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Human Herpesvirus-8 Glycoprotein B Interacts with Epstein–Barr Virus (EBV) Glycoprotein 110 but Fails to Complement the Infectivity of EBV Mutants

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To characterize human herpesvirus 8 (HHV-8) gB, the open reading frame was PCR amplified from the HHV-8-infected cell line BCBL-1 and cloned into an expression vector. To facilitate detection of expressed HHV-8 gB, the cytoplasmic tail of the glycoprotein was tagged with the influenza hemagglutinin (HA) epitope. Expression of tagged HHV-8 gB (gB-HA), as well as the untagged form, was readily detected in CHO-K1 cells and several lymphoblastoid cell lines (LCLs). HHV-8 gB-HA was sensitive to endoglycosidase H treatment, and immunofluorescence revealed that HHV-8 gB-HA was detectable in the perinuclear region of CHO-K1 cells. These observations suggest that HHV-8 gB is not processed in the Golgi and localizes to the endoplasmic reticulum or nuclear membrane. Because both HHV-8 and EBV are γ-herpesviruses, the ability of HHV-8 gB to interact with and functionally complement EBV gp110 was examined. HHV-8 gB-HA and EBV gp110 co-immunoprecipitated, indicating formation of hetero-oligomers. However, HHV-8 gB-HA and HHV-8 gB failed to restore the infectivity of gp110-negative EBV mutants. These findings indicate that although HHV-8 gB and EBV gp110 have similar patterns of intracellular localization and can interact, there is not sufficient functional homology to allow efficient complementation.

INTRODUCTION

Herpesvirus infection is initiated by attachment to a susceptible cell followed by a cascade of interactions between viral glycoproteins and the plasma membrane that ultimately results in penetration of the nucleocapsid into the cytoplasm (reviewed in Spear, 1993; Cooper, 1994). For Epstein–Barr virus (EBV), the interaction of gp350/220 with CD21, a receptor for the complement component C3 days, mediates initial binding (Nemerow *et al.,* 1987, 1989; Tanner *et al.,* 1987, 1988). Glycoprotein 350/220 has no known homolog in any of the other herpesviruses examined to date. Subsequent penetration is mediated in part by gp350/220 (Nemerow and Cooper, 1984; Tanner *et al.,* 1987) and by a complex of viral glycoproteins containing gp85, gp25, and gp42 (Heineman *et al.,* 1988; Miller and Hutt-Fletcher, 1988, 1992; Haddad and Hutt-Fletcher, 1989; Yaswen *et al.,* 1993; Hutt-Fletcher, 1995; Li *et al.,* 1995). EBV gp85 and gp25 are homologues to herpes simplex virus (HSV) gH and gL, respectively (Baer *et al.,* 1984; Heineman *et al.,* 1988; Yaswen *et al.,* 1993). EBV gp42, which has no homolog in HSV, is required for viral entry into B cells but not epithelial cells (Li *et al.,* 1995). Recently, a third protein that interacts with human cytomegalovirus (HCMV) gH and gL has been identified (Huber and Compton, 1997; Li *et al.,* 1997). Interestingly, EBV gp110, the HSV gB homolog, appears to be essential for viral assembly (Herrold *et al.,* 1996; Lee and Longnecker, 1997). In contrast, HSV gB is required for viral penetration (Manservigi *et al.,* 1977; Sarmiento and Spear, 1979; Cai *et al.,* 1988) and can mediate binding to cellular glycosaminoglycans if HSV gC, the principal glycoprotein that mediates initial attachment (Herold *et al.,* 1991), is not expressed (Herold *et al.,* 1994). HSV gB does not appear to be required for viral egress.

Glycoprotein B shows considerable homology among the known herpesviruses, especially in the carboxyl-terminal tail domain and in the relative positions of cysteine and proline residues (Ross *et al.,* 1989; Goltz *et al.,* 1994; Pereira, 1994). All gB glycoproteins tested are essential for herpesvirus infectivity. Engineered herpesvirus mutants that do not express gB are not infectious in the absence of complementation (Cai *et al.,* 1987; Rauh and Mettenleiter, 1991; Miethke *et al.,* 1995; Herrold *et al.,* 1996). Cross-complementation between α-herpesvirus gB glycoproteins can occur but is not always reciprocal. For example, both bovine herpesvirus 1 (BHV-1) and pseudorabies virus (PrV) gB may function in HSV-1 infectivity (Misra and Blewett, 1991; Mettenleiter and Spear, 1994). However, BHV-1 gB (Rauh *et al.,* 1991; Kopp and Mettenleiter, 1992) but not HSV-1 gB (Mettenleiter and

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Spear, 1994) can complement gB-negative PrV while PrV gB cannot complement gB-negative BHV-1 (Miethke *et al.,* 1995). By studying hybrid proteins composed of PrV gB and BHV-1 gB domains, the carboxyl-terminal portion of BHV-1 gB was found to be essential in restoring infectivity to gB-negative BHV-1 (Miethke *et al.,* 1995).

Recently it was shown that, despite significant amino-acid homology, HSV gB and HCMV gB could not complement the infectivity of gp110-negative EBV mutants (Lee *et al.,* 1997). However, differences in the cellular localization or in the function of HSV gB and HCMV gB when compared to EBV gp110 may not precluded efficient complementation (Lee *et al.,* 1997). Substantial differences in cellular processing and localization and in the ability to detect gB in virions exist. For example, EBV gp110 is sensitive to endoglycosidase H treatment, implying high-mannose N-linked glycosylation consistent with gp110 localizing predominantly to the endoplasmic reticulum and nuclear membrane (Qualtiere and Pearson, 1979; Emini *et al.,* 1987; Gong *et al.,* 1987; Gong and Kieff, 1990). In contrast, HSV gB and HCMV gB are processed in the Golgi and are readily detectable in the plasma membrane as well as the endoplasmic reticulum and nuclear membrane (Johnson and Spear, 1982; Britt and Vugler, 1989; Pereira *et al.,* 1989; Zheng *et al.,* 1996). In addition, EBV gp110 is not a major component of the virion envelope (Gong *et al.,* 1987; Gong and Kieff, 1990) while both HSV gB and HCMV gB are readily detectable in virions (Spear, 1976; Britt, 1984; Gretch *et al.,* 1988; Pereira, 1994).

Functionally, EBV gp110 appears to be essential for viral envelopment (Herrold *et al.,* 1996; Lee and Longnecker, 1997) while HSV gB appears to be required for entry. It is not known if EBV gp110 is required for viral entry. HCMV gB is similar to HSV gB in that this glycoprotein appears to be required for viral penetration (Navarro *et al.,* 1993) and has heparin-binding activity (Kari and Gehrz, 1992; Compton *et al.,* 1993). Soluble HCMV gB binds to cells, implying that HCMV gB may mediate viral attachment to susceptible cells by interaction with cellular components, possibly heparan sulfate (Boyle and Compton, 1998).

Glycoprotein B from a closely related herpesvirus that is processed and expressed similarly to EBV gp110 may allow at least partial functional complementation of gp110-negative EBV. Both human herpesvirus 8 (HHV-8) and EBV are γ-herpesviruses, although the former is classified as a member of the Rhadinovirus genus (Moore *et al.,* 1996) while the latter is a member of the Lymphocryptovirus genus (Rickinson and Kieff, 1996). In addition, both viruses infect B lymphocytes (Ambroziak *et al.,* 1995; Rickinson and Kieff, 1996), and, as illustrated in Fig. 1, HHV-8 gB shows 62.1% similarity and 42.6% identity to EBV gp110 (Russo *et al.,* 1996). The gB homologues from three other γ-herpesviruses, bovine herpesvirus 4, and equine herpesviruses 2 and 5, appear to be processed and expressed more similarly to HSV gB than EBV gp110 (Agius *et al.,* 1994; Lomonte *et al.,* 1997). In contrast, murine herpesvirus 68 (MHV-68) gB is processed and expressed similarly to EBV gp110. MHV-68 gB is glycosylated with high mannose N-linked glycans, localizes predominantly to the cytoplasm and perinuclear regions, and is not readily detected in virions (Stewart *et al.,* 1994).

The current studies analyzed the intracellular processing and expression of HHV-8 gB and assessed whether HHV-8 gB can complement gp110-negative EBV mutants. The cellular processing and expression of HHV-8 gB was similar to that previously described for EBV gp110 and the two glycoproteins formed hetero-oligomers *in vitro.* However, there was insufficient functional homology between HHV-8 gB and EBV gp110 to allow efficient complementation.

RESULTS

Construction of HHV-8 gB expression vectors

The predicted HHV-8 gB open reading frame encodes a protein 845 amino acids in length (Russo *et al.,* 1996). This open reading frame was PCR amplified in two independent reactions from the HHV-8-infected cell line BCBL-1, and both products were cloned into the expression vector pSG5. Sequencing of both amplified gB open reading frames revealed the presence of two silent nucleotide substitutions at positions 1705 (T to C) and 2881 (C to T) when compared to the previously published sequence derived from the HHV-8-infected cell line BC-1 (Russo *et al.,* 1996). To facilitate detection of expressed HHV-8 gB, the cytoplasmic tail of the glycoprotein was tagged with the hemagglutinin (HA) epitope, which is recognized by the 12CA5 monoclonal antibody. The HA-tagged gB construct (gB-HA) was deleted for the last four amino acids (Glu-Thr-Gly-Glu) in the wild-type glycoprotein and contained three new amino acids (Ala-Glu-Phe) prior to the HA epitope (Fig. 1).

Glycosylation and oligomerization of HHV-8 gB

Expression of HHV-8 gB-HA was readily detected in CHO-K1 cells after immunoblotting with the anti-HA antibody (data not shown). Human plasma was used to detect the expression of untagged HHV-8 gB and to confirm the expression of HHV-8 gB-HA. Briefly, gB-HA was immunoprecipitated with anti-HA and used to screen human plasma specimens obtained from the Multicenter AIDS Cohort Study (data not shown). Plasma obtained from one person 12 months after the diagnosis of Kaposi's sarcoma was found to be repeatedly reactive against the immunoprecipitated gB-HA. As shown in Fig. 2A, both the untagged and tagged forms of HHV-8 gB were readily detected in CHO-K1 cells after immunoblotting with this human plasma. The untagged HHV-8 gB

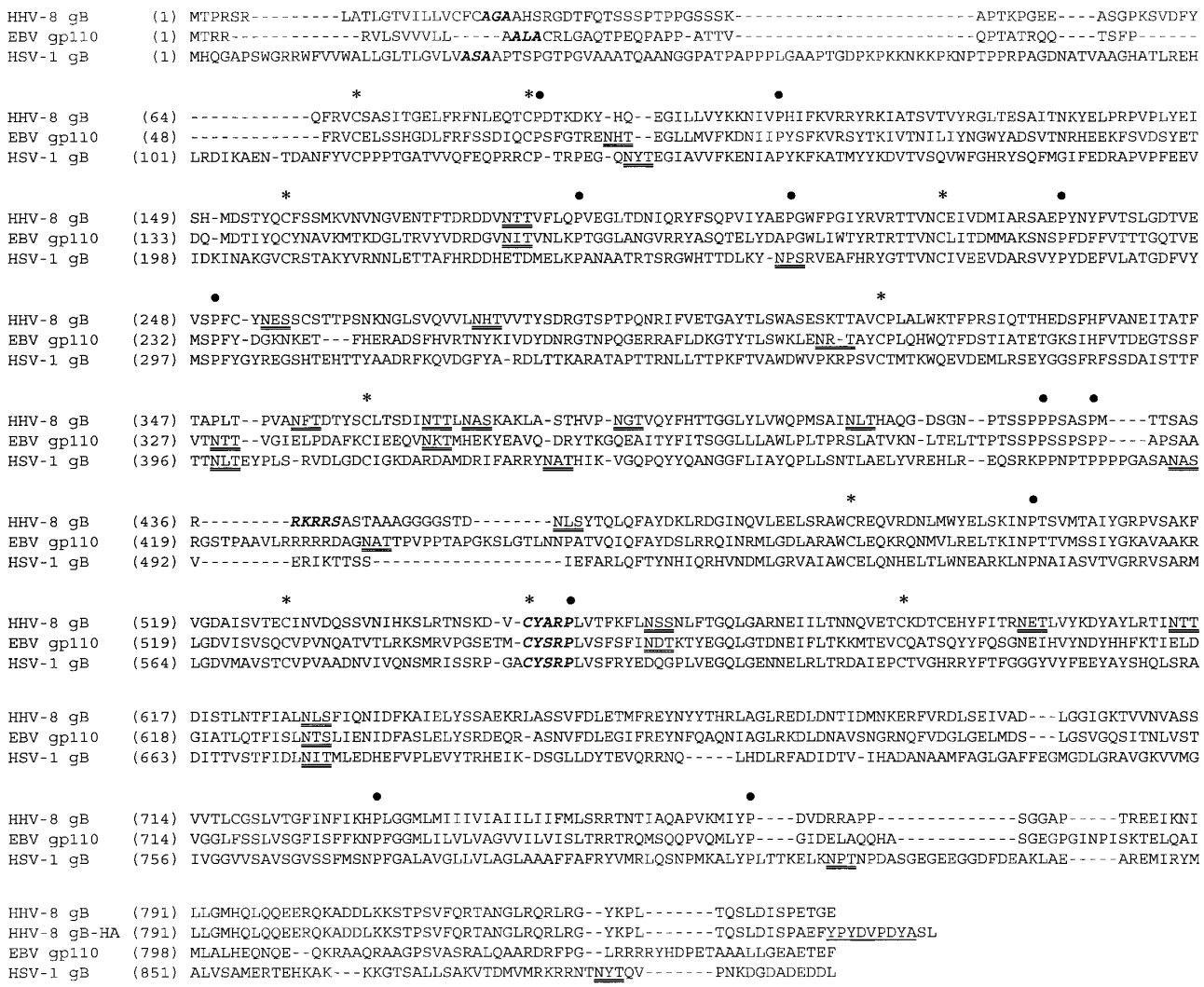

FIG. 1. Predicted amino-acid sequence of HHV-8 gB, EBV gp110, and HSV-1 gB. The amino-acid sequences for HSV-1 (KOS) gB and EBV (B95–8) gp110 were based on nucleotide sequences obtained from published reports (Baer *et al.,* 1984; Bzik *et al.,* 1984; Pellett *et al.,* 1985a). The amino-acid sequence for HHV-8 gB based on the nucleotide sequence obtained in these studies did not differ from that previously published (Russo *et al.,* 1996). Predicted N-linked glycosylation sites (double underline) and conserved cysteine (asterisks) and proline residues (filled circles) are shown. The conserved CY(S/A)RP sequence along with the potential signal peptidase and furin-mediated proteolytic cleavage sites are indicated in bold italics. Also shown is a shorter sequence of HHV-8 gB-HA that highlights the amino-acid changes resulting from addition of the HA-epitope. The HA-epitope is indicated with a single underline. The HHV-8 gB nucleotide sequence from the BIBL-1 cell-line was deposited with GenBank (Accession No. AF092928).

(lane 2) migrated at ~120 kDa while the tagged version (lane 3) was slightly larger due to the addition of 10 amino acids.

Computer-assisted analysis (PCGene and Omiga) of the predicted amino-acid sequence of HHV-8 gB revealed three potential hydrophobic domains (amino acids 7–24, 706–729, and 733–752) (Argos *et al.,* 1982). The initial 23 amino acids are most likely a cleavable signal sequence (Perlman and Halvorson, 1983; von Heijne, 1984). Cleavage at a potential signal peptidase site located immediately following the consensus Ala-X-Ala (where X corresponds to any amino acid) sequence would yield a core protein 822 amino acids in length. Thus the predicted molecular weights of mature core HHV-8 gB and HHV-8 gB-HA would be 91.3 and 92.5 kDa, respectively. The expressed proteins, however, had apparent molecular weights of ~120 kDa (Fig. 2A), consistent with HHV-8 gB undergoing glycosylation. Thirteen potential N-linked glycosylation sites (Asn-X-Thr/Ser motif where X corresponds to any amino acid except proline, which substantially lowers the efficiency of glycosylation) are predicted in the protein sequence of HHV-8 gB (Marshall, 1972; Bause, 1983; Gavel and von Heijne, 1990).

To investigate the glycosylation pattern of HHV-8 gB, the effects of *N*-glycosidase F and endoglycosidase H treatment were studied. As shown in Fig. 2B, the 120-kDa HHV-8 gB-HA protein was reduced to 95 kDa after either

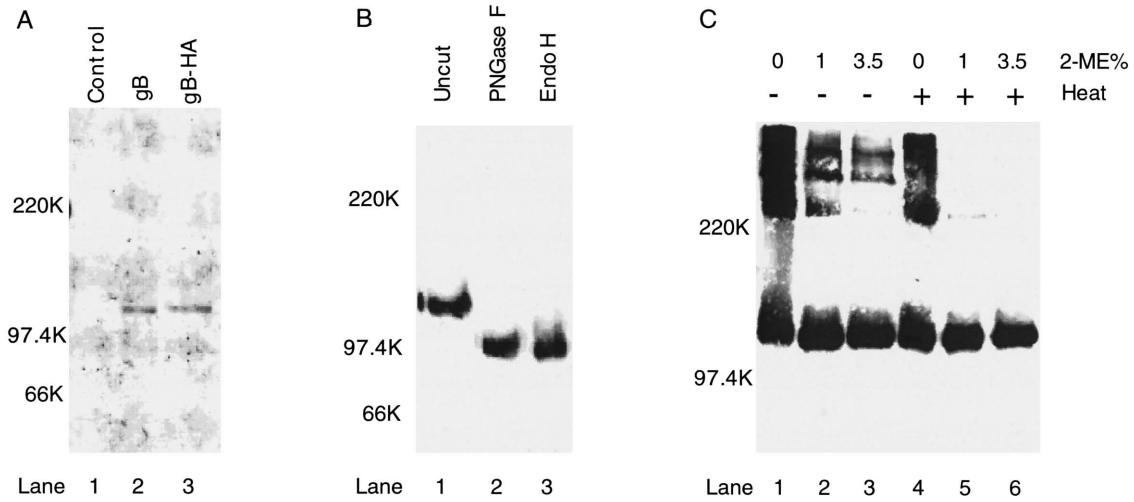

FIG. 2. Expression, glycosylation, and oligomerization of HHV-8 gB. (A) CHO-K1 cells were transfected with HHV-8 gB (lane 2), HHV-8 gB-HA (lane 3), or control expression vectors (lane 1). Twenty-four hours later, the cells were harvested, solubilized, boiled, and electrophoresized on a 7% polyacrylamide prior to transfer to nitrocellulose for immunoblotting. Plasma (diluted 1:1000) obtained from a Kaposi's sarcoma patient enrolled in the Multicenter AIDS Cohort Study and previously shown to react to immunoprecipitated HHV-8 gB-HA (data not shown) was used for immunoblotting. The plasma was preabsorbed with a lysate of CHO-K1 cells transfected with the control vector (pSG5). (B) CHO-K1 cells were transfected with the HHV-8 gB-HA expression vector. The cells were solubilized 24 h later and HHV-8 gB-HA was immunoprecipitated with anti-HA. Immune-complexes were subsequently collected by adding protein G Sepharose. Immunoprecipitated HHV-8 gB-HA was denatured by heating with 0.5% SDS and 1% 2-mercaptoethanol. Carbohydrate modifications were determined by incubating overnight at 37°C with 1 mU of endoglycosidase H (endo H; lane 3) in 50 mM sodium citrate (pH 5.5) or with 200 mU of *N*-glycosidase F (PNGase F; lane 2) in 1% NP-40 and 50 mM sodium phosphate (pH 7.5) or without added enzyme (lane 1). Western blots of control and glycosidase treated samples were immunoblotted with anti-HA. (C) Immunoprecipitated HHV-8 gB-HA was eluted with 3 M potassium thiocyanate (pH 71) and dialyzed against 10 mM NH_4HCO_3-0.01% SDS. Prior to electrophoresis, samples were prepared as follows. Unheated samples in the absence and presence of 2-mercaptoethanol (1% or 3.5% 2-ME) were loaded in lanes 1, 2, and 3 while heated (boiled for 5 min) samples in the absence and presence of 2-mercaptoethanol were loaded in lanes 4, 5, and 6. All samples were kept on ice except when heated.

N-glycosidase F (lane 2) or endoglycosidase H (lane 3) treatment. These results suggest HHV-8 gB is glycosylated with high-mannose N-linked oligosaccharides that are not subsequently modified in the Golgi, similar to results previously described for EBV gp110 (Gong *et al.,* 1987; Gong and Kieff, 1990).

In infected cells and in the viral envelope, HSV gB forms homodimers resistant to dissociation by SDS but sensitive to dissociation by heat (Sarmiento and Spear, 1979; Haffey and Spear, 1980; Claesson-Welsh and Spear, 1986). To determine whether HHV-8 gB forms oligomers, HHV-8 gB-HA expressed in CHO-K1 cells was immunoprecipitated with anti-HA. After elution with 3 M potassium thiocyanate and dialysis against 10 mM NH_4HCO_3-0.01% SDS, the purified HHV-8 gB-HA samples were electrophoresized and then transferred to nitrocellulose for immunoblotting. As shown in Fig. 2C, a significant fraction of unheated HHV-8 gB-HA migrated as oligomers (lane 1), although adding 2-mercaptoethanol to the samples decreased the relative amounts detected (lanes 2 and 3). Heating HHV-8 gB-HA without adding 2-mercaptoethanol had little effect on the amounts of oligomers detected (lane 4). In the samples heated in the presence of 2-mercaptoethanol, however, most of the slowly migrating bands disappeared (lanes 5 and 6). These results indicate that HHV-8 gB forms oligomers and that oligomerization requires disulfide bonds.

Intracellular localization of HHV-8 gB

The intracellular localization of HHV-8 gB was studied by immunofluorescence in CHO-K1 cells following transfection of the expression constructs of interest. As shown in Fig. 3, HHV-8 gB-HA localized to the perinuclear region of the cells but no apparent movement to the periphery was noted. A similar pattern of expression was noted for EBV gp110, consistent with the retention of EBV gp110 in the endoplasmic reticulum and nuclear membrane (Qualtiere and Pearson, 1979; Emini *et al.,* 1987; Gong *et al.,* 1987; Gong and Kieff, 1990). In contrast, the pattern noted for HSV gB is different in that HSV gB staining extended to the edges of each cell. This is consistent with previous reports that HSV gB is expressed in the plasma membrane as well as in the endoplasmic reticulum and nuclear membrane (Pereira *et al.,* 1989).

The lack of apparent movement of HHV-8 gB-HA to the periphery of the cell and the glycoprotein's sensitivity to endoglycosidase H treatment is consistent with HHV-8 gB not trafficking through the Golgi but rather being retained in the endoplasmic reticulum and nuclear membrane. Amino-acid sequence analysis of the cytoplasmic tail of HHV-8 gB revealed numerous lysines and arginines (Fig. 1), including a Lys-Lys-X-X motif (where X corresponds to any amino acid), that may be essential for

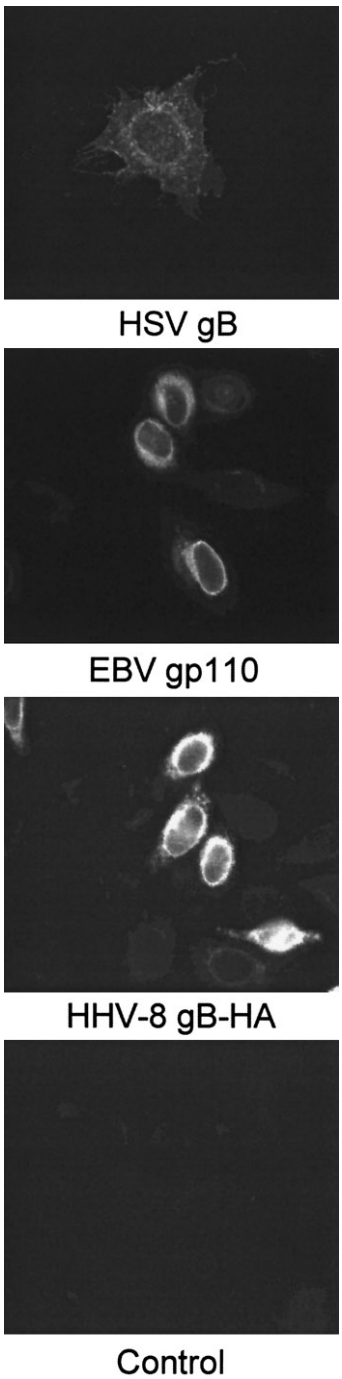

FIG. 3. Intracellular localization of HSV gB, EBV gp110, and HHV-8 gB-HA. CHO-K1 cells were transfected with expression vectors containing the indicated glycoproteins. Twenty-four hours after transfection, the cells were fixed and then incubated with monoclonal antibodies reactive with the indicated glycoproteins. II-105, VCA125, and anti-HA were used to detect HSV gB, EBV gp110, and HHV-8 gB-HA, respectively. Cells transfected with the vector control were incubated with anti-HA. After subsequent incubation with species specific fluorescein isothiocyanate- or indocarbocyanine-conjugated immunoglobulin G, cells were visualized using a Zeiss fluorescence photomicroscope.

retention of the glycoprotein in the endoplasmic reticulum or nuclear membrane (reviewed in Teasdale and Jackson, 1996). However, the di-lysine motif is not near the carboxyl terminus, an apparent requirement for this sequence to function as a retention signal.

Co-immunoprecipitation of HHV-8 gB and EBV gp110

Because both HHV-8 gB and EBV gp110 are γ-herpesviruses and appear to have similar patterns of cellular localization, the ability of the two glycoproteins to form hetero-oligomers was tested. CHO-K1 cells were transfected with one of three combinations: (i) HHV-8 gB-HA and EBV gp110 expression vectors, (ii) EBV gp110 and control expression vectors, or (iii) HHV-8 gB-HA and control expression vectors. Cell lysates were subsequently immunoprecipitated with either an anti-HA antibody or an anti-gp110 antibody. The resulting immune complexes were electrophoresized and then transferred to nitrocellulose for immunoblotting with anti-HA or anti-EBV human serum.

As shown in Fig. 4, EBV gp110 was detected in the anti-HA immunoprecipitate derived from cells transfected with both HHV-8 gB-HA and EBV gp110 (Fig. 4A, lane 1). Similarly, HHV-8 gB-HA was detected in the anti-gp110 immunoprecipitate derived from cells transfected with both glycoproteins, albeit at a lower level (Fig. 4B, lane 4). Thus HHV-8 gB-HA and EBV gp110 co-immunoprecipitated, implying formation of hetero-oligomers. These results could not be explained by immunogenic cross-reactivity between HHV-8 gB and EBV gp110 because no EBV gp110 was detected after immunoprecipitation with anti-HA (Fig. 4A, lane 2) and no HHV-8 gB-HA was detected after immunoprecipitation with anti-gp110 (Fig. 4B, lane 6). As expected, anti-HA did not recognize immunoprecipitated EBV gp110 (Fig. 4B, lane 5) and anti-EBV human serum did not recognize immunoprecipitated HHV-8 gB-HA (Fig. 4A, lane 3).

Similar experiments using cells transfected with HHV-8 gB-HA and HSV gB expression vectors showed that these two glycoproteins did not form hetero-oligomers to any significant extent. Although not visible in Fig. 4, a low level of HSV gB could be detected by an anti-HSV gB immunoblot using R-74 after immunoprecipitating glycoproteins with anti-HA from cells transfected with both HHV-8 gB-HA and HSV gB (Fig. 4C, lane 1). However, no HHV-8 gB-HA was detected by anti-HA after immunoprecipitating glycoproteins with the anti-HSV gB antibody II-105 (Fig. 4D, lane 4). The inability of HHV-8 gB and HSV gB to co-precipitate implies that the noted interaction between HHV-8 gB and EBV gp110 is specific and not the result of aggregation.

Complementation analysis of gp110-negative EBV-infected LCLs with HHV-8 gB

Because the two glycoproteins can form hetero-oligomers, the ability of HHV-8 gB to functionally complement EBV gp110 was examined. To this end, gp110-negative and -positive EBV-infected LCLs were transfected with

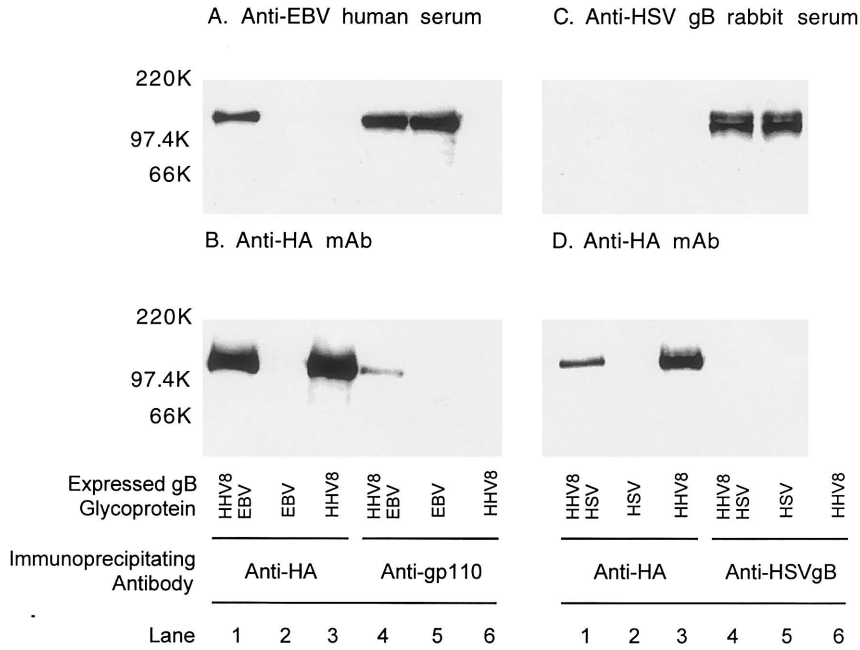

FIG. 4. Co-immunoprecipitation of HHV-8 gB-HA, and EBV gp110. CHO-K1 cells were transfected with combinations of expression vectors containing glycoproteins from the indicated viruses. A total of 2 μg of plasmid DNA (1 μg of each glycoprotein containing expression vector or vector control) was used for each transfection. The cells were lysed 24 h after transfection, and the glycoproteins of interest were immunoprecipitated. Anti-HA was used for the immunoprecipitations loaded in lanes 1–3 of all four panels. Anti-EBV-gp110 was used for lanes 4–6 of (A) and (B) while anti-HSV-1-gB was used for lanes 4–6 of (C) and (D). The immunoprecipitated proteins were separated by 10% SDS–PAGE and transferred to nitrocellulose for subsequent immunoblotting. Proteins were detected with anti-EBV human serum (A), anti-HA (B and D), or R-74 rabbit serum (C).

EBV gp110, HHV-8 gB, or HHV-8 gB-HA expression vectors. The expression of HHV-8 gB-HA was detected in transfected lymphoblastoid cell lines (LCLs) (Fig. 5) while expression of EBV gp110 has been demonstrated previously (Herrold *et al.,* 1996). To induce lytic replication, the LCLs were also transfected with pSVNaeZ (Swaminathan *et al.,* 1991) and then cultured in media containing TPA. Production of infectious virus was assessed by culturing primary human B lymphocytes with lethally γ-irradiated transfected LCLs or cell-free culture supernatants and measuring subsequent EBV-induced transformation (Herrold *et al.,* 1996).

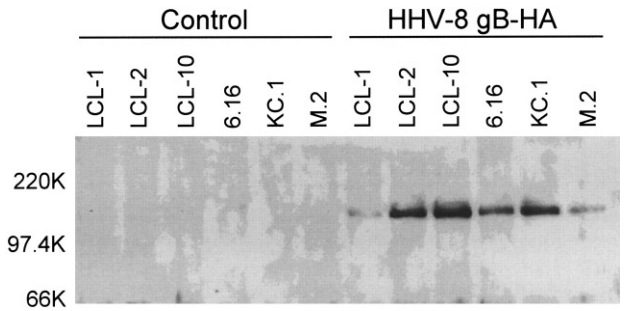

FIG. 5. Expression of HHV-8 gB-HA in LCLs. LCL-1, LCL-2, and LCL-10 contain wild-type EBV while 6.16, KC.1, and M.2 contain gp110-negative EBV mutants. Cells were electroporated with the vector control or the expression vector containing HHV-8 gB-HA. Cells were lysed 3 days after transfection and then the cleared lysates were immunoprecipitated with the anti-HA monoclonal antibody. The immunoprecipitated proteins were separated by 10% SDS–PAGE and transferred to nitrocellulose for subsequent immunoblotting using anti-HA.

As shown in Table 1, transfection of the gp110-positive EBV-infected LCLs with any of the expression vectors had no apparent effect on the frequency of EBV-induced transformation. In contrast, only the gp110-negative EBV-infected LCLs that were transfected with the EBV gp110 expression vector induced transformation of primary B lymphocytes. The lack of transformation after transfection with HHV-8 gB or HHV-8 gB-HA expression vectors indicates that there is not sufficient functional homology between EBV gp110 and HHV-8 gB to allow efficient complementation.

DISCUSSION

Glycoprotein B is the most conserved herpesvirus glycoprotein. Analysis of the HHV-8 gB protein sequence (Fig. 1) reveals that HHV-8 gB shares structural features and motifs with previously characterized herpesvirus gB glycoproteins. HHV-8 gB most likely consists of three domains. Two hydrophobic domains from amino acids 706–752 divide HHV-8 gB into a 93-amino-acid carboxyl terminal tail and a large 705-amino terminal domain. As described for HSV gB (Pellett *et al.,* 1985b), the carboxyl-terminal tail displays features consistent with a cytoplasmic domain in that this region is hydrophilic and posi-

TABLE 1

Transformants Obtained by Passage of gp110-Negative and gp110-Positive EBV Containing LCLs after Complementation with HHV-8 gB

Experimental group	Cell line	Experiment	Glycoprotein used for complementation		
			EBV gp110	HHV-8 gB	HHV-8 gB-HA
Cocultivation					
gp110-positive EBV LCLs	LCL10	1	14	7	19
		2	18	26	30
		3	21	20	34
		4	22	10	12
		5	40	38	50
Total			115	101	145
gp110-negative EBV LCLs	KC.1	1	0	0	0
		2	1	0	0
		3	0	0	0
		4	0	0	0
		5	28	0	0
Total			29	0	0
	M.2	1	5	0	0
		2	6	0	0
		3	1	0	0
		4	2	0	0
		5	4	0	0
Total			18	0	0
Cell free					
gp110-positive EBV LCLs	LCL10	1	2	4	2
		2	3	2	2
		3	1	3	7
		4	0	0	0
		5	2	2	1
Total			8	11	12
gp110-negative EBV LCLs	M.2	1	2	0	0
		2	1	0	0
		3	0	0	0
		4	2	0	0
		5	0	0	0
Total			5	0	0

Note. LCLs were transfected with plasmids containing the indicated glycoproteins. Three to 6 days posttransfection, primary human B lymphocytes were co-cultivated with γ-irradiated transfected LCLs or infected with cell-free virus. The number of wells positive for EBV transformants were scored after 6–8 wk. Each transfection was plated onto one 96-well plate. Each line represents one experiment. The total number of clones obtained in each experimental group is shown. Only clones from which the hygromycin phosphotransferase insert, but not the intact gp110 gene, could be detected by PCR were counted (data not shown). No transformants resulted from infection of primary human B lymphocytes with gp110-negative EBV obtained from LCLs transfected with the expression vector alone (data not shown).

tively charged. The initial 705 residues are most likely a large ectodomain. This region is predominantly hydrophilic, with 13 potential *N*-glycosylation sites and a predicted cleavable signal sequence. In addition, 10 cysteine residues previously shown to be highly conserved among the known herpesvirus gB glycoproteins are present (Ross *et al.,* 1989; Goltz *et al.,* 1994; Pereira, 1994). Interestingly, a highly conserved sequence (Cys-Tyr-Ser-Arg-Pro) unique to herpesvirus gB glycoproteins and of unknown function (Ross *et al.,* 1989) is only partially conserved in HHV-8 gB (Cys-Tyr-Ala-Arg-Pro). The significance of this observation is unclear.

A proteolytic cleavage site (Arg-X-(Lys/Arg)-Arg-Ser), where X can represent any amino acid) is predicted in the HHV-8 gB protein sequence (Spaete *et al.,* 1988). HCMV, PrV, and BHV-1 gB all contain this site and are cleaved during synthesis (Grose *et al.,* 1984; Lukacs *et al.,* 1985; Montalvo and Grose, 1987; Spaete *et al.,* 1988; Whealy *et al.,* 1990; Kopp *et al.,* 1994). Cleavage of HCMV gB is mediated by the human endoprotease furin (Vey *et al.,* 1995). In contrast, both EBV gp110 and HSV gB lack this site and are not cleaved. Inhibition of HCMV gB cleavage may impair release of progeny virus from infected cells (Brücher *et al.,* 1990) while disrupting the cleavage site in BHV-1 gB may impair cell to cell spread of virus (Kopp *et al.,* 1994). Interestingly, HHV-8 gB does not appear to be cleaved. Under reducing conditions, Western blots of transfected CHO-K1 lysates using human plasma for detection only identified bands at ~120 kDa for both HHV-8 gB and gB-HA (Fig. 2A). In addition,

only 120-kDa bands were detected after immunoprecipitating gB-HA from transfected LCLs (Fig. 5). The reason for these observations is not clear but additional HHV-8 proteins may be required for proteolytic cleavage of HHV-8 gB.

The relatively high degree of amino-acid and structural homology among the known herpesvirus gB glycoproteins is consistent with an essential role for this glycoprotein in viral infectivity. All gB glycoproteins tested are required for infectivity in that engineered herpesvirus mutants that do not express gB are not infectious in the absence of complementation (Cai *et al.,* 1987; Rauh and Mettenleiter, 1991; Miethke *et al.,* 1995; Herrold *et al.,* 1996). The high degree of homology among the gB glycoproteins implies that the gBs from different herpesviruses may have similar functions and that cross-complementation may be possible. Indeed, cross-complementation among α-herpesvirus gB glycoproteins can occur but is not always reciprocal (Misra and Blewett, 1991; Rauh *et al.,* 1991; Kopp and Mettenleiter, 1992; Mettenleiter and Spear, 1994; Miethke *et al.,* 1995). However, cross-complementation among the gB glycoproteins from different subfamilies of herpesviruses appears to be considerably less efficient. HSV gB and HCMV gB failed to complement gp110-negative EBV while HCMV gB and EBV gp110 failed to complement gB-negative HSV (Lee *et al.,* 1997). It may be that significant divergence in gB structure and function among the herpesvirus subfamilies may preclude complementation.

The results presented here demonstrate that HHV-8 gB and EBV gp110 appear to have similar patterns of intracellular expression. HHV-8 gB was sensitive to endoglycosidase H treatment and localized to the perinuclear region of transfected cells. Although more definitive localization studies are required, these observations suggest that HHV-8 gB is retained in the endoplasmic reticulum and nuclear membrane, a pattern similar to that previously described for EBV gp110 (Qualtiere and Pearson, 1979; Emini *et al.,* 1987; Gong *et al.,* 1987; Gong and Kieff, 1990). In addition, HHV-8 gB and EBV gp110 co-immunoprecipitated, implying that the domains required for the oligomerization of HHV-8 gB may be conserved in EBV gp110. Based on the results presented here, the oligomerization of HHV-8 gB appears to require disulfide bonds. For HSV gB, an essential site and a nonessential site for oligomer formation have been identified (Highlander *et al.,* 1991; Qadri *et al.,* 1991; Navarro *et al.,* 1993; Laquerre *et al.,* 1996). The essential site can be further divided into two domains (residues 626–653 and 653–675) that are both sufficient for oligomerization (Laquerre *et al.,* 1996). However, the cysteine residues at amino acids 596 and 633 do not appear to be required for the formation of HSV gB dimers (Laquere *et al.,* 1996).

With the noted similarities between HHV-8 gB and EBV gp110, it was somewhat unexpected that HHV-8 gB was unable to efficiently complement gp110-negative EBV. This apparent lack of complementation could be attributed to several reasons. For one, differences in HHV-8 and EBV may prevent efficient complementation. Although similarities in processing and cellular localization suggest that HHV-8 gB and EBV gp110 may have similar functions, no information is currently available on the role of HHV-8 gB in viral infectivity. Also, additional EBV glycoproteins essential for viral infectivity may not interact with HHV-8 gB. If a well-coordinated cascade of interactions between viral glycoproteins and the plasma membrane is needed for efficient viral entry or egress, formation of an entry or egress complex containing some or all of the essential glycoproteins may be required. Essential EBV glycoproteins, such as gp85 or gp25, may be unable to functionally interact with HHV-8 gB.

The above results do not exclude the possibility that HHV-8 gB may be able to complement gp110-negative EBV but only partially. The complementation assay requires the production of virus that can subsequently enter and then transform primary B cells. Although EBV gp110 appears to be essential for viral assembly (Herrold *et al.,* 1996; Lee and Longnecker, 1997), the possibility that gp110 mediates viral entry into cells has not been excluded and is a reasonable assumption based on the known functions of other herpesvirus gB glycoproteins. HHV-8 gB may be able to complement assembly or entry of gp110-negative EBV but not both. EBV gp110 is not required for transformation of primary B cells (Herrold *et al.,* 1996). Similarly, HHV-8 gB may be able to completely complement gp110 but at an efficiency below the sensitivity of the complementation assay. Future studies designed to investigate the functional role of HHV-8 gB in viral infectivity may help to delineate some of these possibilities.

In conclusion, the cellular expression of HHV-8 gB was similar to that previously described for EBV gp110. In addition, HHV-8 gB and EBV gp110 co-immunoprecipitated, implying the formation of hetero-oligomers. However, there was insufficient functional homology between HHV-8 gB and EBV gp110 to allow efficient complementation of gp110-negative EBV mutants.

MATERIALS AND METHODS

Cells and viruses

CHO-K1 cells (American Type Culture Collection) were passaged in medium F12 supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). BCBL-1 is a body-cavity-based lymphoma cell line infected with HHV-8 (Renne *et al.,* 1996) obtained from the AIDS Research and Reference Reagent Program. BCBL-1 cells were passaged in medium RPMI 1640 containing 10% FBS, 5×10^{-5} M 2-mercaptoethanol and antibiotics. All EBV-containing LCLs were passaged in RPMI 1640 medium supplemented with 10% FBS and antibiotics. Lymphoblastoid cell lines LCL-1, LCL-2, and

LCL-10 contain wild-type EBV while 6.16, KC.1, and M.2 contain gp110-negative EBV mutants constructed by inserting hygromycin phosphotransferase into the *Nsi*I endonuclease restriction site of the gp110 (BALF4) reading frame (Herrold *et al.,* 1996; Lee and Longnecker, 1997).

Antibodies

Monoclonal antibodies used were a mouse anti-EBV gp110 antibody VCA125 (Chemicon), a mouse anti-HSV-1 gB antibody II 105 (Para *et al.,* 1985), and a rat anti-HA high affinity antibody (Boeringer-Mannheim). Polyclonal antibodies used were a rabbit anti-HSV-1 gB antibody R-74 (Herold *et al.,* 1994) and previously described anti-EBV human serum (Herrold *et al.,* 1996). R-74 was preabsorbed by incubation at 4°C overnight with a gB-negative HSV-1-infected cell lysate.

Amplification and cloning of viral DNA sequences

PCR was performed using Vent polymerase (New England Biolabs) and purified DNA isolated from BCBL-1 by the Trizol Reagent kit (GibcoBRL). The HHV-8 gB open reading frame was amplified using the *Bam*HI endonuclease restriction site tagged sense primer gB-1 5′-TGAATTCATGACTCCCAGGTCTAGATTGGCC-3′ and the *Eco*RI tagged anti-sense primer gB-2 5′-TTGGATCCGTC-TGTGTGCAACGAGTAGG-3′. (Northwestern University Biotechnology Facility). Thermocycling reactions using a PTC-100 programmable thermal controller (MJ Research) consisted of a 35-cycle step-down protocol in which the annealing temperature was sequentially lowered from 70°C by 3°C increments after every third cycle until a final temperature of 55°C was reached. For each cycle, the DNA was denatured at 94°C for 45 s and the PCR-generated DNA fragment was extended at 72°C for 150 s. A final extension for 5 min at 72°C followed. All PCR products were desalted and concentrated using the QIAEX II gel extraction kit (Qiagen). PCR products were subsequently cloned into the expression vector pSG5 (Stratagene). HHV-8 gB nucleotide sequences were determined from both strands (University of Chicago Cancer Research Center DNA Sequencing Facility).

The cytoplasmic tail of HHV-8 gB was tagged with the HA epitope as follows. Briefly, an *Eco*RI linker was ligated into a blunted *Bsp*EI restriction site. The truncated gB open reading frame was then subcloned into the *Eco*RI site of pMN104 and screened for orientation. The vector pMN104 allows expression of HA-tagged proteins under control of the HCMV immediate-early promoter (Novotny, 1996). The HA-tagged gB construct (gB-HA) was excised by sequential digestion with *Bsp120I*, large (klenow) fragment of DNA polymerase I, and *Bam*HI and then cloned into the *Bam*HI and blunted *Bgl*II sites of pSG5.

Transfections

Transfections into CHO-K1 cells were done using Lipofectamine Reagent (GibcoBRL). Briefly, subconfluent CHO-K1 cells were overlaid with 1–2 μg of purified plasmid DNA and Lipofectamine reagent in OptiMEM (GibcoBRL) and incubated at 37°C for 8 h. After the transfection mixture was removed, complete F12 medium was added, and the cells were incubated for 16 h at 37°C in 5% CO_2. The cells were then harvested or processed for subsequent experiments.

Transfections into LCLs were done by electroporation. Briefly, 5–20 $\times$ 10^6 cells were resuspended in 400 μl RPMI containing the plasmids of interest. The cells were electroporated using a Gene Pulser electroporator (Bio-Rad Laboratories). The cells were then transferred into 25-cm^2 flasks containing medium RPMI 1640 and 20 ng/ml tetradecanoyl phorbol acetate (TPA; Sigma) and subsequently incubated for 3–6 days at 37°C in 5% CO_2.

Western blots

Cell lysates were solubilized in sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 min, and then loaded onto a SDS–polyacrylamide (PAGE) gel (*N,N′*-methylene-bisacrylamide) (Bio-Rad Laboratories). Following electrophoresis, the proteins were transferred to nitrocellulose (Schleicher & Schuell) with a Trans-Blot electrophoretic transfer cell apparatus (Bio-Rad Laboratories). The nitrocellulose blot was incubated in blocking buffer and then sequentially with antibodies reactive against the antigen of interest and species-specific alkaline phosphatase-conjugated antiimmunoglobulin G. Reactive bands were visualized after incubation with chemiluminescence ECL substrate (Amersham Life Sciences) and subsequent exposure to autoradiography film (Amersham Life Sciences).

Immunoprecipitations

Cells were lysed with RIPA buffer containing protease inhibitors for 30 min on ice. After centrifugation, the cleared lysates were preaborbed with protein G sepharose (Pharmacia Biotech) and then immunoprecipitated with either anti-EBV gp110 VCA125 (2 μg/ml), anti-HSV gB II-105 (diluted 1:100), or anti-HA (2 μg/ml) for 1–3 h at 4°C. The immune-complexes were collected by the addition of protein G Sepharose and then washed with lysis buffer prior to further analysis.

Immunofluorescence

CHO-K1 cells were grown and transfected on microscope slides and then fixed with a 1:1 mixture of acetone and methanol for 5 min. After blocking with 20% goat serum, cells were sequentially incubated with monoclonal antibodies (diluted 1:500) reactive against the antigens of interest and fluorescein isothiocyanate-conjugated goat anti-mouse (Pharmingen) or indocarbocya-

nine-conjugated goat anti-rat (Jackson) immunoglobulin G (diluted 1:500). Cells were subsequently observed by using a Zeiss fluorescence photomicroscope.

Lymphocyte transformation assays

Primary human B lymphocytes were isolated by layering whole blood over ficoll and then mixing the resulting buffy coat cells with sheep red blood cells (Bectin Dickinson) to rosette the T cells. As previously described (Herrold *et al.,* 1996), purified B cells were cocultivated with 1–3 $\times$ 10^4 γ-irradiated (5000 rads) transfected LCLs or with cell-free virus obtained from the lymphoblastoid culture supernatant. Subsequently, 5–10 $\times$ 10^4 B cells were plated in each well of 96-well dishes and incubated for 6–8 weeks at 37°C in 5% CO_2. Wells with cell growth were visualized and the presence of the hygromycin phosphotransferase insert and the disruption of the gp110 gene in the newly obtained lymphoblastoid cells verified by PCR as previously described (Herrold *et al.,* 1996).

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