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Cloning and characterization of rhesus cytomegalovirus glycoprotein B

Rachel H. Kravitz,[1] Kathryn S. Sciabica,[2] Kiho Cho,[1] Paul A. Luciw[1] and Peter A. Barry[1]

[1] Department of Medical Pathology, Room 3431 Tupper Hall, University of California, Davis, CA 95616, USA

[2] Department of Microbiology, University of California, Irvine, California, USA

Rhesus cytomegalovirus (RhCMV) infection of rhesus macaques is an important model to investigate critical issues of cytomegalovirus biology. To better understand host immunological responses to viral glycoproteins, the glycoprotein B (gB) gene of RhCMV was molecularly cloned, sequenced and characterized. Transcription analysis revealed that RhCMV gB was transcribed as a late gene. The RhCMV gB gene encoded a predicted protein of 854 amino acids that was 60% identical/75% similar to the human CMV (HCMV) gB protein. The region of HCMV gB proposed to be responsible for virus binding to host cells, fusion and cell-to-cell spread was the most highly conserved region with RhCMV gB (74% identity/85% similarity). Conserved elements included 11 of 12 cysteine residues, 14 of 16 potential *N*-linked glycosylation sites and cross-reactive epitopes. Metabolic labelling experiments demonstrated that RhCMV gB was proteolytically processed similarly to HCMV gB. These results are critical for investigating virus–host relationships in CMV-infected primates.

Pathogenicity of human cytomegalovirus (HCMV) correlates with the immune status of infected individuals. In fully immunocompetent individuals, host antiviral immune responses contain virus replication. In contrast, HCMV can be a serious cause of morbidity and mortality in individuals without an intact immune system (Ho, 1991). Greater understanding of both host antiviral immune responses and mechanisms of viral pathogenesis is required to design strategies which effectively limit HCMV disease. Infection of rhesus macaques (*Macacca mulatta*) with rhesus CMV (RhCMV) facilitates investigation of these key issues. This report describes the molecular cloning and characterization of the RhCMV gB gene at the DNA, RNA and protein levels. This study was undertaken since the gB protein of HCMV constitutes the major target of host neutralizing antibodies in HCMV infection (Britt, 1984). The results demonstrated that the primate CMV gB proteins have been strongly conserved in sequence, processing patterns and immunogenic properties.

Author for correspondence: Peter Barry.
Fax +1 916 752 4548. e-mail pabarry@ucdavis.edu

The GenBank accession numbers for the sequences discussed are U41526, U41527 and U59238.

To isolate the RhCMV gB gene, MRC-5 cells (human lung fibroblasts) were infected with RhCMV strain 68-1 (Asher *et al*., 1974) at a m.o.i. equal to 0·1, and DNA was harvested when the cells showed 100% cytopathic effect (CPE). Viral DNA was cleaved with methyl sensitive restriction endonucleases (i.e. *Cla*I, *Sal*I) and cloned into commercially available plasmid vectors. Termini of inserts from random clones were sequenced, and the DNA and predicted amino acid sequences were aligned with HCMV (Chee *et al*., 1990). One clone, pCla-1, was identified which contained an approximately 21 kb insert with strong sequence homology to the gB gene (UL55) of HCMV (nucleotides 82180 to 82440). The sequenced portion of pCla-1 corresponded to a region immediately upstream of the cleavage site identified for the HCMV protein (Spaete *et al*., 1988) (discussed below). Oligonucleotide primers were designed to sequences within the putative coding region of RhCMV gB (PAB 193 and 194, Table 1), and the 3′ portion of the gene was cloned by the 3′ RACE protocol (Frohman *et al*., 1988). The amplified product was cloned directly into the *Cla*I site within the RhCMV gB gene to produce a full-length RhCMV gB. The gene was sequenced by three methods, all modifications of Sanger dideoxy sequencing. Most of the gene was sequenced using the Sequenase 2.0 (Amersham) protocols. Areas of higher GC content were sequenced with either the Promega *fmol* kit or the Oncor sequencing kit. Both strands of the gene were sequenced in their entirety. Analysis of RhCMV gB sequence motifs, predicted amino acid structure and homology to HCMV gB was performed with the Program Manual for the Wisconsin Package (V.8; Genetics Computer Group, Madison, Wis., USA).

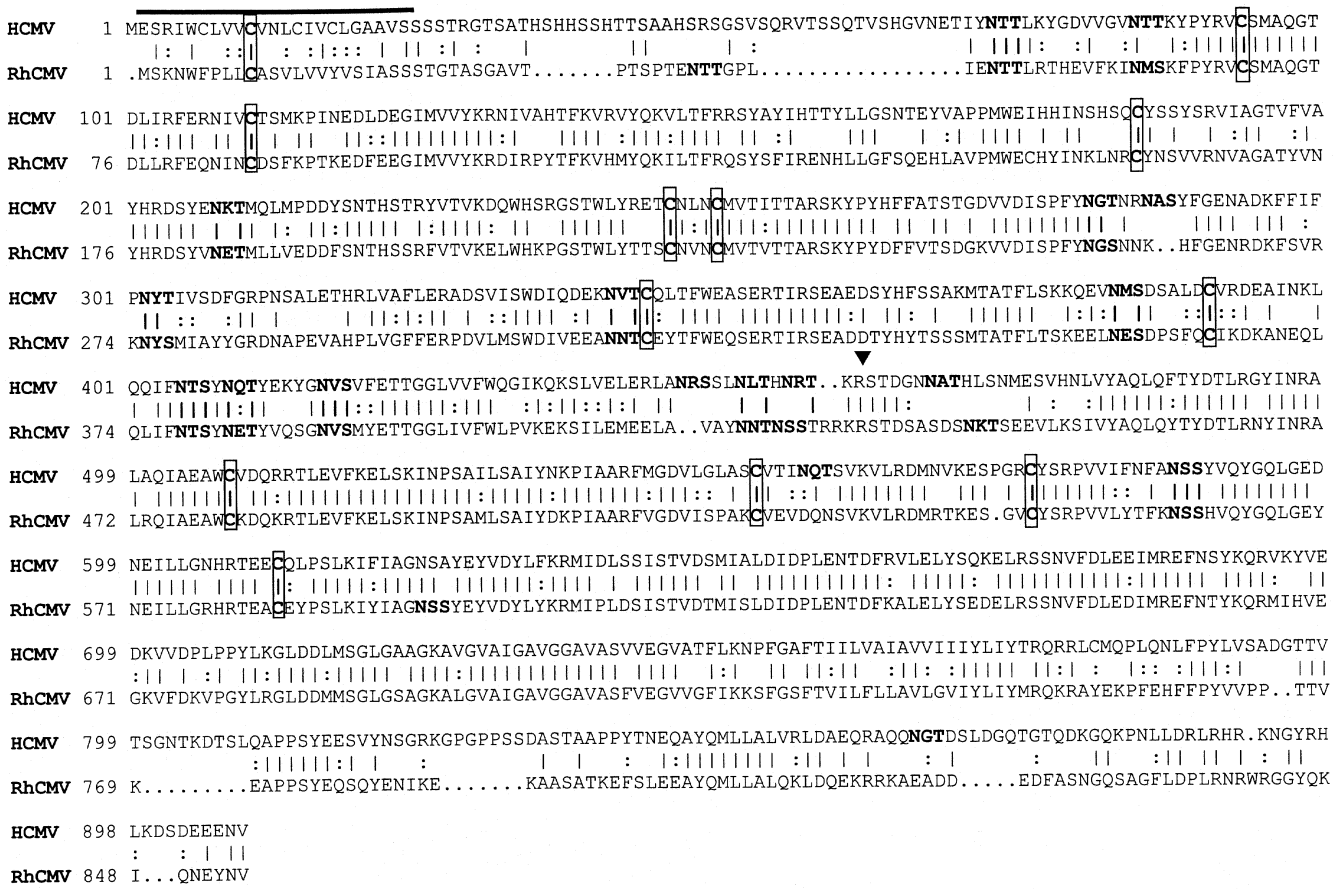
The RhCMV gB gene exhibited significant homology to HCMV gB, and had remarkably little variation between two different RhCMV isolates (discussed below). RhCMV gB DNA was sequenced beginning 418 bases upstream from the start

Table 1. Primers for amplification

Primer	Sequence*	Primer location†
PAB 188	5′ ATAATGATAGGTATCATCAGCC 3′	1295–1274
PAB 191	5′ CTCCGATCTAATTGTGCG 3′	1274–1257
PAB 193	5′ CGAGACATATGTACAATCTGG 3′	1424–1444
PAB 194	5′ CTATGTATGAAACCACTGG 3′	1453–1471
PAB 227	5′ CGTGTTTGCGTGACGACTTT 3′	5–24
PAB 241	5′ CGCCCAGTCTTATGTTTAATGAGC 3′	3014–2991

* All sequences are listed in the 5′ to 3′ orientation.

† Base numbers refer to the primer location within RhCMV 68-1 as defined in GenBank submissions U41526 and U41527, with the start site of transcription (+1) at position 419 of U41526.



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HCMV    1 MESRIWCLVVCVNLCIVCLGAAVSSSSTRGTSATHSHHSSHTTSAAHSRSGSVSQRVTSSQTVSHGVNETIYNTTLKYGDVVGVNTTKYPYRVCSMAQGT
RhCMV   1 .MSKNWFPLLCASVLVVYVSIASSSTGTASGAVT.......PTSPTENTTGPL.................IENTTLRTHEVFKINMSKFPYRVCSMAQGT

HCMV  101 DLIRFERNIVCTSMKPINEDLDEGIMVVYKRNIVAHTFKVRVYQKVLTFRRSYAYIHTTYLLGSNTEYVAPPMWEIHHINSHSQCYSSYSRVIAGTVFVA
RhCMV  76 DLLRFEQNINCDSFKPTKEDFEEGIMVVYKRDIRPYTFKVHMYQKILTFRQSYSFIRENHLLGFSQEHLAVPMWECHYINKLNRCYNSVVRNVAGATYVN

HCMV  201 YHRDSYENKTMQLMPDDYSNTHSTRYVTVKDQWHSRGSTWLYRETCNLNCMVTITTARSKYPYHFFATSTGDVVDISPFYNGTNRNASYFGENADKFFIF
RhCMV 176 YHRDSYVNETMLLVEDDFSNTHSSRFVTVKELWHKPGSTWLYTTSCNVNCMVTVTTARSKYPYDFFVTSDGKVVDISPFYNGSNNK..HFGENRDKFSVR

HCMV  301 PNYTIVSDFGRPNSALETHRLVAFLERADSVISWDIQDEKNVTCQLTFWEASERTIRSEAEDSYHFSSAKMTATFLSKKQEVNMSDSALDCVRDEAINKL
RhCMV 274 KNYSMIAYYGRDNAPEVAHPLVGFFERPDVLMSWDIVEEANNTCEYTFWEQSERTIRSEADDTYHYTSSSMTATFLTSKEELNESDPSFQCIKDKANEQL

HCMV  401 QQIFNTSYNQTYEKYGNVSVFETTGGLVVFWQGIKQKSLVELERLANRSSLNLTHNRT..KRSTDGNNATHLSNMESVHNLVYAQLQFTYDTLRGYINRA
RhCMV 374 QLIFNTSYNETYVQSGNVSMYETTGGLIVFWLPVKEKSILEMEELA..VAYNNTNSSTRRKRSTDSASDSNKTSEEVLKSIVYAQLQYTYDTLRNYINRA

HCMV  499 LAQIAEAWCVDQRRTLEVFKELSKINPSAILSAIYNKPIAARFMGDVLGLASCVTINQTSVKVLRDMNVKESPGRCYSRPVVIFNFANSSYVQYGQLGED
RhCMV 472 LRQIAEAWCKDQKRTLEVFKELSKINPSAMLSAIYDKPIAARFVGDVISPAKCVEVDQNSVKVLRDMRTKES.GVCYSRPVVLYTFKNSSHVQYGQLGEY

HCMV  599 NEILLGNHRTEECQLPSLKIFIAGNSAYEYVDYLFKRMIDLSSISTVDSMIALDIDPLENTDFRVLELYSQKELRSSNVFDLEEIMREFNSYKQRVKYVE
RhCMV 571 NEILLGRHRTEACEYPSLKIYIAGNSSYEYVDYLYKRMIPLDSISTVDTMISLDIDPLENTDFKALELYSEDELRSSNVFDLEDIMREFNTYKQRMIHVE

HCMV  699 DKVVDPLPPYLKGLDDLMSGLGAAGKAVGVAIGAVGGAVASVVEGVATFLKNPFGAFTIILVAIAVVIIIYLIYTRQRRLCMQPLQNLFPYLVSADGTTV
RhCMV 671 GKVFDKVPGYLRGLDDMMSGLGSAGKALGVAIGAVGGAVASFVEGVVGFIKKSFGSFTVILFLLAVLGVIYLIYMRQKRAYEKPFEHFFPYVVPP..TTV

HCMV  799 TSGNTKDTSLQAPPSYEESVYNSGRKGPGPPSSDASTAAPPYTNEQAYQMLLALVRLDAEQRAQQNGTDSLDGQTGTQDKGQKPNLLDRLRHR.KNGYRH
RhCMV 769 K.........EAPPSYEQSQYENIKE.......KAASATKEFSLEEAYQMLLALQKLDQEKRRKAEADD.....EDFASNGQSAGFLDPLRNRWRGGYQK

HCMV  898 LKDSDEEENV
RhCMV 848 I...QNEYNV
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Fig. 1. Comparison of HCMV gB protein with the predicted RhCMV gB protein. Alignments for the gB amino acid sequences of RhCMV (bottom) and HCMV (top) are presented. Identical amino acids are indicated by a vertical line, similar amino acids by two dots. Conserved cysteine residues (boxed), potential *N*-linked glycosylation sites (bold), hydrophobic leader region (overlined) and proteolytic processing site (arrowhead) are indicated. The RhCMV gB isolate 22659 had the following changes at the predicted amino acid level: (1) Asp to Gly at position 513 (RhCMV), (2) Leu to Pro at 789 (RhCMV), (3) Trp to Arg at 841 (RhCMV).

site of transcription (+1) through the site of polyadenylation (+3055); mapping of the mRNA transcript is discussed below. This region of RhCMV gB was 64% identical with the corresponding region of HCMV gB. The region encompassing the promoter regulatory elements (−418 to −1) was 76% identical. A potential TATA box (ATATAA) was located between −30 and −25. The RhCMV gB gene contained a long open reading frame from +189 to +2846, with a consensus start codon at +278 (Kozak, 1989). Coding regions (+278 to +2843) were 62% identical to HCMV.

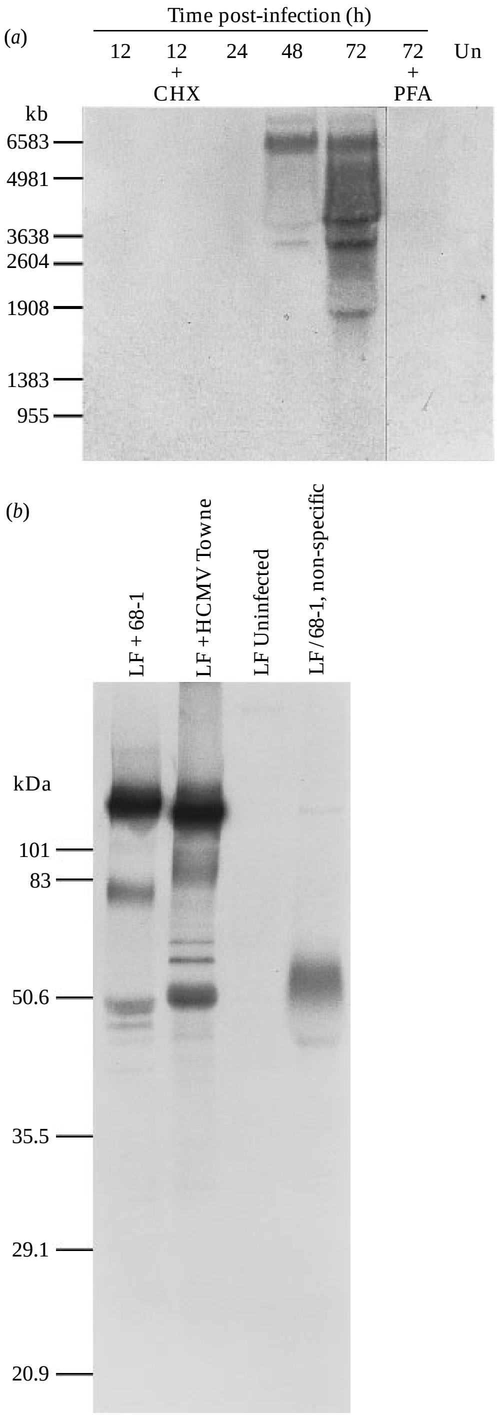

Fig. 2. (*a*) RNA expression kinetics of RhCMV gB. Human lung fibroblasts (LFs) were infected with RhCMV 68-1, and RNA was harvested at 12 h in the absence and presence of cycloheximide (CHX), 24, 48 and 72 h in the absence and presence of phosphonoformic acid (PFA). RNA from uninfected cells (Un) was also analysed. RNA marker sizes are indicated on the left. (*b*) Radio-immune precipitation of RhCMV gB. Metabolically labelled LF cell extracts from cultures infected with RhCMV (lanes 1 and 4), HCMV (lane 2) or uninfected cells (lane 3) were immunoprecipitated with anti-HCMV monoclonal antibodies (lanes 1 to 3). RhCMV-infected cells were also immunoprecipitated with a non-CMV-specific monoclonal antibody (lane 4). Sizes of protein markers (kDa) are indicated.

The gB gene from primary isolate RhCMV strain 22659 (Alcendor *et al*., 1993), was also sequenced [+6 (PAB 227) to +3013 (PAB 241) relative to strain 68-1 gB]. The nucleotide sequence of 22659 gB had five changes within the coding region compared to 68-1, resulting in three changes in the amino acid (aa) sequence (Fig. 1). RhCMV gB 22659 was 60% identical to HCMV gB strain AD169 at the nucleotide level. GenBank accession numbers for RhCMV strain 68-1 gB are U41526 and U41527. The accession number for the primary RhCMV gB isolate 22659 is U59238. Throughout this paper, the site of transcription initiation (+1) corresponds to base 419 of GenBank sequence U41526.

The 5′ and 3′ ends of the RhCMV gB transcript were mapped using the RACE protocol (Frohman *et al*., 1988). Primers PAB 188 and 191 (Table 1) for 5′ RACE were designed to sequences upstream of the *Cla*I site used to clone the 5′ half of gB. The 5′ end of the transcript was mapped to within one nucleotide, either a guanine or adenine residue, 25 or 26 bases downstream from the TATA sequence, respectively. The initiation site of transcription could not be precisely mapped by the 5′ RACE protocol because a poly(dC) tail was added to the first strand cDNA. The guanine residue 25 bases downstream from the TATA sequence was arbitrarily designated as the start site of transcription (+1). 3′ RACE analysis localized the 3′ end of the RhCMV gB transcript to +3055, 16 bases downstream from the sequence ATTAAA. Identical non-consensus polyadenylation signals (Proudfoot & Whitelaw, 1988) have been identified for the Towne and AD169 strains of HCMV gB (Spaete *et al*., 1988).

RNA expression kinetics of RhCMV gB were examined by Northern blot analysis, and the pattern of transcription was consistent with gB expression at late times of infection (i.e. after DNA replication) (Fig. 2*a*). Lung fibroblasts were infected with RhCMV strain 68-1, and total RNA was harvested at 12 h (in the presence and absence of 200 μg/ml cycloheximide), 24, 48 and 72 h [in the presence and absence of 400 μg/ml phosphonoformic acid (PFA)] post-infection (p.i.). RNA from uninfected cultures was also harvested. RNAs were glyoxylated and electrophoresed in an agarose gel (10 μg RNA per lane) according to published protocols (Sambrook *et al*., 1989). The blot was hybridized with a digoxigenin-labelled anti-sense RNA probe (position +1271 to +2349). The probe length was designed to eliminate overlap with other extended open reading frames. Digoxigenin labelling, blocking, hybridization, washing and detection steps were performed according to the manufacturer's specifications (Boehringer-Mannheim). The chemiluminescent substrate, Lumi-Phos 530 (Boehringer-Mannheim), was used for detection. Duplicate RNA samples were probed with an immediate-early 1 (IE-1) anti-sense RNA probe (Barry *et al*., 1996) as a control.

Glycoprotein B RNA transcripts were first detected 48 p.i., and peaked in intensity 72 h p.i. in the absence of PFA (Fig. 2*a*). No gB transcripts were detected at 72 h in the presence of PFA (400 μg/ml); however, IE-1 RNA was readily detected at 72 h under these conditions (not shown). Sensitivity to the DNA replication inhibitor PFA indicated that RhCMV gB was transcribed as a late gene, similar to murine CMV (MCMV) gB expression (Rapp *et al*., 1992). HCMV gB is transcribed as an early gene, but is translated at late times of infection (Spaete *et al*., 1988). Four bands (approximately 6·5, 4, 3 and 2 kb) were detected at the 48 h and 72 h (−PFA) time-points. The 3 kb transcript exhibited the greatest increase in abundance at 72 h p.i. (−PFA) relative to the other transcripts, and was consistent with a predicted transcript size of 3071 bases (including polyadenylation). Similar results were obtained in experiments using phosphonoacetic acid and ganciclovir as virus replication inhibitors (data not shown). Identities of the other bands were not determined. Multiple RNA transcripts have also been observed for the gB RNA transcripts of HCMV AD169 (Mach *et al*., 1986) and Towne strains (Spaete *et al*., 1988), MCMV (Rapp *et al*., 1992) and guinea-pig CMV (GpCMV) (Schleiss, 1994). No hybridization was observed with RNA from uninfected cells.

There was strong sequence conservation between the primate CMV gBs at the predicted amino acid level, and many structural, modification and processing signals were maintained (Fig. 1). RhCMV gB DNA sequence coded for a predicted protein of 854 aa, compared to 906 for HCMV AD169 (Cranage *et al*., 1986), 907 for HCMV Towne (Spaete *et al*., 1988), 928 for MCMV (Rapp *et al*., 1992) and 901 for GpCMV (Schleiss, 1994). Overall, the gB proteins of RhCMV and HCMV Towne were 60% identical and 75% similar. Homologies were higher (74% identity/85% similarity) for the region of RhCMV gB (aa 374 to aa 700) corresponding to that portion of HCMV gB (aa 401 to 760) implicated in virus binding, fusion and cell-to-cell spread (Navarro *et al*., 1993). Optimal sequence alignment of the RhCMV and HCMV gB proteins required the introduction of several small gaps (Fig. 1). Two gaps near the amino terminus corresponded to a region of HCMV gB coding for linear, strain-specific neutralizing epitopes in HCMV gB (Basgoz *et al*., 1992). Much of the gB sequence difference between the AD169 and Towne strains of HCMV occurs between positions 28 to 67 (Spaete *et al*., 1988).

Most of the amino acid motifs important for post-translational modifications and protein folding have been conserved within the primate CMV gB proteins (Fig. 1). RhCMV gB contained a leader sequence (Met^{1}-Ser^{23}) with 13 hydrophobic amino acids. The predicted cleavage signal for the leader (Ala^{21}-Ser^{22}-Ser^{23}) was similar to the cleavage signal for AD169 and Towne strains (Ala^{22}-Val^{23}-Ser^{24}) (Cranage *et al*., 1986; Spaete *et al*., 1988). Eleven cysteine residues outside the leader sequence were conserved between the RhCMV and HCMV gB proteins, suggesting similar secondary structures. HCMV gB had an additional cysteine residue not found in RhCMV gB. Conservation of cysteine residues within the primate CMV gBs is consistent with the disulfide bonding pattern proposed for all herpesvirus gBs (Norais *et al*., 1996). RhCMV gB contained 16 potential *N*-linked glycosylation sites (NXS or NXT); 14 of these were conserved with HCMV gB. HCMV gB possesses two additional *N*-linked glycosylation sites not found in RhCMV gB. Conservation of cysteine residues and potential *N*-linked glycosylation sites implied conservation of structure and post-translational modifications in the primate CMV family of gB proteins

RhCMV gB protein was proteolytically processed similarly to HCMV gB (Fig. 2*b*). Previous reports demonstrate that the 130 kDa full-length gB protein of HCMV is processed into 55 and 95 kDa cleavage products which associate to form a functional dimer (Britt, 1984). The cleavage signals of HCMV gB Towne (RTKR↓STD) and AD169 (RTRR↓STS) (Cranage *et al*., 1986; Spaete *et al*., 1988) had strong similarity to the corresponding sequences within RhCMV gB (RRKR↓STD), indicating that RhCMV may also be processed within the host cell. Protein extracts from HCMV Towne- and RhCMV 68-1-infected lung fibroblasts exhibiting 75 to 100% CPE were metabolically labelled with ^{35}S-methionine/cysteine Tran^{35}S-label (ICN). Extracts of cells were immunoprecipitated using published protocols (Sawai *et al*., 1994). A pool of monoclonal antibodies to the highly conserved D2b and D3 portions of HCMV gB (CH446-2, CH436-1, CH409-2 generously provided by L. Pereira) (Qadri *et al*., 1992), were used to immunoprecipitate RhCMV gB. D2b and D3 are within the carboxy-terminal gp55 portion of HCMV gB. Electrophoresis of precipitated extracts in 12% SDS–polyacrylamide gel revealed three predominant bands in both HCMV- and RhCMV-infected cells. One band corresponded to a protein size of approximately 50 kDa in RhCMV-infected cells, and 55 kDa (gp55) in HCMV-infected cells (Spaete *et al*., 1988). A 130 kDa band was observed in both HCMV- and RhCMV-infected cells. This band most likely represented full-length gB protein prior to proteolytic processing. A third protein band was observed at 95 kDa in HCMV-infected cells (Cranage *et al*., 1986; Spaete *et al*., 1988) and approximately 80 kDa in RhCMV-infected cells. This size corresponded to the predicted amino-terminal portion of the processed gB protein. While the monoclonal antibodies recognized only the carboxy terminus of gB (Qadri *et al*., 1992), we believe the amino-terminal half of the protein co-precipitated with the carboxy terminus. Specificity of the assay was demonstrated by two controls: (*a*) monoclonal antibodies to HCMV gB did not cross react with cellular proteins of uninfected lung fibroblasts, and (*b*) non-CMV-specific antibodies did not immunoprecipitate proteins corresponding to gB in infected cells, indicating that gB was not precipitated due to non-specific trapping of the protein in immune complexes. The use of anti-HCMV monoclonal antibodies to precipitate RhCMV gB indicated that immunogenic epitopes have been conserved in the primate CMV gBs. Similar results have been observed in immunofluorescence assays (data not shown).

In this study, the RhCMV gB gene has been cloned and characterized. This work represents the first description of a non-human primate CMV gB gene. The high degree of conservation between the primate CMV gBs, particularly within a region containing immunogenic epitopes, makes RhCMV infection in rhesus macaques an excellent model to study HCMV pathogenesis and the development of strategies to prevent or limit HCMV infection and disease.

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