in seropositive macaques (Kaur *et al.*, 1996). This observation, together with our characterization of pp65-2 as a protein expressed with late-gene expression kinetics, would suggest that RhCMV pp65-2 may not have as prominent a role in generating CTL activity as HCMV pp65 does in infected humans. This conclusion should be tempered by recent studies evaluating HCMV-specific CTL activity in the context of HCMV-mediated immune modulation. The breadth and specificity of CTL activity to HCMV proteins are greater when target cells are infected with a variant of HCMV lacking the US2, 3, 6 and 11 ORFs, which downmodulate antigen presentation in the context of MHC class I (Manley *et al.*, 2004). Moreover, studies examining T-cell responses to CD8$^+$ epitopes of 14 HCMV antigens predicted by HLA-binding algorithms or overlapping peptide pools comprising 213 HCMV ORFs also demonstrated that T-cell recognition of HCMV is very broad, including functionally and kinetically diverse ORFs (Elkington *et al.*, 2003; Sylwester *et al.*, 2005). As RhCMV expresses functional homologues of these immunomodulatory ORFs of HCMV (Pande *et al.*, 2005), the relative dominance of RhCMV proteins in stimulating CTL activity should be examined with a modified variant of RhCMV lacking MHC class I-modulating activity or, alternatively, overlapping peptide pools.

The homology between pp65-1 and pp65-2 raises the question of antibody and T-cell cross-reactivity of these two proteins. pp65-2-specific antibodies, generated in pp65-2 expression plasmid-vaccinated mice, do not cross-react by Western blot with an epitope (FLAG)-tagged pp65-1 expressed in transfected mammalian cells (data not shown). Analysis of plasma from 12 monkeys infected naturally with RhCMV (group II) and four experimentally infected animals indicates that only a minority of infected animals generated antibodies to pp65-1 (data not shown). Of the 16 infected monkeys, two animals had weak responses and only

two had robust reactivity (data not shown). The antibody status of pp65-1 in these animals was not correlated to the levels of pp65-2 antibodies, suggesting that there is little cross-reactivity of pp65-1 antibodies to pp65-2. Similar findings have been noted for a lack of cross-reactivity of cellular responses. The cellular immune responses to pp65-1 and pp65-2 are largely independent of each other (L. Picker, personal communication), consistent with the interpretation that our results reflect pp65-2-specific immune responses.

Infection of rhesus macaques with RhCMV strongly recapitulates HCMV persistence and pathogenesis in humans (Barry & Chang, 2005), thereby offering a system to address HCMV vaccine strategies in a non-human primate host that complements other animal models (Schleiss & Heineman, 2005). Together with previous reports demonstrating the conservation of sequence and immunogenic determinants of the glycoprotein B (gB) proteins of primate CMV representatives, the results presented herein enable the macaque model to be used for evaluating the pp65- and gB-based vaccine strategies proposed for humans (Gonczol & Plotkin, 2001).

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