The Cytoplasmic Terminus of Kaposi’s Sarcoma-Associated Herpesvirus Glycoprotein B Is Not Essential for Virion Egress and Infectivity

R. Subramanian,1,2 O. D’Auvergne,1,2 Haixia Kong,2 and K. G. Kousoulas2*

Department of Biological Sciences, Southern University & A&M College, Baton Rouge, Louisiana, 1 and Division of Biotechnology and Molecular Medicine, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana2

Received 19 March 2008/Accepted 5 May 2008

Kaposi’s sarcoma-associated herpesvirus (KSHV)-encoded glycoprotein B (gB) is an important determinant of viral infectivity and virion egress. A small interfering RNA (siRNA)-based strategy was devised to inhibit KSHV gB gene expression. Transient cotransfection of plasmids constitutively expressing gB and anti-gB siRNAs in 293 cells substantially inhibited gB mRNA levels and protein production. Similarly, transient expression of siRNAs into the primary effusion lymphoma cell line BCBL-1 caused a substantial reduction of gB transcripts and protein synthesis. TaqMan real-time PCR assays against the lytic KSHV gene ORF59 and infectivity assays on 293 cells were employed to assess the effect of inhibiting gB synthesis on virion egress from BCBL-1 cells and infectivity on 293 cells, respectively. These experiments showed that gB was essential for virion egress and infectivity. Transfection of a codon-optimized gB gene with the first 540 nucleotides altered, and therefore not recognized by anti-gB siRNAs that target the native but not the codon-optimized sequence, efficiently rescued virion egress and infectivity in BCBL-1 cells in the presence of siRNAs inhibiting wild-type gB expression. To assess the role of the cytoplasmic domain of gB in virion egress, mutant gB genes were generated specifying carboxyl terminal truncations of 25 and 58 amino acids disrupting two prominent predicted α-helical domains associated with virus-induced cell fusion. A third truncation removed the entire predicted cytoplasmic terminus of 84 amino acids, while a fourth truncation removed 110 amino acids, including the terminal most hydrophobic, intramembrane anchoring sequence. Virion egress experiments revealed that all truncated gBs facilitated virion egress from BCBL-1 cells, with the exception of the largest 110-amino-acid truncation, which removed the gB anchoring sequence. Importantly, the gB truncation that removed the entire predicted cytoplasmic domain increased virion egress, suggesting the presence of a egress regulation domain located proximal to the intramembrane sequence within the cytoplasmic domain of gB. All supernatant virions were infectious on 293 cells, indicating that the carboxyl terminus of gB is not essential for either virion egress or virus infectivity.

Kaposi’s sarcoma-associated herpesvirus (KSHV), also referred to as human herpesvirus 8, is a member of the gammaherpesvirus family (genus Rhadinovirus) (46, 59). KSHV is etiologically associated with Kaposi’s sarcoma, primary effusion lymphoma, and multicentric Castleman’s disease (4, 21, 60). KSHV can infect a variety of human cell types, including B, T, endothelial, epithelial, fibroblastic, and keratinocyte cells, and nonhuman cell types, including owl monkey kidney and baby hamster kidney fibroblastic cells (13, 15, 19, 20, 22, 28, 36, 43, 44, 50, 56, 62, 67). All herpesviruses initiate infection by means of direct binding to various receptors on cell surfaces that is mediated by several viral glycoproteins embedded in viral envelopes. Viral glycoproteins play important roles in virus attachment to susceptible cells, fusion of the viral envelope with either cellular or endosomal membranes, and virion morphogenesis and egress (26, 42, 57). Herpesviruses enter into susceptible cells by direct fusion of viral envelopes with cellular membranes via pH-independent or pH-dependent processes, depending on the cell type (7, 16, 40, 47, 48), or via receptor-mediated endocytosis (61), as has been shown for KSHV entry into human foreskin fibroblastic cells and B cells (1, 3).

Herpesviruses assemble their capsids within the nuclei of infected cells and most likely egress by an envelopment-de-envelopment process in which they acquire a primary envelope from the inner lamellae of the nuclear membrane and subsequently de-envelop by fusion of their initial viral envelopes with the outer nuclear lamellae, releasing capsids into the cytoplasm. Final cytoplasmic envelopment is thought to occur in the cytoplasm by the budding of cytoplasmic capsids into intracellular membranes, most likely derived from the trans-Golgi network (37–39, 45). KSHV codes for a number of glycoproteins, some of which have significant homology to glycoproteins of other herpesviruses. These include glycoprotein B (gB) (open reading frame 8 [ORF8]) (49), gH (ORF22), gM (ORF39), gL (ORF47) (46, 59), and gN (ORF53) (14, 30, 59). KSHV also encodes additional glycoproteins which do not have homologs in other herpesviruses, including gpK8.1A, gpK8.1B, K1, K14, and K15, which are expressed during lytic replication (59).

gB is one of the most conserved herpesvirus glycoproteins. It is an essential virion component for members of the alpha- and betaherpesvirus subfamilies and thought to function in virion
attachment and virus entry into susceptible cells (10, 17, 52), Epstein-Barr virus (EBV), a gamma-1-herpesvirus (genus Lymphocryptovirus) (25), and murine gammaherpesvirus 68, a member of the gamma-2-herpesvirus (genus Rhadinovirus) subfamily, do not incorporate detectable levels of gB into their virions (24). However, virions of bovine herpesvirus 4, a gamma-2-herpesvirus (9, 34), contain gB (34). KSHV virions incorporate gB in the viral envelope, which is important for attachment to cell surfaces and entry via an RGD-dependent binding to integrins. Initially, the α3β1 integrin was implicated (2), but more recently, it was shown that αvβ3 is the integrin involved in gB RGD-mediated virus entry (23). KSHV gB is a type 1 membrane glycoprotein of 845 amino acids in length (6, 53, 55). It contains a predicted signal sequence of 23 amino acids, an extracellular domain containing multiple glycosylation sites, and multiple hydrophobic regions, of which the most carboxy-terminal region serves to anchor gB in membranes (6, 53–55). gB has been shown to be involved in the egress of herpesviruses, such as alphahepesvirus, pseudorabies virus, and EBV (8, 29, 32, 51, 52). KSHV gB was also shown to be essential for virion egress in 293 cells, since a KSHV mutant virus carrying a deletion of the gB gene was unable to egress from 293T cells, while the defect was rescued by exogenously provided gB (31). In contrast to these examples, herpes simplex virus type 1 (HSV-1) gB is not required for virion egress (11). More recently, it was shown that the carboxy terminus of HSV-1 gB is ubiquitinated and it appears to regulate virion egress (12).

RNA interference (RNAi) is the process by which double-stranded RNA silences gene expression, either by inducing the sequence-specific degradation of complementary mRNA or by inhibiting translation (41). Chemically synthesized short double-stranded RNA molecules of 21 or 22 nucleotides known as small interfering RNAs (siRNAs) were shown to cause mRNA degradation via the RNAi mechanism while evading the interferon response (18). Furthermore, endogenous expression of siRNA in the form of short hairpin RNAs, which bear a foldback stem-loop structure (8–11), induced target gene silencing in mammalian cells. RNAi and siRNA technologies have been instrumental as molecular biological tools for the silencing of specific gene expression under a variety of experimental settings (27). For KSHV, siRNA approaches have been used to investigate specific viral functions (64, 66).

We employed an siRNA approach to conditionally silence KSHV gB gene expression in 293 and BCBL-1 cells. In this system, rescue of gB synthesis was accomplished by providing a synthetic gB with an altered DNA sequence which could not be recognized by the anti-gB siRNAs. This siRNA-based conditional silencing system was utilized to investigate the role of the cytoplasmic terminus of KSHV gB in virion egress. We found that the carboxy terminus of gB contains a domain that regulates virion egress, but overall, the cytoplasmic domain of gB is not essential either for virion egress from BCBL-1 cells or for virus infectivity.

MATERIALS AND METHODS

Cells and virus propagation. BCBL-1 cells harboring the rKSHV.152 genome constitutively expressing the green fluorescent protein (GFP) (gift from J. Vieira) were cultured in RPMI 1640 (Hyclone) medium with 10% heat-inactivated fetal bovine serum (Hyclone) with 2 mM l-glutamine, 1% penicillin-streptomycin (Gibco), and 250 mg of G418/ml (Gibco). 293A cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 1% penicillin-streptomycin. The KSHV lytic cycle was induced in the BCBL-1 cells by adding 25 ng/ml of 12-O-tetradecanoylphorbol-13-acetate (TPA; Sigma, St. Louis, MO). Virus from the supernatant was collected after 16 h and treated with DNase I before the extraction of viral DNA as previously described (35). For infectivity studies, the supernatants were collected 48 h after induction with TPA.

Antibodies. Rabbit polyclonal antibodies generated in our laboratory against KSHV gB peptides were used to detect gB protein by Western blot analysis as previously described (6). Peptide antibodies specific to amino acids 167 to 191 of the gB protein were used in this study. Mouse anti-HuGAPDH (human glyceraldehyde-3-phosphate dehydrogenase) monoclonal antibodies were used to detect the GAPDH.

Vector construction. siRNAs were designed targeting the 5′ region of the KSHV gB expression sequence by use of Ambion’s online siRNA target finder. Two siRNAs, si18 (sense strand, HindIII 5′ AGCTTCAAGATGTA 3′), and si19 (antisense strand, HindIII 5′ ATTCGAGTGTCCTTTTTG 3′), were used in these experiments. siRNA approaches have been used to investigate specific viral functions (64, 66).

Codon optimization and construction of gB truncations. The region consisting of the first 540 bases of KSHV gB from ATG to the HpaI restriction site, which carried the siRNA target sequences, was codon optimized using the DNAWorks website at http://helixweb.nih.gov/dnaworks. The wild-type KSHV gB was cloned in the expression vector pXFLAG within the KpnI and BamHI sites. The codon-optimized fragment was used to replace the first 540-bp region between the KpnI and HpaI sites of the wild-type gB gene. The gB carboxy-end truncations were generated by designing forward primers for the 5′ end of the codon-optimized gB and reverse primers carrying stop codons 75, 174, 252, and 330 bp from the 3′ end of gB to generate truncation gBp, gBc, gBtrm, and gBtrm gene constructs, respectively. The forward primers for codon-optimized gB and reverse primers carrying the opal mutations are indicated in Table 1. A 577-bp encephalomyocarditis virus internal ribosome entry site (IRES) sequence was inserted in frame downstream of the gB trm vector between the KpnI and BamHI sites. The gB gene sequences coding for the gB truncations were cloned downstream of the IRES at the BamHI site.

Plasmid vectors expressed siRNA individually or in combination with any one of the codon-optimized gBco, gBp, gBc, or gBtrm constructs.

Transient transfection of 293 and BCBL-1 cells. 293 cells were seeded onto 12-well plates at 0.5 × 10⁵ cells/well for 8 hours, medium was replaced with 500 µl of fresh medium, and cells were used for transfection experiments. BCBL-1 cells (0.5 × 10⁵ cells/well) were suspended in fresh medium and used for each transfection. Transfections were carried out using SuperFect (Qiagen, Inc.) transfection agent according to the manufacturer’s instructions.

Immunohistochemical studies. To detect the expression of gB mutant genes, 293 cells were transiently transfected with plasmid vectors carrying gBco, gBp, gBc, and gBtrm as described earlier. Forty-eight hours posttransfection, the cells were fixed, reacted with anti-gB peptide antibody, and stained using the Vector NovaRED substrate kit (Vector Laboratories Inc., California), as per the manufacturer’s instructions.

RT-PCR. Transiently transfected 293 cells were harvested 48 h after transfection and total RNA was extracted. Specifically, medium was aspirated and cells were scraped and resuspended evenly in 200 µl of ice-cold phosphate-buffered saline. Seven hundred microliters of Triz-Reactant (Molecular Research Center, Inc.) were added to the cells and mixed thoroughly to lyse the cells. After 15 min, 200 µl of chloroform was added and mixed thoroughly. The tubes were placed on ice for 15 min and centrifuged at 13 × 10⁶ rpm for 15 min at 4°C. The supernatant was collected, precipitated in an equal volume of isopropanol, and left on ice for 30 min. The mix was centrifuged at 4°C for 20 min at 13 × 10⁶ rpm. The
resultant pellet was washed in 70% ethanol made with nuclease-free water, dried, and resuspended in 40 μl of nuclease-free water. The RNA was treated with Turbo DNase I (Ambion, Inc.) and quantified. First-strand cDNA was made using the high-capacity cDNA reverse transcription (RT) kit (Applied Biosystems, Inc.). Equal quantities of first-strand cDNA were used for PCR using the universal gB primers and the primers for GAPDH. The PCR products were resolved by electrophoresis on a 1% agarose gel.

For RT-PCR quantification of RNA derived from BCBL-1 cells, lytic replication of KSHV was induced by adding 25 ng/ml TPA to transiently transfected BCBL-1 cells 24 h after transfection. Induction was carried out in serum-free medium and the supernatant and cells were collected 16 h postinduction. Harvested cells were resuspended in 500 μl of ice-cold phosphate-buffered saline and split into two parts for RNA extraction (200 μl) and Western blot analysis (300 μl). Total RNA was extracted and first-strand cDNA was prepared as described earlier. Equal amounts of first-strand cDNA were used for PCR using the universal gB primers and the primers for GAPDH (Table 1) in the case of 293 cells. RT-PCR of RNA derived from BCBL-1 cells was carried out using primers specific to full-length wild-type gB and the carboxyl-terminal region of gB (Table 1) in addition to primers for universal gB and GAPDH. The PCR products were resolved by electrophoresis on a 1% agarose gel.

**TABLE 1. List of primers**

<table>
<thead>
<tr>
<th>Primer name (description)</th>
<th>Sequence (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>gBco (gB optimized; forward)</td>
<td>ACAGGAAAGCTTATGACCCCAAGGACGGCTTG</td>
</tr>
<tr>
<td>gBp (25-aa gB truncation; reverse)</td>
<td>TTGTATGGACCTCATTGCGGCTAGGCTAAACACCCGA</td>
</tr>
<tr>
<td>gBg (58-aa gB truncation; reverse)</td>
<td>ATAGAAGGAGCTCAGATTTCCTCCGTTGTTGGG</td>
</tr>
<tr>
<td>gBc (84-aa gB truncation; reverse)</td>
<td>GGATCCTCTTCACTCGGACTGGA</td>
</tr>
<tr>
<td>gBBrm (110-aa gB truncation; reverse)</td>
<td>TGGATCCTCAGGATACCTAGGGGTGTTT</td>
</tr>
<tr>
<td>gBUNI (gB universal; forward)</td>
<td>TTCAGACTAACCCGAGGAC</td>
</tr>
<tr>
<td>gBUNI (gB universal; reverse)</td>
<td>GTCAGGTATATCGCGCACAT</td>
</tr>
<tr>
<td>gBwt (forward)</td>
<td>GACACCTTTTGACAGT</td>
</tr>
<tr>
<td>gBwt (reverse)</td>
<td>TCCTGGGAGTTCAATCCTG</td>
</tr>
<tr>
<td>gB (carboxy; forward)</td>
<td>ATCGTTATAGCAATC</td>
</tr>
<tr>
<td>gB (carboxy; reverse)</td>
<td>TCACCTCCCGGTTCGG</td>
</tr>
<tr>
<td>ORF59 (forward)</td>
<td>TCGACTTCAGGAAATACGTCG</td>
</tr>
<tr>
<td>ORF59 (reverse)</td>
<td>GGCTATGCGGCTCGAGTA</td>
</tr>
<tr>
<td>GAPDH (forward)</td>
<td>GATTCCACCACGGAATT</td>
</tr>
<tr>
<td>GAPDH (reverse)</td>
<td>AAAGATTGGTAGGATTTCATT</td>
</tr>
</tbody>
</table>

| *FAM*, 6-carboxyfluorescin.

**RESULTS**

Construction and testing of a conditional silencing system for KSHV gB expression. The siRNAs si18 and si22, which target gB mRNA sequences starting at nucleotides 403 and 484, respectively (Fig. 1A), were designed as described in Materials and Methods. These two siRNAs were selected from a list of more than 25 siRNAs based on their specificity for the KSHV gB mRNA after extensive homology searches using BLAST and CLUSTAL algorithms. To develop conditional inhibition of gB expression using the si18 and si22 siRNAs, the first 540 bp of the DNA coding sequence of gB was codon optimized as described in Materials and Methods. Codon optimization substantially altered the 5′-most DNA sequence of the gB gene, effectively destroying recognition of the codon-optimized gB mRNA by the si18 and si22 siRNAs, while the encoded amino acid sequence was unaltered (Fig. 1B). The wild-type KSHV gB (gBw) and the codon-optimized gB (gBco) were cloned into plasmid p3XFLAG (Sigma, Inc.) The siRNAs si18 and si22 were cloned either individually in the 3′ XFLAG plasmid (ps18, ps122) or together in tandem with the gBco gene separated by a 577-bp encephalomyocarditis virus IRES situated immediately downstream from the siRNA sequences (ps18/22Bco) (see Materials and Methods).

Conditional silencing of gB expression was tested in transient expression experiments with 293 cells. Cells were cotransfected with equimolar mixtures of plasmids containing gBw mixed with either ps18 or ps22 or with an equimolar mixture of plasmids containing gBco with either ps18 or ps22 plasmids. RT-PCR analysis using primers which are equally specific for gBw and gBco (universal gB primers; gBUNI) showed a significant reduction in the levels of gB transcripts in 293 cells cotransfected with wild-type gB and siRNAs 18 and 22, as also evidenced after densitometric analysis using levels of GAPDH transcripts for normalization purposes (Fig. 2). In contrast, cotransfection of gBco with either ps18 or ps22 did not reduce the levels of...
gB transcripts (Fig. 2). gB transcripts were not detected in RNA derived from untransfected 293 cells or cells transfected with the transfecting agent alone (Fig. 2). Both gBw and gBco appeared to produce similar amounts of gB transcripts in the presence of a nonspecific siRNA provided for control purposes (Fig. 2).

Similar experiments were performed as outlined above, with the exception that cellular extracts were processed to detect gB expression in Western blots. gB expression was quantified by densitometry and normalized to GAPDH protein levels. Significant inhibition of gB expression was observed for 293 cells cotransfected with plasmids expressing gBw and either si18 or si22 in comparison to cells transfected with gBw and a nonspecific siRNA (Fig. 3). In contrast, the amount of gB detected in samples derived from cotransfections of 293 cells with gBco and either si18 or si22 was similar to that seen for gB from cells cotransfected with gBco and the nonspecific siRNA, indicating that the siRNAs did not inhibit gBco expression (Fig. 3).

Role of the carboxyl terminus of gB in virion egress from BCBL-1 cells. To investigate the role of the carboxyl terminus of gB in virion egress, a panel of mutated gB genes was constructed, specifying different gB truncations by using the gBco gene as the template for mutagenesis. The carboxyl terminus of gB was truncated at specific sites to disrupt predicted α-helical structures (Fig. 4). The gB truncations include gBp, which has 25 amino acids deleted; gBg, with a deletion of 58 amino acids; gBc, with 84 amino acids deleted; and gBtrm, with 110 amino acids deleted. gBp disrupts the predicted α-helical domain 1, while the gBg truncation disrupts the predicted α-helical domain 2. gBc removed the entire predicted cytoplasmic domain of gB, while gBtrm removed the entire carboxyl terminus, including part of the predicted transmembrane domain (Fig. 4). Plasmids coding for the gB truncations were transfected into 293 cells and gBs were detected by immunohistochemistry. All gB constructs, including the gBco full-copy gB, were detected by anti-gB antibodies, except for gBtrm, which was not detectable (Fig. 5). Low levels of gBtrm were detected in supernatants of infected cells, suggesting that gBtrm was secreted into extracellular spaces (not shown).
To investigate the ability of si18 and si22 siRNAs to inhibit gB expression in BCBL-1 cells, similar experiments were performed as outlined earlier for the transient expression experiments with 293 cells by use of RT-PCR to detect gB mRNAs and GAPDH for normalization purposes. In these experiments, BCBL-1 cells were transfected and 24 h posttransfection cells were induced with TPA as detailed in Materials and Methods. Transfection of psi18 or psi22 in BCBL-1 cells significantly reduced endogenous gBwt mRNA levels detected by RT-PCR using primers that amplify a 338-bp gBwt-specific DNA fragment in comparison to what was seen for transfection with the nonspecific siRNA (Fig. 6A). In contrast, in BCBL-1 cells transfected with plasmids psi18gBco and psi22gBco, which coexpress the codon-optimized gB with si18 and si22, respectively, gB levels were approximately the same as those detected for BCBL-1 cells transfected with the nonspecific siRNA (Fig. 6A). In contrast, in BCBL-1 cells transfected with plasmids psi18gBco and psi22gBco, which coexpress the codon-optimized gB with si18 and si22, respectively, gB levels were approximately the same as those detected for BCBL-1 cells transfected with the nonspecific siRNA (Fig. 6B). RT-PCR products shown in the row labeled gBUNI were produced using gB primers, which amplify a 240-bp DNA fragment situated outside the codon-optimized region of gBco and are therefore predicted to amplify both gBwt and gBco sequences with equal efficiency.

Similar transfection experiments were performed with each of the gB constructs encoding carboxyl-terminal truncations of gB. Coexpression of gBp, gBg, gBc, and gBtrm with plasmids expressing either si18 or si22 elevated gB expression in BCBL-1 cells to levels similar to those observed for BCBL-1 cells transfected with the nonspecific siRNA (Fig. 6B). As expected, PCR primers which specifically amplify a 317-bp DNA fragment encoding the gB carboxyl terminus did not detect the elevated levels of the truncated gB transcripts obt-
served with the universal gB primers, because the targeted gB gene sequence was absent from all gB mutant genes (Fig. 6C). The amount of gB transcripts detected is similar to that observed for si18- or si22-inhibitory conditions (Fig. 6C).

Similar experiments were performed with BCBL-1 cells as described above, with the exception that gB expression was detected using Western blot assays. Transfection of BCBL-1 cells with either psi18 or psi22 plasmids reduced the amount of gB detected using anti-gB specific antibody in comparison to gB levels in cells transfected with the nonspecific siRNA (Fig. 7A and B). As observed previously for the gB mRNA levels, coexpression of gBco, gBp, gBg, and gBc with plasmids expressing either si18 or si22 elevated gB expression in BCBL-1 cells to levels similar to those observed for BCBL-1 cells transfected with the nonspecific siRNA (Fig. 7A and B). Coexpression of gBtrm with either si18 or si22 did not elevate the overall amount of gB expression, as noted previously for the transient transfection experiments with 293 cells (Fig. 7A and B).

It has been previously shown that gB is essential for virion egress from 293 cells (31). Therefore, we investigated whether the inhibition of gB mRNA levels by the si18 and si22 siRNAs could reduce the amount of virion particles in the supernates of BCBL-1 cells. The number of virion particles in the supernates of BCBL-1 cells was quantified by real-time TaqMan PCR after DNase treatment of extracellular virions to specifically detect fully encapsidated viral DNA, as described above (see Materials and Methods) and previously (35). Transfection of BCBL-1 cells with either si18- or si22-expressing plasmids substantially reduced the amount of viral genomes detected in the supernates of BCBL-1 cells (Fig. 8). Coexpression of the gBco truncation with either si18 or si22 reproducibly increased virion egress by nearly 20% in comparison to what was seen for the nonspecific siRNA control sample, while coexpression of gBtrm with either si18 or si22 failed to rescue virion egress (Fig. 8).

To determine the effect of the gB truncations on virion infectivity, supernatant virions were used to infected 293 cells. Infectivity was assayed by the expression of the EGFP gene, which is constitutively expressed by the rKSHV.152 genome (62). These results showed a correlation between the numbers of virion particles and infectious virions found in the supernatants of BCBL-1, revealing that all truncated gBs were able to increase production of infectious virions in the supernatants of BCBL-1 cells, with the exception of the gBtrm-transfected BCBL-1 cells (Fig. 8).

FIG. 5. Immunohistochemistry for detecting expression of gB mutants in 293 cells. Anti-gB antibodies were utilized to detect gB expression in transiently transfected cells at 48 h posttransfection as described in Materials and Methods.

DISCUSSION

The cloning of the KSHV genome as a BAC has enabled the rapid generation of KSHV mutant viruses (22, 67, 68). Virus mutants lacking specific gene functions have been generated and characterized in cell culture systems permissive for viral replication (31, 35, 65, 68). However, viral yields from cells transfected with KSHV mutant genomes are relatively low, hindering the elucidation of gene functions involved in virion infectivity, assembly, and egress from infected cells. In this study, we utilized anti-gB siRNAs to inhibit gB synthesis in BCBL-1 cells, which are known to produce large amounts of infectious virions after induction of the dormant KSHV genome to lytic replication. Transfection of BCBL-1 cells under anti-native gB siRNA conditions with a codon-optimized gB not susceptible to siRNA inhibition restored gB expression to near-native levels. We utilized this system to demonstrate that the cytoplasmic terminus of gB from amino acid residues 761 to 787 contains a domain that regulates virion egress; overall,
FIG. 6. Detection of gB mRNA in BCBL-1 cells. Total RNA was extracted from transiently transfected BCBL-1 cells and first-strand cDNA was used for PCR using primers. Shown are wild-type gB (gBWT) (A), universal gB primers amplifying both wild-type and codon-optimized gB (gBUNI) (B), and gB carboxy primers amplifying the 3' end of gB (gBCarb) (C) and GAPDH primers producing an 80-bp amplicon. The levels of gB transcripts obtained after densitometric analysis and normalization with GAPDH are shown aligned below for each sample.
however, this domain is not essential for either virion egress or infectivity.

Transient expression of either si18 or si22 siRNAs reduced gB mRNA levels and gB expression in both 293 and BCBL-1 cells by approximately 40 to 60%. Typically, more than 40% of 293 cells can be routinely transfected with plasmids by use of SuperFect (Qiagen, Inc.), as evidenced by transfection rates obtained with an EGFP-expressing plasmid (not shown). Therefore, the observed siRNA-mediated inhibition of mRNA levels and gB expression indicates that the majority of the 293 cells were cotransfected with both siRNA- and gBw-expressing plasmids. In BCBL-1 cells, approximately 20% of the cells can be routinely transfected with an indicator EGFP-expressing plasmid. Furthermore, approximately 15% of BCBL-1 cells can be reproducibly induced to KSHV lytic gene expression after induction with TPA (not shown). Therefore, if the transfection rate of BCBL-1 cells is independent of the rate of KSHV lytic gene expression, only 3% of BCBL-1 cells could be

FIG. 7. Western blot analysis for detection of gB in transiently transfected BCBL-1 cells. (A) Cells were transfected with plasmid vectors expressing si18 and various gB genes as indicated. (B) Cells were transfected with plasmid vectors expressing si22 and various gB genes as indicated. The levels of gB protein detected by Western immunoblot analysis and quantified by densitometric analysis and normalization with GAPDH protein are shown aligned below for each sample.

FIG. 8. Determination of virion egress by use of real-time PCR. Transiently transfected BCBL-1 cells were induced with 25 ng/ml TPA for 12 h in serum-free medium. Two hundred microliters of supernatant was collected and treated with Turbo DNase for 2 h. Viral DNA was extracted from equal volumes of supernatants by use of a DNeasy tissue and blood kit and used for real-time PCR with primers for ORF59 and the 6-carboxyfluorescein/TAMRA TaqMan probe (Table 1). Serial dilutions of BAC 36 plasmid containing the wild-type KSHV genome were used to generate the standard curve.
both transfected and induced to lytic gene expression. However, gB mRNA levels were reduced by 40 to 60%, and gB synthesis was reduced by more than 30%, indicating that a disproportionate number of BCBL-1 cells were both transfected and reactivated. Recently, it was shown that BCBL-1 cells in the S phase appeared to be more likely to undergo lytic reactivation than were those in G0/G1 phase, and these cells exhibited a smooth surface topology in comparison to the G0/G1-phase cells (63). Therefore, it is possible that BCBL-1 cells in the S phase may be more amenable to transfection using SuperFect (Qiagen, Inc.), accounting for the observed inhibition of gB mRNA levels and gB expression. Cotransfection with the codon-optimized gB, which was not susceptible to siRNA inhibition, effectively increased gB mRNA levels and gB expression. In these experiments, the siRNAs were cloned in tandem with the codon-optimized gB, which ensured their coexpression into the same transfected 293 and BCBL-1 cells. As discussed earlier, similar arguments can be made to explain the observed recovery of gB mRNA levels and gB synthesis encoded by the codon-optimized gB constructs.

Earlier studies established that KSHV gB was essential for KSHV egress from 293 cells. In these studies, the KSHV gB gene was deleted via mutagenesis of the KSHV BAC pBAC36. Lack of gB led to inhibition of virion egress, while complementation of gB expression by transient expression of plasmids coding for gB recovered virion egress (31). Our results with BCBL-1 cells are in agreement with these published studies and suggest that egress pathways in 293 and BCBL-1 cells are similar with respect to gB functions in virion egress. Complementation of BCBL-1 cells with truncated gBs revealed that the cytoplasmic carboxyl terminus of gB was not essential for either virion egress or virus infectivity. Furthermore, it appeared that the cytoplasmic terminus contained a domain located proximal to the predicted intramembrane region that regulated virion egress, since deletion of the entire gB cytoplasmic domain significantly increased virion egress in comparison to what was seen for the shorter gB truncations. It is possible that the long carboxyl terminus of KSHV gB may bind to one or more other viral proteins and that this binding may affect the rate of cytoplasmic envelopment and virion egress.
Apparently, gB functions in virion egress are not entirely conserved among different herpesviruses. For HSV-1, it was originally shown that gB is not essential for virion egress, since virions devoid of gB could egress out of cells but were not infectious (10). In contrast to these results, it was recently shown that the cytoplasmic terminus of gB may be required for efficient virion egress, since gB carboxyl-terminal truncations appeared to reduce virion egress by two- to threefold. This egress phenotype was attributed to the ubiquitination of the cytoplasmic terminus of HSV-1 gB (12). gB has also been shown to be essential for the egress of other herpesviruses, such as pseudorabies virus and EBV (8, 29, 32, 51, 52).

The process of final virion envelopment most likely occurs in the cytoplasm, and it is mediated by interactions between tegument proteins and the cytoplasmic termini of viral membrane proteins and glycoproteins (37, 38). All gB homologs have a rather large cytoplasmic domain ranging from 84 amino acids for KSHV to 109 amino acids for HSV-1. The KSHV gB was shown to interact with the ORF64 tegument protein, which could facilitate virion envelopment and infectious virus production (58). Potential ubiquitination of the carboxyl terminus of KSHV gB, as demonstrated for HSV-1 gB (12), or the presence of arginine-rich regions, as demonstrated for EBV (33), may affect intracellular transport and the localization of KSHV gB within intracellular membranes, indirectly affecting virion envelopment and egress.

All KSHV gB truncations with the exception of the largest (110-amino-acid) truncation were able to complement virion egress and virion infectivity under siRNA inhibitory conditions. The siRNA-mediated inhibition of KSHV gene synthesis of the human cytomegalovirus glycoprotein B gene and induction of neutralizing antibodies via its expression in recombinant vaccinia virus. EMBO J. 8:3057–3063.