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Glycoprotein B of Human Herpesvirus 8 Is a Component of the Virion in a Cleaved Form Composed of Amino- and Carboxyl-Terminal Fragments

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Human herpesvirus 8 (HHV-8) or Kaposi's sarcoma-associated herpesvirus (KSHV) is the only known human member of the *Rhadinovirus* genus of the gammaherpesvirus subfamily. Antibodies against peptides representing portions of the amino- and carboxyl-termini of HHV-8 gB were produced and used to detect gB expression in Vero cells transfected with the gB gene, in the HHV-8-harboring cell line, BCBL-1, and in purified virions. Expression of gB was detected in approximately 3% of uninduced BCBL-1 cells, while up to 30% of the cells expressed gB after 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induction of virus replication. Indirect immunofluorescence assays and confocal microscopy showed that gB was distributed throughout the cytoplasm of BCBL-1 cells and transfected Vero cells. Immunoblot analyses of virion preparations revealed the presence of full-length as well as two smaller than full-length gB-derived species corresponding to the amino- and carboxy-terminal portions of gB, respectively. Biochemical analysis of the gB carbohydrate moieties using glycosylation inhibitors revealed that gB contained N-linked oligosacharides of the high-mannose type, characteristic of precursor carbohydrate chains added in the endoplasmic reticulum.

INTRODUCTION

Glycoprotein B (gB) is one of the most conserved herpesvirus glycoproteins. It is an essential virion component for members of the alpha- and betaherpesvirus subfamilies. It is thought to function in virion attachment and virus entry into susceptible cells (Cai *et al.,* 1988; Cranage *et al.,* 1986; Pereira, 1994; Roizman and Sears, 1996; Spear, 1993a). Epstein-Barr virus (EBV), a gamma-1 herpesvirus (genus *Lymphocryptovirus*) (Honess, 1984), and murine gammaherpesvirus 68 (MHV-68), which is a member of the gamma-2 herpesvirus (genus *Rhadinovirus*) subfamily, do not incorporate detectable levels of gB into their virions (Gong and Kieff, 1990; Stewart *et al.,* 1994). However, bovine herpesvirus 4, also a gamma-2 herpesvirus (Bublot *et al.,* 1992; Lomonte *et al.,* 1997), incorporates gB to its virion (Lomonte *et al.,* 1997).

Human herpesvirus 8 (HHV-8), or Kaposi's sarcoma-associated herpesvirus, is associated with Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castlesman's disease (Cesarman *et al.,* 1996; Chang *et al.,* 1994; Said *et al.,* 1996; Soulier *et al.,* 1995). Phylogenetic analyses of viral DNA sequences revealed that HHV-8 belongs to the gamma-2 sublineage of the gammaherpesvirus subfamily, and thus represents the first human gamma-2 herpesvirus identified (Moore *et al.,* 1996).

The present study was carried out to determine whether gB is a component of the HHV-8 virion and whether certain biochemical properties of HHV-8 gB are similar to those reported for other gB homologs. Antibodies against peptides representing portions of the amino- and carboxyl-termini of HHV-8 gB were produced and used to detect gB expression in Vero cells transfected with the gB gene, in the HHV-8-harboring lymphoma cell line, BCBL-1, and in purified virions. We found that gB is part of the HHV-8 virion as full-length and cleaved protein and shares biochemical characteristics with other gB homologs.

RESULTS

Expression of HHV-8 gB in Vero cells transfected with the gB gene and in BCBL-1 cells. To facilitate the detection of gB, anti-peptide antibodies were produced against synthetic peptides representing immunogenic portions of gB. Peptides were coupled to keyhole limpet hemocyanin (KLH) before immunization of rabbits (see Materials and Methods). To validate the specificity of the rabbit antisera generated against HHV-8 gB peptides, Vero cells were transfected with the pcDNA-gB plasmid carrying the entire gB gene under the promoter control of the human cytomegalovirus immediate early promoter, and gB expression was detected by IFA at 48 h post-transfection. Antibodies gB-N1 and gB-C detected gB in

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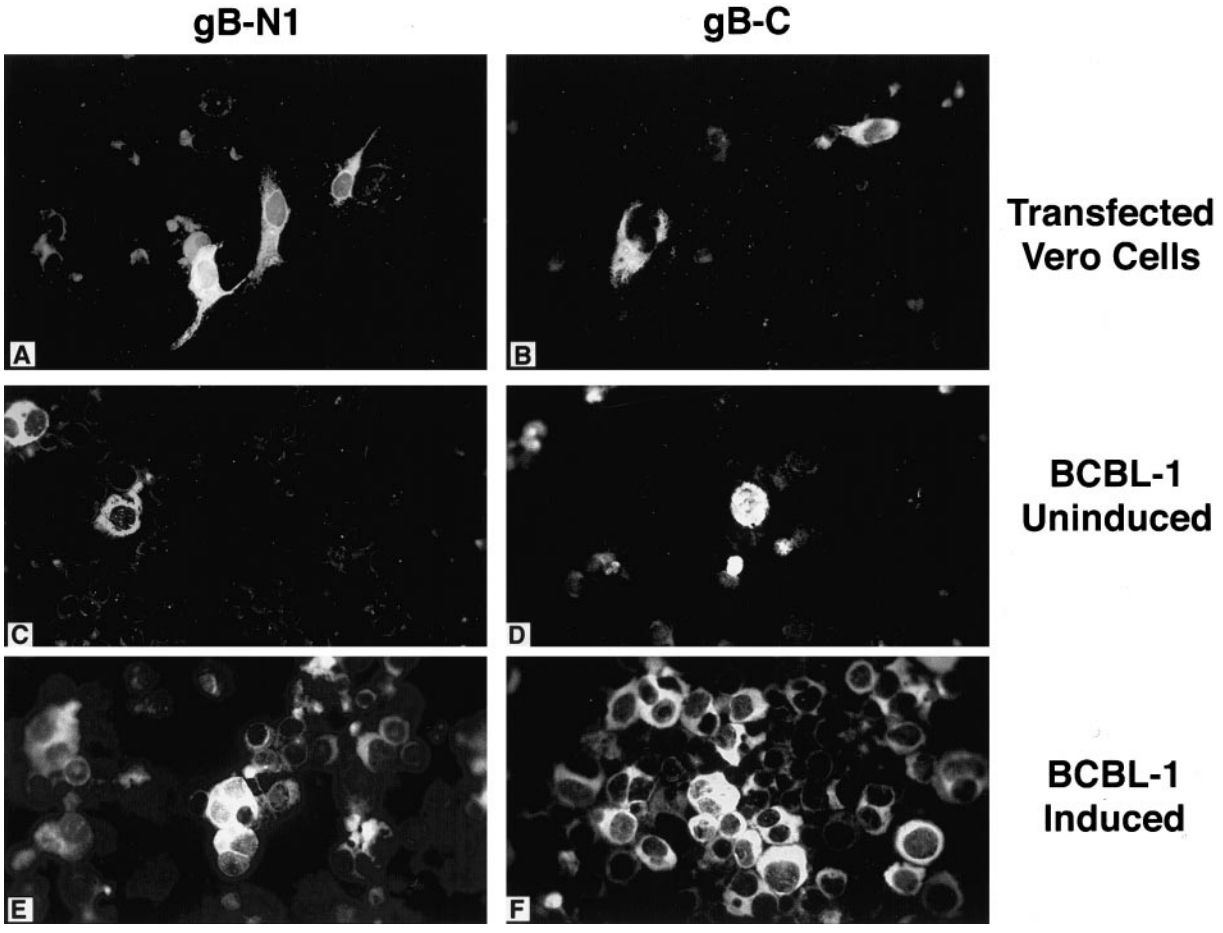

FIG. 1. Immunofluorescence detection of HHV-8 gB using anti-gB-peptide antibodies. Vero cells transfected with the pcDNA-gB plasmid were fixed with methanol at 48 h after transfection (A, B). Indirect immunofluorescence was performed with the gB-N1 antibody (A, C, E) and with the gB-C antibody (B, D, F). Uninduced BCBL-1 cells (C, D) and TPA-induced BCBL-1 cells (E, F) reacted with gB-N1 (C, E) or gB-C (D, F) at 2 days after TPA treatment. Cells were visualized by fluorescence microscopy at 400X magnification.

approximately 5% of the cells, and fluorescent signals were dispersed throughout their cytoplasm (Fig. 1A, B). The reactivities of the antibodies were then tested against BCBL-1 cells. Antibodies gB-N1 and gB-C reacted strongly with approximately 3% of uninduced (Fig. 1C, D) and up to 30% of TPA-induced BCBL-1 cells (Fig. 1E, F). Fluorescent signals were distributed throughout the cytoplasm and perinuclear spaces. No reactivity was detected in Vero cells transfected with the plasmid vector alone or with preimmune sera (not shown).

To improve the visualization of gB distribution within cells, BCBL-1 cells were fixed with paraformaldehyde and labeled with the gB peptide antibodies as well as with a nuclear counterstain as described under Materials and Methods and then examined by confocal microscopy (Fig. 2). Although paraformaldehyde is sometimes used to crosslink the surface of cells to prevent antibody penetration, induced BCBL-1 cells that had been treated with paraformaldehyde were permeable to antibodies directed against nuclear membrane and endoplasmic reticulum proteins (not shown), thus the reactivity seen here was not limited to the cell surface. Reactivity with antibody gB-N1 and gB-N2 was detected in the cytoplasm of TPA-induced BCBL-1 cells (Fig. 2, panels 1 and 2, respectively); however, gB-N1 produced a more intense fluorescent signal than the gB-N2 antibody. Antibody gB-C reacted strongly with paraformaldehyde-fixed BCBL-1 cells (Fig. 2, panel 3). Antibodies gB-N1 (Fig. 2, panel 4), gB-N2, and gB-C (not shown) produced very weak signals against uninduced cells. In addition, confocal microscopy of TPA-induced BCBL-1 cells revealed cellular structures that contained gB but without any detectable nuclear material. The largest of these structures was approximately the size of mononuclear BCBL-1 cells (Fig. 2, panel 1). These structures may represent cytosolic portions of cells that blebbed off after TPA induction.

Synthesis and processing of HHV-8 gB in COS cells transfected with the gB gene and in BCBL-1 cells. COS cells were transfected with the gB gene and gB was detected by immunoblot analysis at 48 h posttransfection in the presence or absence of the N-glycosylation inhib-

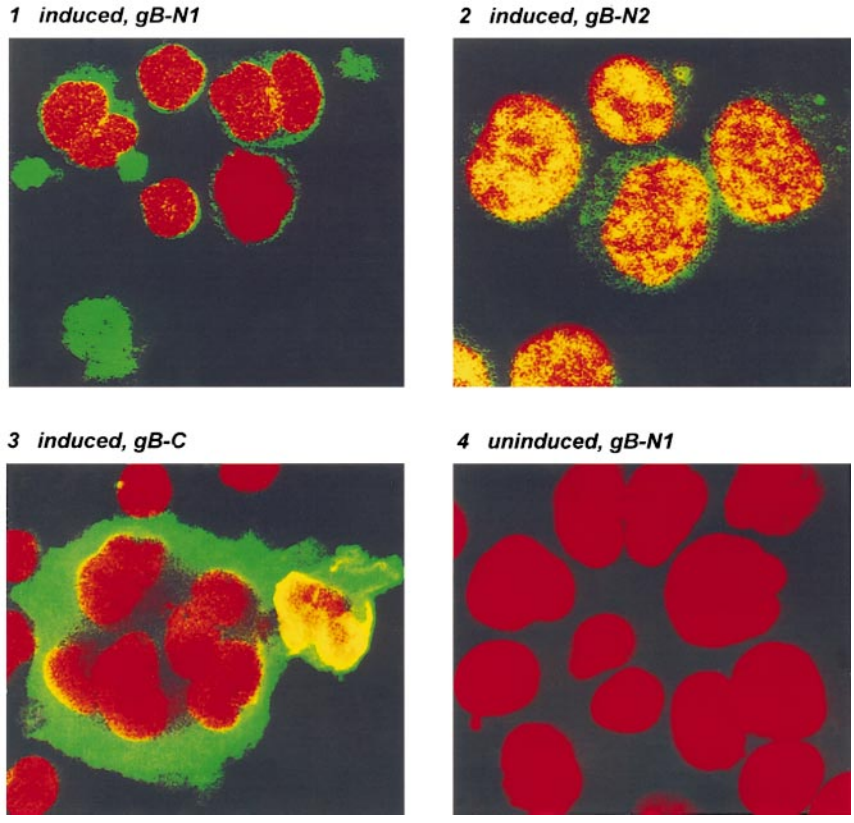

FIG. 2. Confocal immunofluorescence detection of gB in BCBL-1 cells. TPA-induced and uninduced BCBL-1 cells were harvested 5 days after induction and mounted onto slides after fixing with 3% paraformaldehyde. Indirect immunofluorescence was performed with the indicated antibodies. Panel 3 shows a representative syncytium structure. Blebbing structures containing gB have no visible nucleus in panel 1.

itor tunicamycin (TM) (Fig. 3). Antibody gB-C reacted with a 107-kDa protein (Fig. 3A, lane 2). In the presence of TM, the 107-kDa protein species was not detected, while a new protein species of approximately 85 kDa appeared (Fig. 3A, lane 3). For comparison, antibody gB-C reacted against extracts from purified HHV-8 virions detected 112-, 107-, and 59-kDa proteins (Fig. 3, lane 4).

Similar experiments were performed to detect gB in uninduced and TPA-induced BCBL-1 cells in the presence and absence of glycosylation inhibitors (Fig. 3B). Antibody gB-C reacted with major protein species of 112-, 107-, and 59-kDa in TPA-induced cells (Fig. 3B, lane 2). Proteins of similar sizes were present in substantially lower amounts in uninduced cells (Fig. 3B, lane 1). The relative amounts of other protein species did not change after TPA induction and thus were considered to represent background reactivity (Fig. 3B, lanes 1 and 2). To inhibit N-linked glycosylation, TM was added to BCBL-1 cells concurrent with TPA induction, and cells were harvested 2 days later. The amounts of the 112-, 107-, and 59-kDa proteins were drastically reduced in the presence of TM, while a new gB-C-reactive species of approximately 90 kDa was detected (Fig. 3B, lane 3). The processing pathway of gB in induced BCBL-1 cells was further examined using endoglycosidase H (endo-H), which specifically cleaves high mannose, non-Golgi processed sugars (Tarentino *et al.,* 1989; Maley *et al.,* 1989). Digestion by endo-H of extracts from cells induced by TPA in the absence of TM produced gB species ranging in molecular mass from 112–90 to 51–46 kDa (Fig. 3B, lane 4 compared to lane 2). Peptide N-glycosidase F (PNGase F) is an amidase that cleaves between the innermost GlcNAc and asparagine residues of high mannose, hybrid, and complex oligosacharides from N-linked glycoproteins (Maley *et al.,* 1989; Tarentino *et al.,* 1990). Digestion with PNGase F of extracts of BCBL-1 cells induced by TPA in the absence of TM produced two major gB-C-reactive species of approximately 98–90 and 46 kDa (Fig. 3B, lane 5).

Detection of gB and gB-related peptides in HHV-8 virions. The gB-C and gB-N1 antibodies were used to detect gB in purified virion samples obtained from supernatant fluids by differential centrifugation or by purifying virions through density gradients (see Materials and Methods). Antibody gB-C reacted specifically with the proteins of 112-, 107-, and 59-kDa apparent molecular masses in immunoblots of electrophoretically separated proteins from purified virions obtained from supernatants (Fig. 3A, lane 4; Fig. 3B lane 6; Fig. 4A, lane 1) as well as from gradient-purified virions (Fig. 4A, lane 2). The amounts of the 112- and 107-kDa proteins were decreased in gradient-purified virion samples in compari-

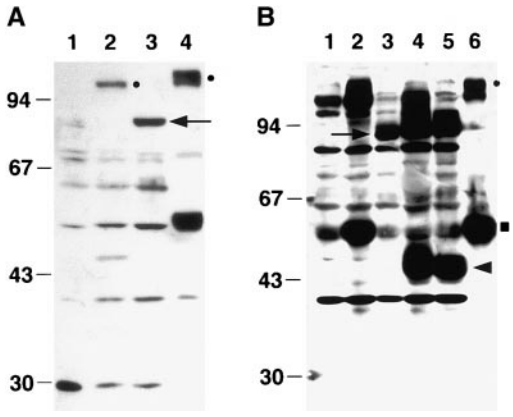

FIG. 3. The effect of glycosylation inhibitors on gB expressed in COS and BCBL-1 cells. (A) COS cells were transfected with the HHV-8 gB gene-expressing plasmid and incubated in the presence or absence of TM. Cellular extracts were collected at 48 h posttransfection and tested for the presence of gB in immunoblots using the gB-C antibody. Lane 1: mock-transfected COS cells. Lanes 2 and 3: COS cells transfected with the gB gene in the absence and presence of TM, respectively. Lane 4: extracts of virions purified from BCBL-1 supernates. The location of the 107-kDa gB species in lane 2 and the location of the gB-doublet of 112- and 107-kDa species in lane 4 are marked with a solid dot. The location of the 85-kDa species produced in the presence of TM is marked by an arrow in lane 3. (B) BCBL-1 cells incubated in the presence of TPA alone or in the presence of TPA and TM were harvested at 48 h post-TPA induction. Cellular extracts derived from TPA-induced BCBL-1 cells were treated with either endo-H or PNGase F. Immunoblot analysis was used to detect gB using antibody gB-C. Lane 1: BCBL-1 cells in the absence of TPA induction. Lane 2: BCBL-1 cellular extracts after TPA induction. Lane 3: BCBL-1 cellular extracts after TPA and TM treatment. Lane 4: sample from TPA-induced cells digested with endo-H. Lane 5: TPA-induced cellular extracts digested with PNGase F. Lane 6: virion extracts reacted with anti-gB-C antibody. The location of the 112-kDa species in lane 6 is marked with a solid dot. The location of the 59-kDa species in lane 6 is marked with a solid square. The location of the 90-kDa species produced in the presence of TM is marked by an arrow in lane 3.

son to the purified virion samples obtained from supernatant fluids, while a smaller protein of approximately 40-kDa apparent molecular mass was present in virions obtained from supernatant fluids but not present in density gradient-purified virion samples (Fig. 4A, lanes 1 and 2). Antibody gB-N1, which reacts with the amino-terminal portion of gB, detected specifically a 75-kDa protein species in virion samples purified from either supernatant fluids or through density gradients. Decreased amounts of the 75-kDa protein were detected in gradient-purified samples in comparison to virion samples obtained from supernatant fluids (Fig. 4B, lanes 1 and 2, respectively).

The processing pathway of gB in purified virions was further examined using enzymes endo-H and endo-F. Antibody gB-C reacted specifically with proteins of 112–107 and 59 kDa (apparent molecular masses) in immunoblots of electrophoretically separated proteins from purified virions (Fig. 5, lane 4), which exhibited similar mobilities to gB-C antibody-reactive protein species from TPA-induced BCBL-1 cells that served as controls (Fig. 5, lane 3). For comparative purposes, the gB-C antibody was also reacted against extracts from both BJAB cells as well as BCBL-1 cells in the absence of TPA induction. The gB-C antibody did not react with proteins of 112–107 kDa; however, it did react with a protein species of approximately 57 kDa that was present in samples from both BJAB and uninduced BCBL-1 cells (Fig. 5, lanes 1, 2). The fact that the 57-kDa protein was detected in both BJAB and BCBL-1 cells without TPA induction indicates that this reaction was nonspecific. Digestion by endo-H of extracts from purified virions produced gB species with faster electrophoretic mobilities ranging in apparent molecular mass of approximately 107–90 and 51–45 kDa (Fig. 5, lane 5 compared to lane 4). Digestion of purified virions with PNGase F produced two major gB-C-reactive species of approximately 95–90 and 45 kDa (Fig. 5, lane 6).

DISCUSSION

We produced specific antibodies against HHV-8 gB and demonstrated that unlike EBV gB, HHV-8 gB is a major component of the virion particle. Furthermore, we demonstrated that gB is incorporated in virions as full-length and cleaved forms and that it is predominantly N-glycosylated.

gB homologs specified by varicella zoster virus (VZV),

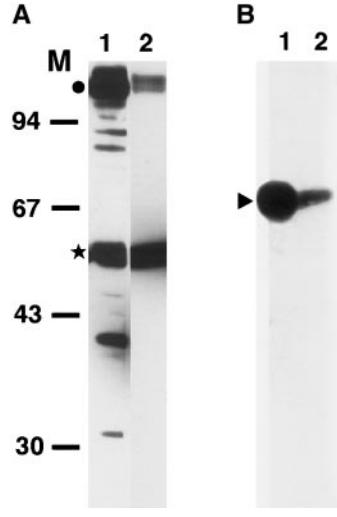

FIG. 4. Detection of gB in purified virions. Virions were purified from the supernatant fluids of BCBL-1 cells prepared 2 days after TPA addition. Purified viral antigens were reacted in immunoblots with gB-C and gB-N1 antibodies and visualized using chemiluminescence. (A) Lanes 1 and 2: extracts of virions purified from either supernatant fluids or density gradients, respectively, reacted with the gB-C antibody. The location of the 112-kDa gB species is marked with a solid dot and the 59-kDa gB species is marked with an asterisk. (B) Lanes 1 and 2: extracts of virions purified from either supernatant fluids or density gradients, respectively, reacted with the gB-N1 antibody. The location of the 75-kDa gB species in B is marked with an arrowhead. Molecular mass markers are as shown and are the same for both A and B.

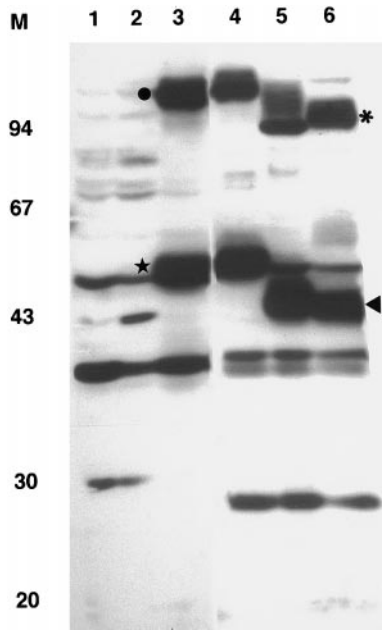

FIG. 5. The effect of glycosylation inhibitors on gB expressed in purified virions. (A) Immunoblot analysis was used to detect gB in samples prepared from cellular extracts and virions purified from supernatant fluids of BCBL-1 cells using antibody gB-C. In addition, purified virion samples were digested with either endo-H or PNGase F. Cellular extracts obtained from either BJAB or BCBL-1 cells without TPA induction were reacted with the gB-C antibody and served as background controls. Lane 1: BJAB cells. Lane 2: BCBL-1 cells in the absence of TPA induction. Lane 3: BCBL-1 cellular extracts after TPA induction. Lane 4: purified virion samples in the absence of any enzymatic treatment. Lane 5: purified virions digested with endo-H. Lane 6: purified virion samples digested with PNGase F. The location of the 112-kDa species in lane 6 is marked with a solid dot. The location of the 59-kDa species in lane 6 is marked with a star. The locations of the 45-kDa gB species produced after either endo-H or PNGase F treatment is marked with an arrowhead, while the 90-kDa gB species is marked with an asterisk.

human cytomegalovirus, human herpesvirus 6, bovine herpesvirus 1 (BHV-1), bovine herpesvirus 4 (BHV-4), pseudorabies virus, Marek's disease virus, equine herpesvirus 1, equine herpesvirus 4 (EHV-4), murine cytomegalovirus, and feline herpesvirus 1 are proteolytically cleaved into two major parts after digestion between the R and S amino acids of the consensus site RX(R/K)RS which is located near the middle of the extracellular domain of the molecule (Pereira, 1994). The cleaved domains retain their covalent association via disulfide bonds (Pereira, 1994; Spaete *et al.,* 1988). However, cleavage of the BHV-1 gB molecule was shown not to be required for gB function (Kopp *et al.,* 1994). The predicted primary sequence of HHV-8 gB includes a potential proteolytic cleavage site (RKRR/S) located between amino acids 440 and 441 of the HHV-8 gB predicted amino acid sequence. The predicted molecular weight of the amino acid backbone of HHV-8 gB is 93,978 and 91,307 Da without its predicted signal sequence (aa 1–26). The predicted molecular weights of the amino and carboxyl terminal portions obtained after proteolytic cleavage are 45,879 and 45,445 Da, respectively, which correspond to the probable peptide backbones of the 75- and 59-kDa N-terminal and C-terminal gB species detected in cells and virions.

Glycosylation inhibition experiments revealed that HHV-8 gB contains predominantly N-linked carbohydrates in agreement with a previous report describing the gB specified by the closely related BHV-4 (Lomonte *et al.,* 1997) as well as with HHV-8 gB transiently expressed in Chinese hamster ovary (CHO) cells (Pertel *et al.,* 1998). The amino-terminal portion of HHV-8 gB contains 9 potential N-linked glycosylation sites, while the carboxy-terminal portion contains 6 sites. Therefore, the 59-kDa species detected in immunoblots of antigens derived from both BCBL-1 cells and purified virions with the gB-C antibody may be obtained by glycosylation of the 45-kDa carboxyl-terminal portion of gB. The 75-kDa species detected in purified virions with the gB-N1 antibody most likely represents the glycosylated version of the 46-kDa amino terminal portion of gB. The 112-, 107-, and 59-kDa gB species contain N-linked sugars because their apparent molecular masses were reduced after endo-H treatment. This conclusion was confirmed by the TM experiments with BCBL-1 cells in which the 112- and 107-kDa gB species were replaced by a species of 90 kDa. In these experiments, the amount of 59-kDa protein was reduced drastically in the TM-treated sample, indicating that the potential precursor protein of the 59-kDa protein may be rapidly degraded in the absence of N-glycosylation.

In contrast to results obtained by transiently expressing gB in CHO cells where it was found that gB was localized in the rough endoplasmic reticulum (RER) and perinuclear spaces (Pertel *et al.,* 1998), gB expressed in BCBL-1 cells as well as in Vero cells was distributed throughout the cytoplasm. These differences in gB distribution detected by immunofluorescence assays may be due to the different cell lines used in these studies, the relative amounts of gB expressed under different conditions, or the different antibodies used to detect gB.

In the presence of TM, a 85-kDa protein was detected in COS cells transfected with the gB gene instead of the 90-kDa gB expressed in the presence of TM in BCBL-1 cells indicating that the nonglycosylated gB precursor protein may be partially digested in COS cells. Similar transient expression experiments in CHO cells indicated that the full-length gB migrated with an apparent molecular mass of approximately 120 kDa, while gB in the presence of TM was approximately 95 kDa (Pertel *et al.,* 1998). The discrepancies in molecular masses between COS, CHO, and BCBL-1 cells for gB and gB-related peptides may be due to differences in the extent of gB glycosylation by the different cells used in these studies. It is important to note that under transient expression conditions, gB is expressed as full-length, while in

BCBL-1 cells is produced as amino- and carboxyl-terminal portions as well as full-length gB. Thus, it seems that there is a protease that is responsible for cleavage of gB in BCBL-1 cells.

Apparently, gB exists in virions as a full-length protein as well as in a cleaved form consisting of a 75-kDa amino terminal portion and a 59-kDa carboxyl-terminal portion. Addition of the apparent molecular masses of these two gB species should produce a full-length gB of approximately 134 kDa instead of the 112-kDa gB detected by gB-C and gB-N1 antibodies. This discrepancy between predicted and observed molecular masses for full-length gB may be due to slower electrophoretic migration of gB fragments in comparison to the full-length gB. Double-gradient virions appeared to have less full-length gB than virions produced from BCBL-1 supernates. We considered that this difference between the two virion preparations may be due to either contaminating full-length gB in BCBL-1 supernates or partial processing of gB after virion purification. However, double-gradient purification should eliminate any specific proteases, and therefore, it is unlikely that specific cleavage of gB could be achieved after virus purification. Therefore, our results are consistent with the hypothesis that gB is found in HHV-8 virions predominantly as 75- and 59-kDa fragments. Furthermore, the amino-terminal portion of gB does not contain any predicted membrane spanning amino acid sequences; therefore it is likely to be retained on virions through association with the gB-carboxyl-terminal portion.

Addition of protease inhibitors such as phenylmethylsulfone fluoride (PMSF) or a mixture of *N*-tosyl-L-phenylananine chloromethyl ketone (TPCK) and *N-p*-tosyl-L-lysine chloromethyl ketone (TLCK) protease inhibitors prior and during cellular disruption failed to inhibit proteolytic cleavage of gB, indicating that this activity does not occur after lysis due to lysosomal enzymes that are released by cellular disruption (not shown). Transient expression of gB in COS cells revealed the presence of full-length gB, while the 59-kDa portion of gB detected in BCBL-1 cells was not detected (Fig. 3A). Similar results were obtained in experiments in which gB was expressed in BHK-1 cells under transient conditions using plasmid-based expression vectors or the Semliki Forest Virus expression System (DiCiommo and Bremner, 1998). Moreover, extracts prepared from uninduced BCBL-1 or BJAB cells, when incubated with gB expressed via transient expression in COS cells, failed to cleave gB and produce the 59-kDa gB species (not shown). Thus, gB cleavage seems to be intimately associated with gB expression in BCBL-1 cells.

Amino acid sequences of gB are highly conserved among herpesviruses. Furthermore, gB specified by alpha- and betaherpesviruses function in virus entry and virus-induced cell fusion (Pereira, 1994; Spear, 1993a,b). Apparently, these membrane fusion functions of gB are not shared by the EBV gB homolog, because EBV gB is not found in EBV virions and is not expressed on the surface of infected cells, but it is strictly localized on the rough endoplasmic reticulum and nuclear membranes (Emini *et al.,* 1987; Gong *et al.,* 1987; Gong and Kieff, 1990; Qualtiere and Pearson, 1979). In contrast, both BHV-4 (Lomonte *et al.,* 1997) and HHV-8 contain gB in their virions, and HHV-8 expresses gB throughout the cytoplasm of BCBL-1 cells. The presence of gB in HHV-8 virions suggests that it may function in virion entry.

MATERIALS AND METHODS

Cells and virus purification. BJAB cells were obtained from Naoki Inoye (Centers for Disease Control and Prevention, Atlanta, GA). BCBL-1 cells (Renne *et al.,* 1996) were obtained from the NIH AIDS Reference and Reagent Program and cultured according to the instructions provided. The lytic cycle was induced by treatment with 20 ng/ml of the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (Sigma Chemical Corp., St. Louis, MO). Vero cells were grown in Dulbecco's modification of Eagle's medium supplemented with 7% heat-inactivated fetal calf serum and penicillin and streptomycin. For virus purifications, 5 liters of induced BCBL-1 cell supernatants was harvested at 8 days postinduction (d.p.i.) and filtered through a 0.45-μm filter and then virions were pelleted at 22,000 *g* for 16 h. Virion pellets were allowed to resuspend overnight on ice in TNE buffer (0.1 M Tris, pH 7.4, 0.1 M NaCl, 0.001 M EDTA) containing protease inhibitors. Pellets were pooled and layered onto a 20–35% Accudenz (Accurate Chemical Corp., Westbury, NY) step gradient and then centrifuged at 82,500 *g* in a swinging bucket rotor for 2 h at 5°C. The band visible at the interface was collected, diluted with cold TNE, layered onto a 15–50% continuous Accudenz gradient, and centrifuged as described above. A single-wide band of 1.5 ml was collected, diluted with TNE, layered onto a second 15–50% continuous Accudenz gradient, and centrifuged under identical conditions. The final band was collected and diluted in 10 ml of Tris-buffered saline (TBS), and then virions were pelleted at 77,000 *g* in a swinging bucket rotor for 1 h at 5°C. Electron microscopic examination of purified virions revealed a high concentration of apparently intact virions and very little visible cellular debris.

Alternatively, viruses were partially purified by separately harvesting cells and supernatants obtained from two 250-ml cell cultures. Supernatant fluids were centrifuged at 1500 *g* to remove any cells and kept in ice. Cells were collected by centrifugation, suspended into 10 ml of PBS buffer, freeze-thawed three times, and subsequently centrifuged at 1500 *g* in a preparative centrifuge for 15 min. Supernates from these preparations were combined with clarified supernates from the original cell cultures.

These fluids were cleared by centrifugation at 5000 *g* for 30 min and passed through a 0.2-μm filter. Filtered virions were pelleted at 27,000 *g* for 3 h and then viral pellets were resuspended in 40 ml of phosphate-buffered saline (PBS) and repelleted as above. Pellets were resuspended in 200–300 μl of PBS before processing for SDS-PAGE. Electron microscopic examination showed that these preparations contained high concentrations of virions and low amounts of cellular debris (not shown).

Plasmid construction (pcDNA-gB). HHV-8 glycoprotein B was amplified by PCR from BCBL-1 cells with primers 5′-GGAATTCCGCTAGCTGGGTATTTAAGGACCTGTAC and 5′-CGGGATCCAAGCTTAGAGGCGGGCCGT GTTTC-CTA based on the BCBL-1 gB DNA sequence (Meng *et al.,* in press). The resulting PCR product, which contains 59 bp of the 5′ nontranslated sequence, the entire open-reading frame, and the stop codon, was cloned into pcDNA 3.1/Zeo (+) (Invitrogen, Inc., Carlsbad, CA) using the unique restriction sites *Nhe*I and *Bam*HI, yielding the recombinant plasmid pcDNA-gB. The ends of the cloned gB DNA fragment were sequenced using the T7-forward and pcDNA3.1/BGH-reverse primers (Invitrogen, Inc.) to confirm that gB was correctly inserted for expression. $CsCl_2$-purified plasmid DNA was used for transient expression experiments.

Peptide synthesis and immunizations. Peptides representing three regions of the predicted translation product of the HHV-8 gB gene were synthesized using an automated peptide synthesizer (Model 432A, Applied Biosystems, Foster City, CA) according to the manufacturers instructions. Peptide gB2575 (gB-N1) contained aa 27–42, peptide gB2518 (gB-N2) contained aa 167 to 191, and peptide gB2523 (gB-C) contained aa 828 to 845. Synthetic peptides were coupled to keyhole limpet hemocyanin, emulsified with Freund's complete adjuvant for the primary immunization and Freund's incomplete adjuvant for two booster immunizations. Two New Zealand white rabbits per peptide were immunized subcutaneously with each conjugated peptide.

Transfection and immunofluorescence assays. Vero cells were seeded in 24-well plates on glass coverslips. When cells reached 50–60% confluency, they were transfected with the plasmid pcDNA-gB using Lipofectamine reagent according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD). Transfected cells were incubated for 48 h at 37°C, and then coverslips were fixed with methanol for 20 min, rinsed three times with PBS, and then processed for immunofluorescence staining. Rabbit antisera to gB peptides (see below) were used in IFA at a dilution of 1:100 in 10% normal goat serum in PBS. BCBL-1 cells were centrifuged onto a slide using a cytospin centrifuge and processed as detailed above for the gB gene-transfected Vero cells. Fluorescein-conjugated goat anti-rabbit antibody (ICN Pharmaceuticals, Inc., Aurora, OH) was used as the secondary antibody.

Confocal microscopy. Cytospin-prepared cells were fixed in paraformaldehyde (3% in PBS) and then incubated with the indicated antiserum for 1 h at 4°C followed by washing three times with ice-cold PBS. Incubation with the secondary antibody, Oregon Green 488-conjugated goat anti-rabbit IgG (Molecular Probes, Inc., Eugene, OR) at a 1:200 dilution in 20% horse serum in PBS) was used at 4°C for 1 h. Microscopic examination was with a Zeiss LSM 410 inverted confocal microscope equipped with an argon/krypton laser and a 40X (1.2 N.A.) objective using the appropriate excitation and emission filters.

Immunoblot analysis. Cell lysates or purified virions were diluted in loading buffer (2% SDS, 1% 2-mercaptoethanol, 10% glycerol, and 0.001% bromphenol blue in 0.5 M Tris-HCl, pH 6.8) and separated by SDS-PAGE (Laemmli, 1970) in 10% polyacrylamide gels. Proteins were electrotransferred onto nitrocellulose membranes, blocked with BLOTTO (5% nonfat milk in 0.01 M PBS/0.05% Tween 20), and then reacted 1 h with the indicated gB peptide antiserum at a dilution of 1:400 at room temperature. Membranes were washed three times with the same buffer and proteins were visualized by either alkaline phosphatase or chemiluminescence (Alkaline Phosphatase Conjugate Substrate Kit, Bio-Rad, Inc., Hercules, CA; SuperSignal Substrate Western blotting chemiluminescence detection kit, Pierce, Inc., Rockford, IL).

Glycosylation studies. Tunicamycin was purchased from Boehringer Mannheim Corp. (Indianapolis, IN). Endoglycosidase H and peptide endoglycosidase F (PNGase F) were purchased from New England BioLabs (Beverly, MA). Approximately, 10^6 BCBL-1 cells were obtained from cell cultures grown in the presence or absence of TPA for 48 h. Other BCBL-1 cell cultures were incubated in the presence of TM (1 μg/ml) added simultaneously with TPA. Cells were collected by low-speed centrifugation (5 min at 500 *g*), washed by resuspending and pelleting in PBS, and then lysed in 750 μl of double-distilled water. Cellular extracts were sonicated for 7 s (Sonifier Cell Disruptor 200, Branson, Inc., Danbury, CT) and centrifuged (5 min at 1000 *g*), and then supernatants were collected. Endo-H and PNGase-F digestions were performed on 100 μl of cellular extracts using 1000 U of each enzyme per reaction for 1 h at 37°C, and otherwise according to the manufacturer's instructions (New England BioLabs). Enzyme reactions were stopped by the addition of loading buffer to samples for SDS-PAGE analysis.

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