Characterization of Human Herpesvirus-8 K8.1A/B Glycoproteins by Monoclonal Antibodies

Liangjin Zhu, Veena Puri, and Bala Chandran

Department of Microbiology, Molecular Genetics and Immunology, The University of Kansas Medical Center, Kansas City, Kansas 66160

Received May 7, 1999; returned to author for revision June 7, 1999; accepted July 12, 1999

Human herpesvirus-8 K8.1 gene encodes for two immunogenic class I glycoproteins, K8.1A and B, originating from spliced messages ([1998] Virology 243, 208–217). The 228-amino-acid-long K8.1A open reading frame (ORF) contains four N-glycosylation sites and the 167-amino-acid-long K8.1B ORF contains three N-glycosylation sites, sharing similar amino- and carboxyl-termini with ORF K8.1A but with an in-frame deletion ([1998] Virology 249, 140–149). To characterize the K8.1A and B glycoproteins in the infected body cavity-based B cell lymphoma (BCBL-1) cells and in the virion envelopes, monoclonal antibodies (MAbs) recognizing only K8.1A protein or both K8.1A and B proteins were generated. These antibodies reacted with the infected cell membranes and virion envelopes. Stable COS-1 transformant cells expressed the K8.1A and B proteins independently on the plasma membranes. MAbs recognized multiple proteins with molecular weights ranging from 23 to 72 kDa from the BCBL-1 cells and COS-1 cells and the 72 to 68 kDa molecular-weight proteins from the virion particles. The K8.1A is the predominant protein affinity purified from the infected BCBL-1 cells. Digestion with glycosidases show that these proteins contain both N- and O-linked sugars, suggesting that the multiple proteins recognized by the MAbs represent the precursor and product forms of K8.1A and B proteins, and the 72 to 68 kDa molecular-weight proteins represent the virion particle-associated mature forms of these glycoproteins.

INTRODUCTION

Human herpesvirus-8 (HHV-8) or Kaposi’s sarcoma-associated herpesvirus (KSHV) is associated with acquired immune deficiency syndrome (AIDS)-KS and non-AIDS KS. HHV-8 DNA has been detected in more than 95% of Kaposi’s lesions in all clinical subtypes of KS (Chang et al., 1994; Schulz et al., 1998). HHV-8 sequences have been also detected in primary effusion lymphomas or body cavity-based B cell lymphomas (BCBLs) (Cesarman et al., 1995a,b; Moore et al., 1996). BCBL-1 and BC-3 cells carry only HHV-8, and a lytic cycle can be induced by phorbol ester-TPA (Renne et al., 1996; Zhong et al., 1996). HHV-8 DNA is about 160 kb, encoding 80 complete open reading frames (ORFs), and the gene arrangements and protein sequences deduced from these sequences show close similarity to the members of γ-herpesvirus group (Russo et al., 1996; Neipel, 1997). These HHV-8 ORFs are currently identified as ORFs 1–75 by their similarity to herpesvirus saimiri ORFs. Russo et al. (1996) also identified 15 ORFs unique for HHV-8 designated K1–K15. Neipel et al. (1997) identified four additional HHV-8 unique ORFs and assigned decimal K numbers for them (K4.1, K4.2, K8.1, and K10.1).

Herpesviruses encode at least 5–10 virion envelope-associated glycoproteins, and these glycoproteins are crucial to the viral replication cycle (Kieff, 1996; Spear, 1993). They mediate several functions in the interaction between virus and host cells such as the attachment to cellular receptors, fusion of membranes, and egress from the cell. Importantly, many of these envelope glycoproteins serve as major targets for the host immune system. Sequencing data show that HHV-8 has counterparts to other herpesvirus glycoproteins gB, gH, and gL. In addition to these conserved glycoproteins, HHV-8 genes K1 and K8.1 encode for glycoproteins unique for HHV-8 (Lee et al., 1998; Neipal et al., 1997; Russo et al., 1996). Knowledge about HHV-8 glycoproteins is limited. In our studies, human sera recognized several HHV-8 glycoproteins with molecular weights ranging from 29 to 128 kDa from uninduced and 12-0-tetradecanoylphorbol-13-acetate (TPA)-induced BCBL-1 cells (Chandran et al., 1998a). We have initially identified cDNAs originating from the HHV-8 K8.1 gene, which encoded two ORFs generated from spliced messages (Chandran et al., 1998a). One cDNA encodes for a 228-amino-acid protein designated K8.1A with a cleavable signal sequence, a transmembrane domain, and four N-glycosylation sites. The splicing event has generated the transmembrane domain in the K8.1A ORF not seen in the genomic K8.1 ORF. Another cDNA encodes for an ORF of 167 amino acids designated K8.1B with three N-glycosylation sites, sharing similar amino- and carboxyl-termini with ORF K8.1A but with an in-frame deletion (Chandran et al., 1998b). K8.1A and B ORFs possess several serine and threonine residues (>20), which are the potential sites for O-glycosylation. In the presence of microsomal mem-

1 To whom reprint requests should be addressed at 3901 Rainbow Blvd. Fax (913) 588-7295. E-mail: bchandra@kumc.edu.
branes, the primary translation products of ORF K8.1A 34 kDa and ORF K8.1B 20 kDa in the in vitro transcription/translation experiments shifted into glycosylated species of 43 and 32 kDa, respectively (Chandran et al., 1998b). This suggested that the ORFs K8.1A and B encode for glycoproteins. Riboprobes from the K8.1A cDNA insert hybridized with a HHV-8-specific 0.9-kb abundant transcript from the BCBL-1 cells. Synthesis of this RNA was eliminated in the presence of a DNA synthesis inhibitor, suggesting that this RNA was a late gene transcript (Chandran et al., 1998b). Our studies with human sera also demonstrated the immunogenic nature of K8.1A/B glycoproteins (Chandran et al., 1998b; Zhu et al., 1999).

Rabb et al. (1998) suggested that the glycoproteins gp35kDa–37kDa in the BCBL cells were encoded by the K8.1 ORF. While the current work was in progress, the use of polyclonal rabbit antibodies against the genomic ORF K8.1 protein (amino acids 26–142). Li et al. (1999) identified the K8.1 protein as a virion envelope-associated glycoprotein. However, the identity of the K8.1/B glycoproteins in the infected cells and the virion envelope was not defined. To characterize the K8.1/B glycoproteins, we expressed the K8.1A and B proteins in the insect cells and in the COS-1 cells and developed MAbs against the baculovirus-expressed proteins. These antibodies were used in the identification and characterization of these glycoproteins in the infected BCBL-1 cells and in the transfected COS-1 cells. Our studies show that these glycoproteins are associated with the virion envelopes and the surfaces of BCBL-1 and COS-1 cells. MAbs recognized multiple proteins with molecular weights ranging from 23 to 72 kDa from the BCBL-1 and COS-1 cells and the proteins of 68 to 72 kDa from the virion particles. The K8.1A form is the predominant protein affinity purified from the infected BCBL-1 cells. Digestion with glycosidases shows that these proteins contain both N- and O-linked sugars, suggesting that the multiple proteins recognized by the MAbs represent the precursor and product forms of K8.1A and B proteins and the proteins of 68–72 kDa represent the virion particle-associated mature forms of these glycoproteins.

RESULTS

MAbs against K8.1A/B proteins show differential reactivities

GST-K8.1A and -K8.1B proteins expressed in the insect cells were purified by glutathione–Sepharose 4B. The purity of these proteins was verified by SDS–PAGE followed by staining with Coomassie blue (Fig. 1, lanes 1 and 2) or with BioRad zinc stain kit (data not shown). The purified GST protein was about 29 kDa, and the observed sizes of K8.1A and B fusion proteins were 62 and 54 kDa, respectively (Fig. 1, lanes 1 and 2). Purified GST-K8.1A and -K8.1B fusion proteins were recognized by the rabbit anti-peptide antibodies (Chandran et al., 1998b) recognizing both N- and O-linked sugars, suggesting that the multiple proteins recognized by the MAbs represent the precursor and product forms of K8.1A and B proteins and the proteins of 68–72 kDa represent the virion particle-associated mature forms of these glycoproteins.

FIG. 1. Reactivities of K8.1A and K8.1A/B MAbs in the Western blot reactions with the affinity-purified K8.1A and B proteins. Full-length K8.1A and B ORFs were subcloned from the cDNAs (Chandran et al., 1998a,b). These were expressed as GST fusion proteins in the baculovirus-infected insect cells and purified by glutathione–Sepharose columns (Zhu et al., 1999). Equal concentrations of the purified GST-K8.1A, GST-K8.1B, and GST proteins were run on 12% acrylamide gel, Western blotted, and reacted with the different MAbs. Prestained molecular weight markers were run in parallel lanes; the numbers on the right indicate the molecular masses in kDa. Lanes 1, 3, 5, 7, and 9, GST-K8.1B protein. Lanes 2, 4, 6, 8, and 10, GST-K8.1A protein. Lanes 1 and 2, Coomassie stain of the gel. Lanes 3 and 4, reactivities of K8.1A/B MAb 4A4. Lanes 5 and 6, reactivities of K8.1A/B MAb 6A5. Lanes 7 and 8, reactivities of K8.1A MAb 3B5. Lanes 9 and 10, reactivities of K8.1A MAb 2A3. No reactivities were detected with the GST protein.
reacted with the control BJAB cells. No reactivities were detected with the control BJAB cells. About 1–2% of uninduced and 10–15% of TPA-induced HHV-8-positive P3HR-1 cells (data not shown). About 10–15% of the TPA-induced BCBL-1 cells were tested in the IFA with the K8.1A and K8.1A/B MAbs and showed moderate cytoplasmic fluorescence with sera from HIV + KS-positive patients and with the K8.1A/B MAbs. No reactivity was seen with the acetone fixed control COS-1 cells (data not shown). These MAbs showed very bright fluorescence predominantly localized in the cytoplasm and plasma membranes (Fig. 2A). Our previous studies showed that HHV-8-carrying BCBL-1 cells are virus producer cells with a certain percentage of cells entering spontaneously into lytic cycle. About 3% of uninduced BCBL-1 cells showed moderate cytoplasmic fluorescence with sera from HIV + KS-positive patients and with the MAbs 11D1 recognizing the HHV-8 early lytic ORF 59 protein and the number of cells recognized by HIV + KS-positive sera and ORF 59 MAb increased to 15–25% after 5 days post-TPA induction (Chandran et al., 1998b; Smith et al., 1997). Similarly, the K8.1A and B MAbs recognized about 1–3% of uninduced BCBL-1 cells and about 15% of TPA-induced cells. The absence of K8.1A/B MAB reactivities with the majority of the BCBL-1 cells also served as an internal control for the specificity of these MAbs. Our earlier experiments showed that the K8.1 transcript is a true HHV-8 late gene product requiring viral DNA replication (Chandran et al., 1998b). The absence of MAb reactivity with the majority of BCBL-1 cells latently infected with HHV-8 demonstrated that the MAbs recognized proteins from BCBL-1 cells entering lytic cycles spontaneously and with cells induced by TPA, most likely the lytic cycle HHV-8 K8.1A/B proteins.

To determine whether the K8.1A/B proteins were expressed on the infected cell plasma membranes, TPA-induced BCBL-1 cells (4 days postinduction) were tested in the surface IFA with the K8.1A and K8.1A/B MAbs and with the rabbit anti-K8.1A/B peptide antibodies. No reactivity was observed with uninduced and TPA-induced BJAB cells (data not shown). About 10–15% of the TPA-induced BCBL-1 cells showed surface IFA with the anti-K8.1A and anti-K8.1A/B MAbs demonstrating that the K8.1A/B proteins were expressed on the infected cell plasma membranes (Fig. 2B). Similar reactivities were observed with the rabbit anti-peptide antibodies recognizing amino acids 140–156 of K8.1A and amino acids 82–97 of K8.1B proteins (Chandran et al., 1998b; data not shown). These results suggested that the amino-terminus of the K8.1A/B protein was exposed to the exterior of the cells.

K8.1A and B proteins are expressed independently on the plasma membranes of COS-1 cells transfected with expression plasmids

For the further characterization of K8.1A and B proteins, COS-1 cells were transfected with the eukaryotic expression plasmids containing the K8.1A and B genes, and stable transformants were obtained by G418 drug selection. Subsequently, single-cell clones of COS-K8.A and COS-K8.1B were established. Cells permeabilized by acetone fixation were tested in the IFA with the K8.1A/B or K8.1A MAbs. No reactivity was seen with the acetone fixed control COS-1 cells (data not shown). These MAbs
showed very bright cytoplasmic fluorescence (Figs. 2C and 2D), and the K8.1A-specific MAbs reacted only with the COS-1 cells expressing the K8.1A protein but not with the cells expressing the K8.1B protein (data not shown). To determine whether the K8.1A and K8.1B proteins were expressed on the transfected plasma membranes, MAbs were tested with the unfixed cells. The anti-K8.1A and -K8.1A/B MAbs showed surface immunofluorescence with the transfected COS-1 cells (Figs. 2E and 2F). These results demonstrated that the K8.1A and K8.1B proteins can be expressed independently on the plasma membranes. These COS-1-expressed proteins were used for the further characterization of K8.1A and B proteins.

K8.1A/B MAbs recognize multiple glycoproteins from the HHV-8-infected BCBL-1 cells

To identify the HHV-8 proteins recognized by the anti-K8.1A/B MAbs, we used the uninduced and TPA-induced cells in radioimmunoprecipitation (RIP) and Western blot reactions. The K8.1A/B MAb 4A4 did not react with the TPA-induced 35S-methionine/cysteine-labeled uninfected BJAB cells (Fig. 3a, lane 1). In contrast, it recognized several 35S-labeled polypeptides of about 28–72 kDa from TPA-induced BCBL-1 cells (Fig. 3a, lane 3). Longer exposure of the autoradiographs also showed reactivities with similar molecular weight polypeptides from the uninduced BCBL-1 cells (Fig. 3a, lane 2), suggesting the reactivity with proteins from BCBL-1 cells entering lytic cycles spontaneously. No reactivity was seen with the 3H-glucosamine-labeled TPA-induced BJAB cells (Fig. 3b, lane 1). In contrast, several 3H-glucosamine-labeled glycopolypeptides of about 28–72 kDa were recognized by the K8.1A/B MAbs from the TPA-induced BCBL-1 cells (Fig. 3b, lane 1). Similar size multiple polypeptides were recognized by the K8.1A MAbs (data not shown).

Immunoprecipitation of multiple glycoproteins by the K8.1A/B MAbs could be due to the reactivity with polypeptides exhibiting a precursor and product relationship sharing common antigenic epitopes or due to the precipitation of polypeptides in a complex not sharing antigenic epitopes. To determine the identity of the various polypeptides precipitated by the MAbs, uninduced and TPA-induced (24–96 h postinduction) BCBL-1 and BJAB cells were collected, washed, and lysed in distilled
Samples were adjusted to equal protein concentrations and boiled in the sample buffer. About 10 μg of each protein was loaded per lane and tested in the Western blot reactions; Figs. 3c and 3d show the reactivity with adjacent lanes in the same gel. The K8.1A/B MAbs recognized a number of proteins with the molecular weights ranging from about 28 to 72 kDa from the TPA-induced BCBL-1 cells (Fig. 3c, lane 3). A weak reaction was seen with similar size proteins from the uninduced BCBL-1 cells (Fig. 3c, lane 2) but not with the BJAB cells (Fig. 3c, lane 1). Because equal protein quantities were tested, the increased reactivities with TPA-induced cells further support the IFA data (Fig. 2A) that MAbs recognized the lytic cycle K8.1A/B proteins induced by TPA. Reactivity with uninduced cells suggests the reactivity with proteins from BCBL-1 cells spontaneously entering lytic cycles. These results suggested that the multiple proteins recognized by the MAbs in the RIP and Western blot reaction were due to the reactivity with the various forms of K8.1A/B glycoproteins in the BCBL-1 cells such as the underglycosylated precursors, glycosylated intermediates, and the final mature glycosylated products sharing common antigenic epitopes.

Lytic expression of K8.1A/B glycoproteins

Our previous studies demonstrated that the K8.1A/B transcript is a late gene transcript (Chandran et al., 1998b). To examine the kinetics of K8.1A/B protein synthesis, BCBL-1 cells were stimulated with TPA for 24, 48, and 96 h, and equal quantities of proteins were tested in the Western blot reactions with the K8.1A/B MAbs. Rabbit antibodies against the HHV-8 latency-associated ORF 73 protein (V. Puri et al., manuscript in preparation) was used as a control. Only weak reactivities with K8.1A and K8.1A/B MAbs were detected with the uninduced and 24-h TPA-induced cells (Fig. 3d, lanes 1, 5, and 9). These MAbs recognized increasing quantities of the K8.1A/B glycoproteins from the BCBL-1 cells induced with TPA for 48 h (Fig. 3d, lanes 3, 7, and 10). In general, the K8.1A/B MAbs (Fig. 3d, lanes 1–4) reacted more strongly than the K8.1A MAbs (Fig. 3d, lanes 5–8). In contrast, the synthesis of the HHV-8 latency-associated ORF 73 protein was constant during the 24- to 96-h TPA induction, and there was no change in the kinetics of the multiple forms of ORF 73 proteins recognized (Fig. 3e, lanes 9–11). These results confirmed
that the K8.1A/B glycoproteins are late lytic gene products whose expression is stimulated greatly by the TPA treatment.

Virion-associated forms of K8.1A/B glycoproteins

To confirm the localization of K8.1A/B glycoproteins on the surfaces of infected BCBL-1 cells, immune electron microscopy was done. Unfixed BCBL-1 cells stimulated with TPA for 24 or 92 h were incubated with K8.1A and K8.1A/B MAbs followed by anti-mouse antibodies conjugated with 10-nm gold particles. These cells were fixed with glutaraldehyde and embedded in resins; thin sections were made and observed under an electron microscope. No reactivity was observed with TPA-induced BJAB cells (data not shown). In contrast, gold particles were detected on the surfaces of about 10–15% of TPA-induced BCBL-1 cells (Figs. 4A and 4B). MAbs recognizing the K8.1A protein (Fig. 4A) or the K8.1A/B proteins (Fig. 4B) showed similar reactivities. A majority of the latently infected BCBL-1 cells (Fig. 4A, large arrow) were negative, demonstrating the specificity of the MAbs recognizing the lytic cycle-associated K8.1A/B proteins. These results confirmed the presence of K8.1A/B proteins on the infected BCBL-1 cell membranes.

Examination of the cell surfaces by immune electron microscopy after 24 h post-TPA induction revealed only a few virus particles (data not shown), and more virus particles were detected after 92-h TPA induction. However, the architecture of cells at 92 h was considerably deteriorated. Despite this, gold particles were detected on the surfaces of virion particles in samples incubated with the K8.1A/B (Fig. 4D) and K8.1A MAbs (data not shown). These virus particles were found on the external surfaces of the plasma membranes. Overall, more gold particles were detected with the anti-K8.1A/B MAbs than with the anti-K8.1A MAbs. No reactivity was seen with MAb 11D1 recognizing the HHV-8 early non-envelope-associated ORF 59 protein (Fig. 4D) These results dem-
onstrated that the MAbs recognized the virion envelope-associated K8.1A/B glycoproteins.

The virion envelopes of herpesviruses contain the fully glycosylated final product of the glycoproteins (Kieff, 1996; Spear, 1993). To determine which of the multiple species of HHV-8 K8.1A/B glycoproteins detected in the infected cells (Figs. 3a–3d) were incorporated in the virion envelopes, HHV-8 particles from TPA-induced KS-1 cells purified by two cycles of density gradient centrifugation (Chatlynne et al., 1998) were solubilized with sample buffer with and without 2-mercaptoethanol and tested with the MAbs in the Western blot reactions. The sequence of KS-1 virus K8.1 gene amplified by PCR was identical to the sequences of BCBL-1 virus DNA (data not shown). In contrast to the multiple proteins seen in the TPA-induced BCBL-1 cells, the K8.1A MAb 4A4 (Fig. 5a, lane 1) and the K8.1A MAb 2A3 (Fig. 5a, lane 3) recognized the virion particle-associated broad protein band of 68–72 kDa. The migration of these proteins remained unchanged in the absence of 2-mercaptoethanol (Fig. 5a, lane 2), suggesting the lack of disulfide linkages between the virion-associated K8.1A/B proteins. The lower-molecular-weight bands detected in the infected cells (Figs. 3a–3d) were clearly absent in the virus particles. This suggested that the virion-associated 68–72 kDa proteins probably were the fully glycosylated mature form of K8.1A/B proteins, and the reactivity of K8.1A MAb showed that the virion particles predominantly contain the K8.1A protein.

K8.1A and K8.1 B glycoproteins contain both N- and O-linked sugars

To determine whether the virion-associated 68–72 kDa K8.1A/B glycoproteins represent the mature form of K8.1A/B proteins, virus particles were denatured, treated with glycosidase enzymes, Western blotted, and reacted with the K8.1A/B MAb 4A4. As a control, HHV-8 particles were also incubated at 37°C for 24 h without any enzymes. Under this condition, in addition to the prominent 68–72 kDa region, slightly smaller bands were also detected (Fig. 5b, lane 1), probably due to proteolytic breakdown. In contrast, after digestion with the neuraminidase and O-glycosidase, which removed only the O-linked sugars (Fig. 5b, lane 2), or with the N-glycosidase removing only the N-linked sugars (Fig. 5b, lane 3), the 68–72 kDa band (Fig. 5b, lane 1) disappeared; instead, lower-molecular-weight bands of about 54–46 kDa appeared. However, when the virus was incubated with all three enzymes, the higher-molecular-weight bands of about 54–46 kDa appeared. This results demonstrated that the K8.1A/B glycoproteins contain both N- and O-linked sugars, suggesting that the multiple proteins recognized by the MAbs represent the precur-
isor and product forms of K8.1A/B proteins and the 68–72 kDa proteins represent the virion particle-associated mature form of these glycoproteins.

To characterize the infected cell-associated K8.1A/B glycoproteins, TPA-induced BCBL-1 cell lysate was passed over Sepharose 4B covalently coupled with K8.1A-specific 4D6 MAb and the flowthrough was passed over Sepharose 4B covalently coupled with K8.1A/B-specific 4A4 MAb. The unbound proteins were removed, and the bound proteins were eluted and tested in the Western blot reactions with K8.1A/B- or K8.1A-specific MAbs. The profiles of protein eluted from the 4D6 (K8.1A) column or 4A4 (K8.1A/B) columns and the proteins recognized by the K8.1A and K8.1A/B MAbs were identical. The K8.1A protein affinity-purified from the BCBL-1 cells exhibited multiple forms with an intense band of about 36 kDa and diffused bands of 40 to 72 kDa high-molecular-weight glycoproteins (Fig. 5c, lane 1). There were no low-molecular-weight bands at 23 kDa corresponding to the predicted precursor form of the K8.1B protein. After N-glycosidase treatment, the 36-kDa band disappeared and a 26-kDa band appeared (Fig. 5c, lane 2, +N). The intensity of the bands in the 40–70 kDa region was reduced but not eliminated. The predicted molecular weight of the unglycosylated K8.1A protein is about 25 kDa, which will be reduced to about 22 kDa after the cleavage of the predicted signal peptide (amino acids 1–28) (Chandran et al., 1998b). After the N-glycosidase treatment, one molecule of N-acetyl glucosamine is still attached to the asparagine residue of the glycoprotein. The protein bands detected in the 26 kDa region after the N-glycosidase treatment (Fig. 5c, lane 2) could represent the precursor forms of K8.1A protein, and the higher-molecular-weight protein bands in the 40–72 kDa region (Fig. 6a, lane 2) could represent the K8.1A proteins containing O-linked sugars without N-linked sugars due to N-glycosidase treatment. These results further strengthened the notion that the HHV-8 K8.1A glycoprotein contains both N- and O-linked sugars.

The K8.1B protein purified from the COS-1 cells also appeared as multiple species of highly diffused protein bands ranging from 23 to 72 kDa (Figs. 6b, lane 1, and 6c, lane 1). After N-glycosidase treatment, the 23-kDa band disappeared and diffused bands at 16–18 kDa appeared (Figs. 6b, lane 2, and 6c, lane 2). The predicted molecular weight of unglycosylated K8.1 B protein is about 18 kDa, which will be reduced to about 16 kDa after the cleavage of the predicted signal peptide (amino acids 1–28). The distinct protein band detected in the 18 kDa region after the N-glycosidase treatment (Figs. 6b and 6c, lane 2) suggests that these could represent the precursor forms of K8.1B protein, and the higher-molecular-weight protein bands in the 40 to 62 kDa (Figs. 6b and 6c, lane 2) region could represent the K8.1B protein containing O-linked sugars lacking N-linked sugars due to N-glycosidase treatment. These results suggested that K8.1B protein also contains both N- and O-linked sugars. The treatment of COS1- K8.1B protein with neuraminidase followed by O-glycosidase and N-glycosidase resulted in the disappearance of most of the high-molecular-weight bands with an increase in the intensity of the 16–18 kDa band (Fig. 6c, lane 3). These results suggested that K8.1B glycoprotein also contains both N- and O-linked sugars.

**DISCUSSION**

In this study, we developed MAbs against HHV-8 K8.1A/B glycoproteins and identified and characterized the infected cells and the virion envelope-associated forms of these glycoproteins. While these studies were completed, using rabbit antibodies against the genomic ORF K8.1 protein (amino acids 26–142), Li et al. (1999) reported that the K8.1 protein is a virion envelope-associated glycoprotein. However, the identity of the K8.1 protein in the infected cells and virions was not defined. In the COS-1 cells expressing the K8.1A cDNA, Li et al. (1999) identified a 37-kDa protein as a major species, a 27-kDa as a minor species, and 60–75 kDa proteins. In the presence of tunicamycin, the 37-kDa and 60–75 kDa proteins disappeared, and the 27-kDa protein band was the major band detected. Our studies with TPA-induced BCBL-1 cells, COS-1 cells expressing K8.1A and B proteins, and the purified virions show that the K8.1A and B proteins undergo extensive post-translational modification. Our studies also demonstrate that the 60–75 kDa forms identified by us and Li et al. (1999) represent the mature form of K8.1A/B glycoproteins containing both N- and O-linked sugars.

Our previous in vitro transcription/translation experiments (Chandran et al., 1998b) and the treatments with
glycosidases show the precursor–products relationship between the low- and higher-molecular-weight K8.1A and B proteins. Attempts to perform pulse-chase experiments were not successful, yet the levels of newly synthesized K8.1A/B proteins available for labeling were low due to the low percentage (20%) of BCBL-1 cells expressing viral proteins after 72–96 h post-TPA induction and due to continuous cellular death. The better detection of K8.1A/B proteins in the Western blot reactions could be due to the quantitative accumulation of these proteins during TPA induction despite the degeneration of cells. Because the K8.1B protein is identical to the K8.1A protein except for the absence of 61 amino acids deleted by the in-frame deletion of the transcript (Chandran et al., 1998b), the production of specific antibodies against the K8.1B protein was not possible. Moreover, the molecular weights of the K8.1A and K8.1B proteins expressed independently in the COS-1 cells were closely similar. Hence, we were unable to determine the identity of the K8.1B protein among the various glycoproteins recognized by the K8.1A/B-specific proteins in the BCBL-1 cells and in the virion envelopes. MAb affinity purification from the BCBL-1 cells demonstrates that the K8.1A glycoprotein is the predominant species, and the reactivity of K8.1A MAb showed that the virion particles contain predominantly the K8.1A protein. Affinity purification of the gpK8.1A alone also suggests the absence of complex formation between K8.1A and B proteins, which is similar to the absence of complex formation between the EBV envelope glycoproteins gp350 and gp220.

Among the different glycoproteins encoded by the HHV-8, ORFs 8, 22, and 47 are homologous to the glycoproteins gB, gH, and gL, respectively, conserved among the other herpesviruses (Neipel et al., 1997; Russo et al., 1996). In contrast, comparison with the human or animal herpesvirus sequences to date show that the ORFs K8.1A and B are unique for HHV-8. This suggested that K8.1A/B glycoproteins may be mediating

FIG. 6. K8.1A and K8.1B proteins expressed in the COS-1 cells contain both N- and O-linked sugars. Reactivities of K8.1A/B MAb 4A4 with the K8.1A and B proteins affinity purified from the COS-1 K8.1A and K8.1B cells. Total cell lysates from the stable COS-1 transformant cells expressing K8.1A and K8.1B proteins were passed over Sepharose 4B covalently coupled with 4D6 MAb (K8.1A) or with 4A4 MAb (K8.1A/B). The bound K8.1A protein was eluted with pH 2.5 buffer, and the bound K8.1B protein in the 4A4 column was eluted with pH 11.5 buffer. Samples were tested in the immunoblots with the K8.1A/B-specific MAb 4A4. Molecular weight markers were run in parallel lanes; the numbers on the right indicate the molecular masses in kDa. (a) Lane 1, untreated affinity-purified K8.1A protein. Lane 2, affinity-purified K8.1A protein treated with N-glycanase (−N). (b) Lane 1, untreated affinity-purified K8.1B protein. Lane 2, affinity-purified K8.1B protein treated with N-glycanase (−N). Samples were analyzed on 12% acrylamide gels. (c) Lane 1, untreated affinity-purified K8.1B protein. Lane 2, affinity-purified K8.1B protein treated with N-glycanase (−N). Lane 3, affinity-purified K8.1B protein treated with neuraminidase (NU), O-glycosidase (O), and N-glycanase (N). HM-GPS, high-molecular-weight glycoproteins. Samples were analyzed on 8–16% gradient acrylamide gels.
important biological function or functions specific for HHV-8. The location of the HHV-8 ORF K8.1A gene in the genome clearly suggests an important role of K8.1A and B proteins in the biology of HHV-8. The K8.1A gene is positionally colinear to the glycoprotein genes in the members of the gammaherpesvirus group such as the EBV gene encoding the major EBV glycoproteins gp350/gp220 (Kieff, 1996), gp150 gene of murine gammaherpesvirus 68 (MHV68) (Rabb et al., 1998), herpesvirus saimiri ORF 61 gene (Russo et al., 1996), and the BORFD1 gene of bovine herpesvirus-4 (BHV-4) (Rabb et al., 1998). Some of these glycoproteins are involved in the binding of the virus to the target cells; among these, the EBV gp350/gp220 has been studied extensively. HHV-8 gpK8.1A and B proteins show several similarities with the EBV glycoproteins. EBV gp350/gp220 elicits a strong humoral immune response (Kieff, 1996; Richardson and Kieff, 1996), and our studies with human sera show that the HHV-8 K8.1A and B are immunogenic proteins (Chandran et al., 1998b; Zhu et al., 1999). Similar to the specificity of our MAbs recognizing only the K8.1A protein or both K8.1A and B proteins, MAbs against gp350 react with gp350 only or both gp350 and gp220 (Kieff, 1996), and there are no gp220-specific MAbs. Like EBV gp30, the gpK8.1A/Bs are virion envelope- and infected cell membrane-associated glycoproteins. The EBV gp220 arises from an in-frame deletion of gp350 transcripts and differs only in the loss of 197 acids (Kieff, 1996; Tanner et al., 1987). The K8.1B arises from an in-frame deletion of the K8.1A transcript (Chandran et al., 1998a,b). The nascent proteins of gp350 and gp220 are 135 and 100 kDa, respectively, and the mature glycoproteins of about 350 and 220 kDa arise by the addition of extensive N- and O-linked sugars (Kieff, 1996). Similar to this, our studies show that the HHV-8 gpK8.1A and B also are highly glycosylated molecules with the nascent proteins of 25 and 18 kDa, respectively, containing both N- and O-linked sugars, and the HHV-8 virion-associated 68–72 kDa proteins represent the mature form of K8.1A and B glycoproteins.

The ability of anti-K8.1A/B MAbs to interfere in the HHV-8 infectivity must be evaluated. HHV-8 appears to infect a variety of cell lines of human and animal origin but does not establish a sustained productive infection (Renne et al., 1998; Vieira et al., 1997). To monitor the expression of K8.1A and B proteins, anti-peptide antibodies were generated. The peptide designated K8.1A-CP (FSGSYSSGEPSSRTTRIR) corresponds to the carboxyl-terminus of K8.1A and B proteins and is absent in the genomic HHV-8 ORF K8.1. New Zealand White male rabbits were immunized with the peptide, and the IgG antibodies (Ra 8.1A-CP) were purified from the sera by affinity chromatography on a protein A-Sepharose column (Pharmacia, Piscataway, NJ). The DNA sequence of KS-1 virus K8.1 gene amplified by PCR was identical to the sequences of BCBL-1 virus DNA.

Rabbit anti-peptide antibodies

To monitor the expression of K8.1A and B proteins, anti-peptide antibodies were generated. The peptide designated K8.1A-CP (FSGSYSSGEPSSRTTRIR) corresponds to the carboxyl-terminus of K8.1A and B proteins and is absent in the genomic HHV-8 ORF K8.1. New Zealand White male rabbits were immunized with the peptide, and the IgG antibodies (Ra 8.1A-CP) were purified from the sera by affinity chromatography on a protein A–Sepharose column (Pharmacia, Piscataway, NJ). The cDNAs encoding monkey kidney cells, and baby hamster kidney fibroblasts (BHK-21). All these in vitro infections were extremely inefficient, and abortive (Renne et al., 1998; Vieira et al., 1997). Recently, dermal microvascular endothelial cells have been infected by HHV-8 from BCBl-1 in a very limited fashion (Flore et al., 1998). Studies are in progress to test the ability of K8.1A and B MAbs to neutralize the infection of the endothelial and 293 cells, and preliminary studies show that the K8.1A/B MAbs prevent HHV-8 binding to the target cells. MAbs against gp350/gp220 neutralize the EBV infectivity by preventing the virus binding to the CD21 molecule. EBV gp350 and gp220 are the most abundant of the EBV glycoproteins and are responsible for attaching the virus to the complement receptor CR2 or CD21 on B lymphocytes. HHV-8 appears to have broader tropism than EBV because HHV-8 DNA and transcripts have been identified in human B cells (Cesarman et al., 1995; Schulz et al., 1998), macrophages (Orenstein et al., 1997; Schulz et al., 1998), and endothelial cells (Boshoff et al., 1995; Staskus et al., 1997). HHV-8 has been shown to infect, albeit abortively, owl monkey kidney cells and BHK-21 cells (Renne et al., 1998). Further studies are in progress to define the host molecule or molecules recognized by the HHV-8 K8.1A and B glycoproteins.

MATERIALS AND METHODS

Cell lines and virus

HHV-8-positive and EBV-negative BCBL-1 cells (Renne et al., 1996) and HHV-8- and EBV-negative BJAB cells were grown in RPMI 1640 medium with glutaMAX I (Gibco-BRL) supplemented with 10% heat-inactivated FBS and antibiotics. HHV-8 from TPA-induced KS-1 cells purified by density gradient (Chatlynne et al., 1998) was a gift from Dr. D. V. Ablashi (ABI, Rockville, MD). The DNA sequence of KS-1 virus K8.1 gene amplified by PCR was identical to the sequences of BCBL-1 virus DNA.

Expression of recombinant HHV-8 K8.1A/B proteins in the baculovirus–insect cell system

A baculovirus GST expression and purification kit was used to prepare the recombinant HHV-8 proteins (PharMingen, San Diego, CA). The cDNAs encoding
HHV-8 ORFs K8.1A and K8.1B were previously identified by screening a cDNA library from TPA-induced BCBL-1 cDNA with serum from a patient positive for HIV and KS (Chandran et al., 1998a,b). The identified cDNAs were released into the phagemid forms by in vivo excision using the EαSSist helper phage. The full-length cDNAs of ORFs K8.1A and K8.1B were amplified by PCR with the following primers: K8.1A/B, 5’-TCCCGGTGACATGC-3’ and 5’-GGGGGGTACCTTACACTATGAGG-3’. The restriction sites in the primers used for cloning purposes are underlined. The PCR products were cloned into the SacI and KpnI sites of the pAcGHLT-A baculovirus transfer vector and verified by sequence analyses. To generate the recombinant baculovirus, recombinant plasmids were cotransfected with Baculogold DNA (PharMingen) into Sf9 cells. Recombinant virus was passaged three times before use. Expression and purification of fusion proteins were performed according to the manufacturer’s recommendations (PharMingen).

Production and characterization of MAbs against HHV-8 K8.1A and K8.1B glycoproteins

BCBL-1 cells were collected 4 days post-TPA induction and washed twice with PBS, and 1 × 10^7 cells were injected per each Balb/c mouse intraperitoneally (Chan et al., 1998). After two such injections, mice were boosted subcutaneously with purified HHV-8 K8.1A and K8.1B proteins expressed in the baculovirus–insect cell system. Mice were immunized with proteins mixed with an equal amount of Freund’s adjuvant. Mice were bled by tail vein, and dilutions of sera were tested in the Western blot reaction with the K8.1A and B proteins. Spleen from a mouse with the highest level of antibodies was used for fusion with Sp2/0.Ag14 myeloma cells (Balachandran et al., 1982, 1989, 1992; Chan et al., 1998).

Hybridoma supernatants were screened by ELISA, IFA, and Western blot reactions. The IFA and ELISA using uninduced and TPA-induced BCBL-1 and BJAB cells were done according to the procedures described previously (Chan et al., 1998; Chandran et al., 1998a,b). The Western blot reactions with K8.1A, K8.1B, and GST proteins expressed in the insect cells were performed according to procedures described below. Clones secreting antibodies reactive only with the BCBL-1 cells by IFA and ELISA and with the K8.1A/B proteins in the Western blots were selected for further testing. These were further expanded, frozen, and single cell cloned twice. Monoclones were rescreened according to the same techniques described above. Monoclonal culture supernatants were used in an ELISA to determine the isotype of antibodies (Pierce, Rockford, IL). High titer antibody containing ascitic fluids were made by injecting hybridoma cells intraperitoneally into Pristane-primed Balb/c mice. IgGs were purified by protein A–Sepharose column and coupled with Sepharose 4B (Pharmacia) according to the manufacturer’s recommendations.

Western blot assays

Uninduced and TPA-induced (24–96 h postinduction) BCBL-1 and BJAB cells were collected, washed, and lysed in distilled water. Samples were adjusted to equal protein concentrations and boiled in the sample buffer. Equal amounts of these total cell lysate proteins (about 10 μg/ml) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Standard prestained molecular weight markers (GIBCO-BRL) were included in the parallel lanes. The membranes were blocked in solution (10 mM Tris–HCl, pH 7.2, 150 mM NaCl, 5% skim milk, 0.02% NaNO₃) at 4°C overnight, and then reacted with MAbs or rabbit anti-peptide antibodies for 1 h at room temperature. The membranes were washed three times with washing buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.3% Tween 20) and incubated for 1 h with alkaline phosphatase-conjugated goat anti-mouse IgG or anti-rabbit IgG (KPL, Gaithersburg, MD). Bound enzyme-labeled antibodies were detected by color reaction of alkaline phosphatase with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrates (Sigma Chemical Co., St. Louis, MO). The reactions were stopped by washing the membranes in distilled water.

Fixed cell IFA

IFA was performed according to procedures described previously (Chan et al., 1998; Chandran et al., 1998a; Smith et al., 1997). Briefly, 10⁶ BCBL-1 cells in 10 ml of medium were induced with 20 ng/ml TPA (Sigma Chemical Co.). Uninduced and TPA-induced BCBL-1 and BJAB cells or uninduced COS-1 cells and COS-1 cells expressing K8.1A and K8.1B proteins were collected, washed in PBS (pH 7.4), spotted onto slides (5-mm inner diameters, 10 circles per slide), air dried in a laminar flow hood under UV light, and fixed with cold acetone for 10 min. Fixed cells were incubated for 30 min at 37°C with hybridoma culture supernatant. After incubation, slides were washed rigorously in PBS. The slides were incubated further for 30 min at 37°C with a prestandardized dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG. After washing, the slides were counterstained with 1:10,000 dilution of Evans blue (Sigma) for 5 min at room temperature, washed, and mounted with 50% (v/v) glycerol in PBS. Slides were examined under a fluorescence microscope.

Surface IFA and immune electron microscopy

Uninduced and TPA-induced (2–5 days postinduction) BCBL-1 and BJAB cells or uninduced COS-1 cells and COS-1 cells expressing K8.1A and K8.1B proteins were collected and washed once with RPMI. Cells (10⁶) were
added with 10 ml of 0.1% para-formaldehyde in PBS, pH 7.4, and centrifuged at 1000 rpm for 10 min. These cells were washed twice, and cell concentrations were adjusted to 10⁶ cells/ml. Then 1 ml of cells in Eppendorf tubes were centrifuged for 10 min at 125 g, and the supernatant was discarded. Dilutions of antibodies (200 μl) were added, mixed, and incubated for 30 min at 37°C. The tubes were centrifuged at 400 g, and the supernatants were discarded. The cells were washed five times with RPM containing 0.1% Na azide and then incubated for 30 min at 37°C with the prestandardized dilutions of FITC-conjugated goat anti-mouse or anti-rabbit IgG antibodies or with goat anti-mouse or anti-rabbit IgG antibodies conjugated with 10-nm gold particles (B.B. International). The cells were washed five times as described above. Cell pellets incubated with FITC antibodies were mounted onto a glass slide and examined under a fluorescence microscope. Cells incubated with gold-conjugated antibodies were fixed with 2% glutaraldehyde and embedded in resins. Thin sections were made, stained, and examined under an electron microscope (Balachandran et al., 1992).

RIP

Uninduced and TPA-induced (72–96 h postinduction) cells (10⁶) were labeled for 20 h with 250 μCi of ³⁵S-methionine and cysteine (Tran²¹⁵S label; specific activity, 1177 Ci/mmol; ICN, Irvine, CA) or with 500 μCi of ³H-glucosamine (specific activity, 25 Ci/mmol; American Radiolabeled Chemicals Inc., St. Louis, MO) in deficient DMEM (Sigma). Immunoprecipitation was carried out according to procedures described previously (Balachandran et al., 1982, 1989; Smith et al., 1997). Briefly, cells were solubilized with RIPA buffer (0.05 M Tris–HCl, pH 7.5, 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 100 U of aprotinin/ml, 0.1 mM phenylmethylsulfonyl fluoride), sonicated, and centrifuged at 100,000 g for 1 h. Equal trichloroacetic acid precipitable counts (5 × 10⁶ cpm) of cell lysates were mixed with 300 μl of hybridoma culture supernatants and 100 μl of protein A-Sepharose beads (Pharmacia, Piscataway, NJ) and were kept rocking at 4°C for 2 h. The precipitates were washed, disociated by boiling in sample buffer, and analyzed by SDS–PAGE in 12% acrylamide cross-linked with 0.28% N,N’-diallyltartardiamide (Sigma). Molecular weight markers (Sigma) were electrophoresed in parallel channels. Gels were stained, destained, infused with 1 M salicyclic acid, dried on filter paper, and placed in contact with XAR-5 film (Kodak, Rochester, NY) at −70°C for fluorography.

Expression of recombinant HHV-8 K8.1A and K8.1B proteins in COS-1 cells

Full-length K8.1A and B cDNA clones were inserted into the BamHI and XhoI sites of the eukaryotic expression vector pTarget (Promega, Madison, WI) containing the cytomegalovirus immediate-early promoter/enhancer. The COS-1 cells were transfected with 2 μg of pTarget-K8.1A/B DNA using CLONexpression (Clontech) according to the manufacturer’s protocols. The COS-K8.1A or B transformants were identified by IFA and Western blot reactions using K8.1A/B-specific monoclonal antibody 4A4. To select the stable transformants, transfected cells were incubated with G418 antibiotics (700 μg/ml) in the growth medium. Once the stable transformants were isolated, they were maintained in the selective medium containing 400 μg/ml active G418. Stable single-cell clones of COS-K8.1A and COS-K8.1B were established subsequently.

Purification of the recombinant HHV-8 K8.1A and B proteins

To purify the K8.1A and B proteins, TPA-induced BCBL-1 cells, COS-1 K8.1A, or COS K8.1B cells were lysed on ice for 1 h with lysing buffer (10 mM Tris–Cl, pH 8.0, 140 mM NaCl, 0.025% NaN₃, 2% Triton X-100, 1% Na-DOC, 0.2 U/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were first passed over Sepharose 4B covalently coupled with K8.1A-specific MAb 4D6 at 4°C, and the flowthrough was passed over Sepharose 4B covalently coupled with K8.1A/B-specific MAb 4A4. The unbound proteins were removed by extensive washing with lysis buffer and monitored by checking the absorbance at 260/280 nm in a spectrophotometer. The bound K8.1A protein was eluted with low pH buffer (50 mM glycine–HCl, pH 2.5, in 150 mM NaCl and 0.1% Nonidet P-40), and the bound K8.1B protein was eluted with high-pH elution buffer (50 mM triethanolamine, pH 11.5, in 150 mM NaCl and 0.1% Nonidet P-40) and immediately neutralized with 1/10 volume of 1 M Tris–HCl, pH 8.0 or 6.5. The peak fractions were pooled, dialyzed against PBS, and stored at −70°C.

Enzymatic treatments of K8.1A and B proteins

Affinity-purified glycoproteins (~5 μg) or the gradient-purified HHV-8 was first denatured by boiling for 4 min in the digestion buffer (50 mM sodium phosphate, pH 7.5, 50 mM EDTA, 20 mM NaN₃, 0.1% SDS, 1% Nonidet P-40) and then treated with glycosidases (Balachandran and Hutt-Fletcher, 1985). To remove N-linked sugars, proteins were incubated with 4 U of N-glycosidase F (Boehringer) for 24 h at 37°C. To remove O-linked carbohydrates, proteins were first incubated with 4 μl of neuraminidase (Boehringer) for 2 h at 37°C, followed by a 22-h incubation with 2.5 μU of O-glycosidase (Boehringer) and 4 U of N-glycosidase F. Equal amounts of digested proteins were separated by SDS–PAGE, transferred onto nitrocellulose membranes, and reacted with K8.1A/B-specific MAb 4A4.
REFERENCES


