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Characterization of the Epstein–Barr virus glycoprotein BMRF-2

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Abstract

Epstein–Barr virus (EBV) BMRF-2 protein interaction with the β1 family of integrins plays an important role in EBV infection of polarized oral epithelial cells. In this work, we characterized BMRF-2 protein expression in EBV-infected B lymphoblastoid and polarized oral epithelial cells, and in hairy leukoplakia (HL) epithelium. BMRF-2 expression in B cells and polarized oral epithelial cells was associated with the EBV lytic infection. In these cells, BMRF-2 is efficiently transported to the cell membrane and its integrin binding Arg–Gly–Asp (RGD) motif is exposed on the cell surface. BMRF-2 is highly expressed in HL epithelium and accumulates at the lateral border of oral keratinocytes. In EBV-infected polarized oral epithelial cells, this protein is transported to the basolateral membranes and co-localized with β1 integrin. These data suggest that BMRF-2 may play an important role in cell-to-cell spread of EBV within the oral epithelium. BMRF-2 is glycosylated through *O*-linked oligosaccharides; it forms oligomers and is associated with the virion envelope. Its C-terminal tail is localized in the cytoplasm. We found that β1, α5, and α3 integrins are present in purified EBV virions. We show that BMRF-2 is a ligand for β1, α5, α3, and αv integrins and our data are consistent with a role for BMRF-2 in viral lytic infection.

Keywords: Epstein–Barr virus; BMRF-2 protein; B lymphoblastoid cells; Polarized oral epithelial cells; Hairy leukoplakia; Integrin

Introduction

Epstein–Barr virus (EBV), a member of the gammaherpesvirus family, is associated with neoplasia of lymphoid and epithelial cell origin. EBV-related diseases include Burkitt's lymphoma, Hodgkin's disease, infectious mononucleosis, nasopharyngeal carcinoma, certain adult T-cell lymphomas, gastric carcinoma, and oral hairy leukoplakia (HL) (Rickinson and E, 2001). EBV tropism is restricted mainly to B lymphocytes and squamous epithelium, causing latent and productive infection, respectively (Rickinson and E, 2001). EBV entry into B lymphocytes or epithelial cells is mediated by the interactions of viral glycoproteins and their cell surface receptors or co-receptors: gp350/220 binds to its cellular CR2-type 2 complement receptor, CD21 (Fingeroth et al., 1984; Tanner et al., 1987), resulting in viral entry into B cells. Complexes of viral glycoproteins–gH/gL/gp42 and gH/gL–are required for infection of B lymphocytes and epithelial cells, respectively (Molesworth et al., 2000; Wang et al., 1998). The binding of EBV gp42 to its co-receptor HLA class II molecule functions as a switching factor for viral tropism between B lymphocytes and epithelial cells (Borza and Hutt-Fletcher, 2002; Li et al., 1997). We have shown that the interactions of BMRF-2 with β1 family integrins play a critical role in the infection of polarized oropharyngeal epithelial cells with EBV (Tugizov et al., 2003).

The mucosal epithelium of the oropharyngeal surface may serve as a portal of entry for EBV during primary infection and as the pathway of exit for progeny virions during systemic infection. Productive EBV infection of the oral mucosal epithelium has been shown in HL (Greenspan et al., 1985), which is associated primarily with HIV/AIDS-mediated immunodeficiency (Greenspan and Greenspan, 1997; Greenspan et al., 1985, 1987; Walling, 2000). EBV infection in HL is restricted to the terminally differentiated part of the mucosal epithelium (Niedobitek et al., 1991; Sandvej et al., 1992; Young et al., 1991) and is absent in the lower basal and parabasal

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layers. The mechanisms of this restriction are not well understood. Analysis of an HL cDNA library showed abundant mRNA expression of the BMRF-2 gene in the oral epithelium of HL, confirmed by in situ hybridization studies showing high-level expression of BMRF-2 in the more differentiated stratum spinosum cell layers (Palefsky et al., 1997; Penaranda et al., 1997).

The BMRF-2 protein was first identified as a glycoprotein by Modrow et al. (1992) in EBV-positive B-cell lines treated with TPA/butyric acid to induce lytic EBV infection. The BMRF-2 open-reading frame (ORF) is conserved throughout the gammaherpesvirus family (Coulter and Reid, 2002), suggesting that it may be important in the viral life cycle. It is located between the BMRF1 and BMLF1 genes in the M region of the EBV genome, defined by restriction digestion with *Bam*HI. Using BMRF-2-specific probes, Becker et al. detected two mRNA species (3.7 and 2.1 kb) in EBV-transformed normal lymphoblasts and in one ataxia-telangiectasia cell line (Becker et al., 1988). The larger mRNA species are bicistronic, encoding both BMRF-1 and BMRF-2 proteins. The smaller species are believed to be transcribed from BMRF-2's own promoter, which is located within the ORF of BMRF-1. We have previously shown that the activity of the BMRF-2 promoter is up-regulated by mechanisms known to induce differentiation of epithelial cells (Lagenaur and Palefsky, 1999).

The BMRF-2 protein contains an Arg–Gly–Asp (RGD) motif that binds to β1 and α5 integrins; this interaction mediates the entry of EBV through the basolateral membranes of polarized oral epithelial cells (Tugizov et al., 2003). Integrins are a large family of heterodimeric cell surface receptors consisting of 18α and 8β subunits (Brakebusch and Fassler, 2005; Giancotti and Ruoslahti, 1999). There are more than 27 heterodimers and each mammalian cell expresses several combinations of αβ subunits. Most cell surface integrins bind to extracellular matrix proteins (ECM) via their RGD motif, and mediate cell–ECM and cell–cell adhesion, interactions that are critical for many functions of mammalian cells including growth, differentiation, apoptosis, endocytosis, attachment and motility (Giancotti and Ruoslahti, 1999; Takagi, 2004). Several viral proteins from either enveloped or non-enveloped viruses also possess the RGD motif, which may be involved in binding virions to cell surface integrins. Enveloped viruses such as human herpesvirus 8 (HHV-8) (Akula et al., 2002), EBV (Tugizov et al., 2003) and hantavirus (Gavrilovskaya et al., 1999) bind to integrins through their RGD-containing glycoproteins. Human cytomegalovirus (HCMV) glycoproteins B (gB) and H (gH) also bind to integrins, but the binding is mediated by RGD-independent motifs (Feire et al., 2004; Wang et al., 2005). Nonenveloped viruses, such as adenovirus (Davison et al., 2001; Huang et al., 1996), rotavirus (Ciarlet et al., 2002; Graham et al., 2003), foot-and-mouth disease virus (Jackson et al., 1997), West Nile virus (Chu and Ng, 2004; Lee et al., 2006), echovirus (Triantafilou and Triantafilou, 2001) and coxsackie virus (Williams et al., 2004), have RGD-containing capsid proteins that bind to various integrin heterodimers. The interactions of viral proteins and integrins activate integrin signaling pathways, which may lead to virus entry into host cells (Krishnan et al., 2006; Medina-Kauwe, 2003; Meier and Greber, 2004; Pietiainen et al., 2004; Sharma-Walia et al., 2004; Wang et al., 2005).

In this study, we investigated BMRF-2 protein expression in B lymphoblastoid cell lines, polarized oral epithelial cells and HL epithelium. Our findings show that BMRF-2 is expressed during EBV lytic infection in B cells and oral epithelial cells. We show that BMRF-2 is glycosylated through *O*-linked oligosaccharides and efficiently transported to the cell surface, and that mature EBV virions are associated with oligomeric forms of BMRF-2. The BMRF-2 RGD domain binds to β1, α5, α3, and αv integrins, indicating that BMRF-2 is a ligand for these integrins.

Results

Expression of the BMRF-2 protein in EBV-infected B lymphocytes and polarized oral epithelial cells

To examine BMRF-2 expression in B lymphoblastoid cell lines, EBV-infected B95-8 and P3HR-1 cells were treated with 30 ng/ml of PMA for 9 days. PMA-treated and untreated EBV-negative BJAB cells were used as a negative control. At 1, 3, 5, 7 and 9 days post-treatment, cells were fixed and immunostained for BMRF-2. To confirm induction of the EBV lytic cycle, these cells were immunostained with EBV gp350/220. Confocal immunofluoresecence analysis showed that BMRF-2 expression was detected in PMA-treated B95-8 and P3HR-1 cell lines, and protein localization was predominantly in the cytoplasm (Fig. 1A). Co-immunostaining of BMRF-2 with gp350/220 in these cells showed co-localization of these two proteins (data not shown). Analysis of B95-8 and P3HR-1 cells not treated with PMA showed that 0.8% and 0.3% of cells, respectively, were weakly positive for BMRF-2 expression. Quantitative analysis of BMRF-2-expressing cells showed that BMRF-2 induction was detected in PMA-treated B95-8 and P3HR-1 cell lines at 24 h post-treatment (Fig. 1B). At this time, about 4.8% and 2.1% of B95-8 and P3HR-1 cells, respectively, were positive for BMRF-2. There was a significant increase in cells expressing BMRF-2 at 3 days post-treatment, reaching 8.5% and 3.4% in B95-8 and P3HR-1 cells, respectively. Expression of gp350/220 in both cell lines was slightly greater (2–3%) than that of BMRF-2 at 3 days post-treatment. Expression of BMRF-2 and gp350/220 in these cells at 7 and 9 days post-treatment decreased by about 10–30% compared with their expression at 3 days post-treatment.

Earlier we established the EBV-permissive polarized HSC-3^{sort} tongue and Detroit^{sort} pharyngeal epithelial cell model (Tugizov et al., 2003). These cells are highly positive for β1- and αv-family integrins and the epithelial differentiation markers involucrin and keratin-1 (manuscript in preparation). The efficiency of EBV infection in these cells is dependent on epithelial cell differentiation and polarization. To study BMRF-2 expression, polarized HSC-3^{sort} tongue and Detroit^{sort} pharyngeal cells were infected with the EBV B95-8 strain; at 1, 3, 5, 7, and 9 days post-infection, cells were immunostained with rat anti-BMRF-2 antiserum. Analysis showed that none of the proteins was detected at 24 h

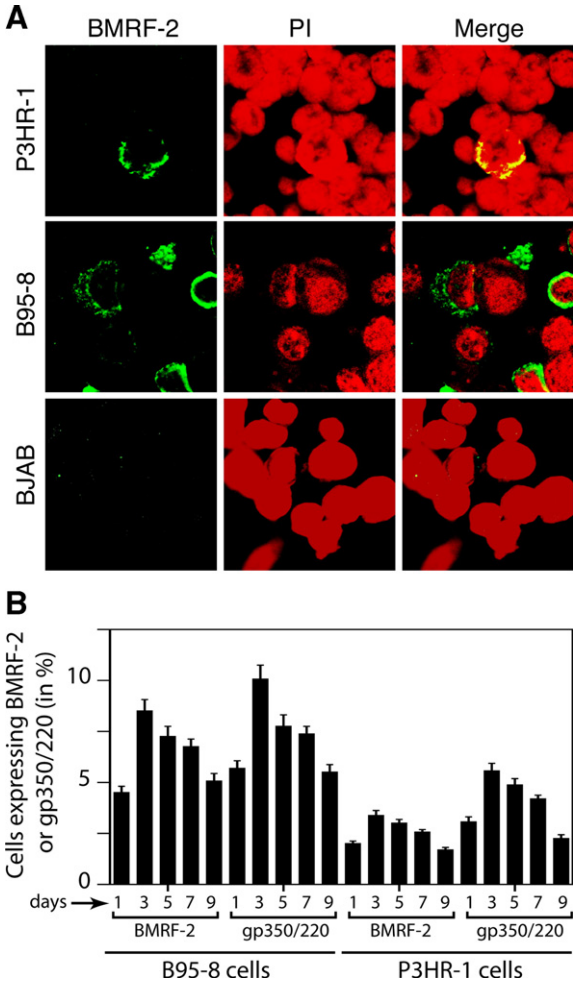

Fig. 1. Expression of BMRF-2 in EBV-infected B lymphoblastoid cells. (A) P3HR-1 and B95-8 cells were treated with 30 ng/ml PMA for 5 days and immunostained with rat anti-BMRF-2 antiserum (green). EBV-negative BJAB cells also were treated with PMA and were used as a negative control. Cell nuclei were stained with propidium iodide (PI) (red). (B) Quantitative analysis of P3HR-1 and B95-8 cells expressing BMRF-2 and gp350/220. P3HR-1 and B95-8 cells were treated with 30 ng/ml PMA for 1, 3, 5, 7 and 9 days and then immunostained for BMRF-2 and gp350/220. EBV-infected cells expressing BMRF-2 and gp350/220 were counted. Error bars show SEM ($n=3$).

post-infection (Fig. 2A). At 2 days post-infection, only a small number of cells (about 0.1–0.5%) showed a weak signal for both proteins (data not shown). Significant expression of BMRF-2 in both Detroit^{sort} and HSC-3^{sort} cell lines was detected at 3 days post-infection (i.e., 5.3% and 2.5% cell were positive for BMRF-2, respectively) (Fig. 2A), at which time the expression level of gp350/220 in Detroit^{sort} and HSC-3^{sort} cell lines was slightly lower than that of BMRF-2. At 5 days post-infection, however, the expression level of gp350/220 was about 2% higher than that of BMRF-2 (Fig. 2A). At 5 days post-infection, BMRF-2 expression was detected in 8.5% and 5.2% of Detroit^{sort} and HSC-3^{sort} cells, respectively. The level of expression of BMRF-2 and gp350/220 in both polarized cells after 7 and 9 days post-infection was stable. The stable expression of BMRF-2 and gp350/220 in Detroit^{sort} cells was observed for 3–4 weeks. Expression of BMRF-2 and gp350/220 in HSC-3^{sort} cells after 2 weeks of infection was significantly reduced, with approximately 0.5% and 0.7% cells being positive for BMRF-2 and gp350/220 proteins, respectively.

Dissociation of these EBV-infected polarized cells from the filters and growing them under non-polarized conditions failed to result in EBV-infected epithelial cell lines. The majority of these cells (about 90%) in the flask were dead within 3–5 days and only 5–10% of the surviving cells generated new cell populations within the next 2–3 weeks. These cells were negative for BMRF-2 and gp350/220 proteins, as well as for EBV-latent proteins LMP1 and EBNA-1 (data not shown). PCR and RT-PCR analysis also did not reveal EBV DNA or RNA (data not shown). Localization of BMRF-2 protein in polarized HSC-3^{sort} (Fig. 2B) and Detroit ^{sort} cells (data not shown) was predominantly in the cytoplasm, and a small amount of protein was detected in the perinuclear area. The staining pattern of

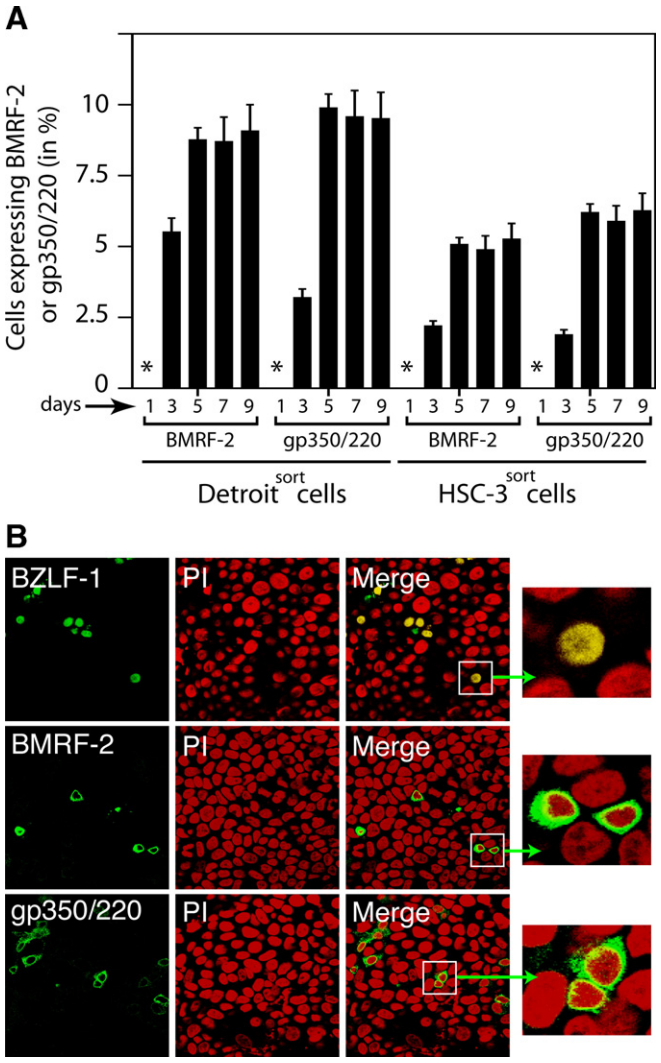

Fig. 2. Expression of BMRF-2 in polarized HSC-3^{sort} tongue cells. (A) Polarized Detroit^{sort} and HSC-3^{sort} cells were infected with EBV from their basolateral membranes for 1, 3, 5, 7 and 9 days and immunostained for BMRF-2 and gp350/220 proteins. Cells expressing BMRF-2 and gp350/220 were counted from triplicate samples and their average number was presented in %. Error bars show SEM ($n=3$). (B) Polarized HSC-3^{sort} cells were infected with EBV for 7 days and immunostained for BZLF-1, BMRF-2 and gp350/220 (green) proteins. The cell nuclei were stained with propidium iodide (red).

gp350/220 in these cells was similar to that of BMRF-2. Co-staining of BMRF-2 and gp350/220 showed co-localization of these two proteins (data not shown). BZLF-1 was localized exclusively in the nuclei (Fig. 2B).

BMRF-2 protein is transported to the cell surface

To determine whether BMRF-2 is expressed on the cell surface in EBV-infected cells, we performed flow cytometry analysis of EBV-infected B95-8 lymphoblastoid cells and EBV-negative BJAB cells as a negative control, treated with 30 ng/ml PMA and 4 mM butyric acid for 5 days. HSC-3^{sort} cells were infected with EBV from basolateral membranes and were studied at 7 days post-infection for flow cytometry. To confirm that the level of EBV infection was sufficient to evaluate the surface transport of EBV glycoproteins, we also studied gp350/220 in the plasma membranes of these cells. The results showed that BMRF-2 protein was present on the surface of both HSC-3^{sort} and B95-8 cells (Fig. 3), indicating that BMRF-2 is efficiently transported to the surface in EBV-infected cells. Because the rat anti-BMRF-2 serum rose against the GST-BMRF-2 RGD domain, these data indicated exposition of the BMRF-2 RGD motif on the cell surface.

BMRF-2 is accumulated within the lateral membranes of the HL epithelium and is transported to the basolateral membranes of polarized oral epithelial cells

To examine BMRF-2 expression in HL lesions, cryosections from three HL biopsies were immunostained for BMRF-2 protein. These data showed that BMRF-2 expression was detected within the stratum spinosum and stratum granulosum layers of HL (Fig. 4A). In parallel experiments, the sections from the same HL biopsies were immunostained for BZLF-1 (Figs. 4B and C), which was detected in the nuclei of spinosum and granulosum cells of the HL

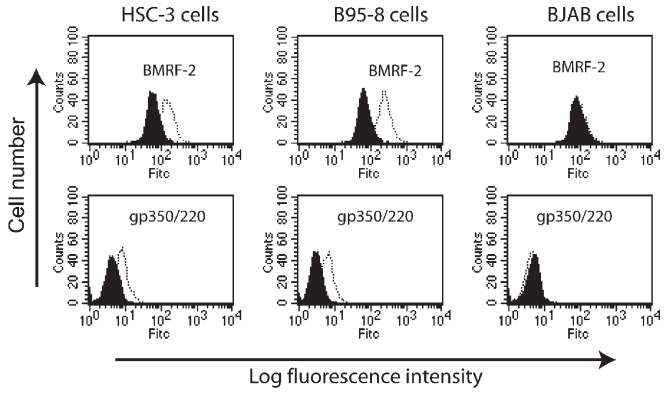

Fig. 3. Transport of BMRF-2 to the cell surface. Polarized HSC-3^{sort} cells were infected with EBV from their basolateral membranes; at 7 days post-infection, BMRF-2 expression was detected by flow cytometry analysis. Black shading indicates uninfected cells, and the dotted line indicates infected cells. EBV-infected B95-8 and EBV-negative BJAB cells were treated with PMA for 5 days and analyzed by flow cytometry. Black shading indicates PMA-untreated cells and the dotted line indicates PMA-treated cells. For flow cytometry assay, rat anti-BMRF-2 antibodies were used.

epithelium, where lytic EBV replication and viral dissemination occur. To determine the distribution of BMRF-2 in the HL epithelium in more detail, the cryosections were costained for BMRF-2 and BZLF-1, and images were analyzed in an *x–z* vertical plane. These experiments showed that BMRF-2 protein accumulated within the lateral membranes of oral epithelial cells within the spinosum (Fig. 4D) and granulosum (data not shown) layers.

Accumulation of BMRF-2 within the lateral borders of the HL epithelium suggested that BMRF-2 might be delivered specifically to the lateral membranes of oral epithelial cells. To confirm this possibility, we examined its localization in EBV-infected polarized Detroit^{sort} cells. Because we found that β1 family integrins transport to the basolateral membranes of polarized epithelial cells (De Luca et al., 1990; Muza-Moons et al., 2003; Tugizov et al., 2003), we co-immunostained β1 integrin with BMRF-2 as a control marker. Non-permeabilized polarized cells were immunostained from apical or basolateral surfaces. Co-immunostaining of BMRF-2 and β1 integrin from the apical membranes of polarized Detroit^{sort} cells did not show apical staining for both proteins (Fig. 5A, upper panel). In contrast, basolateral staining of non-permeabilized Detroit^{sort} cells showed that both BMRF-2 and β1 integrin were present and co-localized at the basolateral membranes (Fig. 5A, lower panel). Analysis of these cells in *x–z* vertical plane also confirmed basolateral transport and co-localization of BMRF-2 and β1 integrin (Fig. 5B).

BMRF-2 is associated with the envelope of mature virions

We and others have shown that BMRF-2 is associated with mature EBV virions (Johannsen et al., 2004; Tugizov et al., 2003); however, the presence and monomeric or oligomeric status of BMRF-2 in the mature virion envelope were not determined. To address these questions, we performed additional experiments using sucrose gradient-purified EBV virions from B95-8 cells. To detect the BMRF-2 protein, the viral proteins were separated on a 4–20% gradient Tris–glycine SDS-PAGE gel and blotted with rat anti-BMRF-2 antiserum (Fig. 6A). These data showed the presence of 55–60 kDa bands and more diffuse bands with a higher molecular mass including a solid band above 220 kDa (Fig. 6A). Normal rat serum did not detect any protein bands in gradient-purified EBV (Fig. 6A), confirming the specificity of the rat anti-BMRF-2 serum to EBV BMRF-2. The electrophoretic mobility of the BMRF-2 protein on a non-gradient 10% or 16% SDS-PAGE gel showed that it migrated poorly in these gels with little entering the separation gel (data not shown). Comparative analysis of BMRF-2 and EBV viral capsid antigen (VCA) p160 in increasing concentration of total viral proteins in the same gel showed that BMRF-2 protein was detectable only when higher concentrations of EBV proteins were loaded (i.e., 9–12 μg/lane) (Fig. 6B).

To examine the presence of BMRF-2 in the virion envelope, the double-gradient purified virions from Nycodenz gradient were extracted with Triton X-100 and 0.5% deoxycholate, and tegument/capsid proteins were separated from the envelope by high-speed centrifugation. Western blot

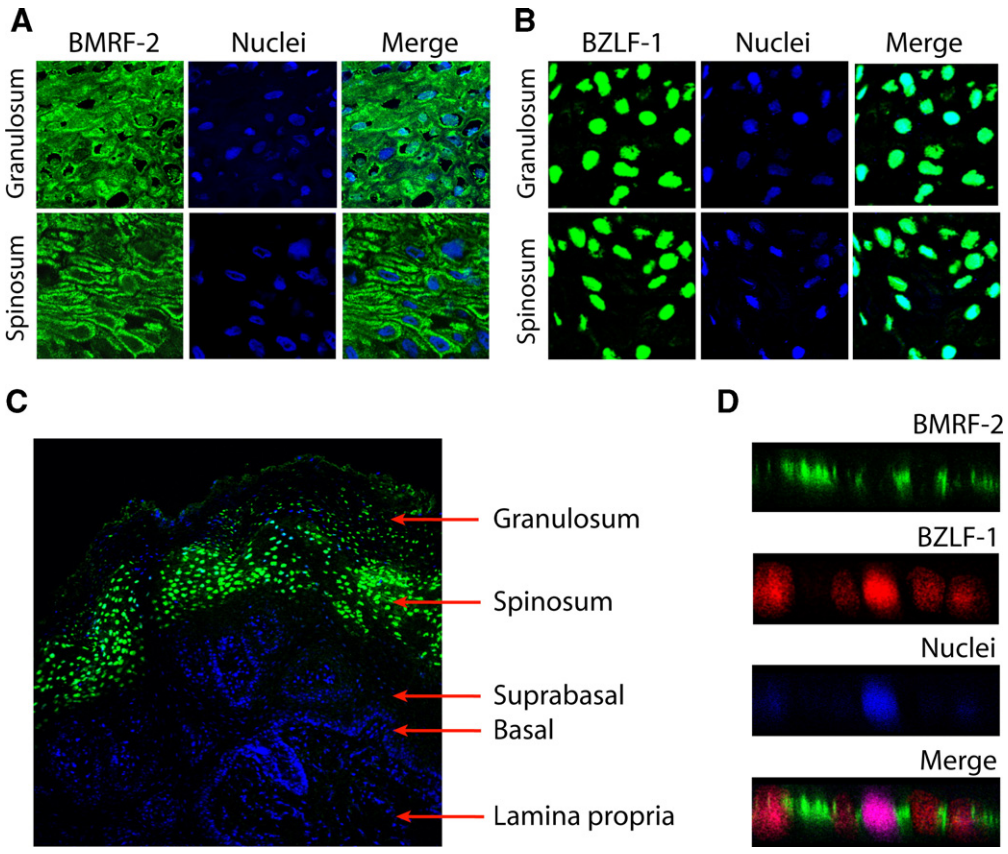

Fig. 4. BMRF-2 expression in oral hairy leukoplakia. (A) Cryosections of HL were immunostained with rat anti-BMRF-2 immune serum (green) and (B) mouse mAb to BZLF-1. Magnifications: ×800. (C) HL section was immunostained for BZLF-1 using mouse mAb. Magnifications: ×200. (D) HL section was co-immunostained for BMRF-2 (green) and BZLF-1 (red). Magnifications: ×1200. (A–C) Images were taken on an *x–z* horizontal plane and (D) images were taken in *x–z* vertical plane. Cell nuclei in all panels were counterstained with TO-PRO-3 (blue).

analysis of pellet and supernatant of the viral extract showed that BMRF-2 was present in the supernatant, which indicates its association with the envelope (Fig. 6C). VCA p160 was present in pellet fractions, as expected. These results indicate that BMRF-2 is a component of mature EBV virions and that it is associated with the virion envelope. To determine the status of BMRF-2 in the virion envelope, EBV proteins were separated on 7.5% native acrylamide gel. Total viral proteins were extracted and separated under native, non-reduced, or reduced conditions, and BMRF-2 protein was detected using a Western blot assay. These results showed that the size of the native (not treated with denaturing agents and heat) BMRF-2 protein was approximately 220–240 kDa (Fig. 6D). Under reduced condition, multiple bands of 55–58 kDa, 120–140 kDa and 220–240 kDa were detected (Fig. 6D). Detection of 220–240 kDa BMRF-2 bands in native EBV virion samples was consistent with the presence of oligomeric forms of BMRF-2 in the virion envelope. The 55–58 kDa and 120–140 kDa bands under reducing and non-reducing conditions represented monomer and dimer forms of BMRF-2, respectively. It is possible that solid bands above 220 kDa detected in denaturing gel (Figs. 6A and B) also may represent an oligomeric form of BMRF-2, but because of the highly hydrophobic nature of the protein, these oligomers may not dissociate completely under denaturing conditions.

Expression of the BMRF-2 protein in transiently transfected cells

To study expression of BMRF-2 protein in absence of other EBV proteins, we cloned the BMRF-2 ORF from the EBV B95-8 strain. The DNA fragment containing BMRF-2 ORF and its upstream 300 bp promoter region was cloned into the expression vector plasmid LNCX downstream of the CMV early promoter. The protein was tagged either with the GFP protein or the HA peptide for convenient visualization and detection. Transfection efficiency of HSC-3^{sort} tongue and Detroit^{sort} pharyngeal cells was very low; therefore we used 293T human kidney epithelial cells for BMRF-2 expression. Protein expression was examined by detection of GFP signals and HA signals, and by indirect immunofluorescence assays using mouse mAb to HA and rat polyclonal antisera against BMRF-2. BMRF-2 expression of both constructs was detected in about 80% of 293T cells. BMRF-2 GFP was detected in the cytoplasm of 293T cells (Fig. 7A), a finding consistent with the localization of BMRF-2 in the cytoplasm in EBV-infected lymphoblastoid and epithelial cells. Anti-HA antibody recognized BMRF-2 expression in BMRF-2-HA transfected 293T cells (Fig. 7B). Co-immunofluorescence analysis of cells transfected with BMRF-2-HA using anti-HA and anti-BMRF-2 antibodies showed that both antibodies localized BMRF-2 to

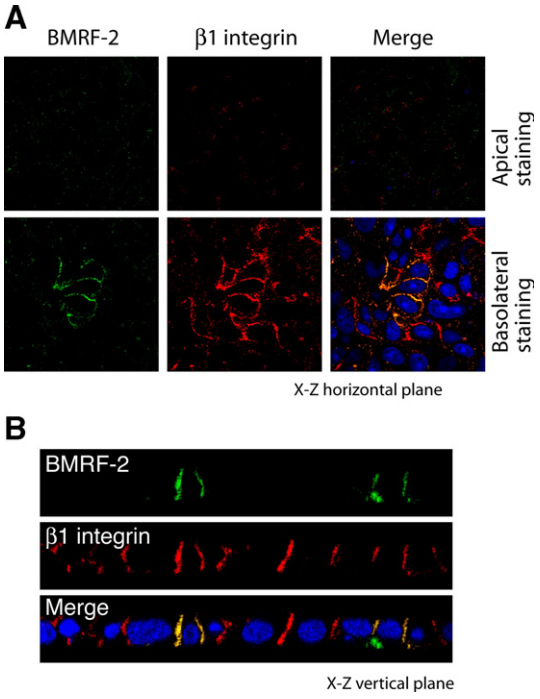

Fig. 5. Basolateral transport of BMRF-2 in polarized Detroit^{sort} epithelial cells. (A) EBV-infected polarized Detroit^{sort} cells were fixed with 3% paraformaldehyde at 7 days post-infection. Cells were reacted with anti-BMRF-2 and anti-β1 integrin antibodies from the apical surface (upper panel) or the basolateral surface (lower panel). The images corresponding to the apical and basolateral membranes were obtained in *x–z* horizontal plane. (B). Analysis of BMRF-2 and β1 integrin co-staining in the *x–z* vertical plane. (A and B) Cell nuclei were stained in blue and are shown in the merged panel. Yellow in the merged panel indicates co-localization of BMRF-2 (green) and β1 integrin (red) signals. Magnification: ×1200.

the cytoplasm, indicating that anti-HA antibody recognizes authentic BMRF-2 protein (Fig. 7B).

Western blot analysis of BMRF-2 protein in 293T cells using denatured Tris–glycine SDS-PAGE showed that the majority of the protein was detected only as high molecular-weight bands of more than 220 kDa (data not shown). This was also noted in the detection of BMRF-2 from purified virions, possibly due to the highly hydrophobic nature of this protein. To improve detection of BMRF-2, we purified the membrane fraction from BMRF-2 transfected cells and separated the membrane proteins on a 7 M urea denaturing gel. Blotting of this gel showed that membrane-associated BMRF-2 was detected as two bands of about 55 and 60 kDa (Fig. 7C). However, a large portion of BMRF-2 protein also was detected above 220 kDa, i.e., did not enter into separation gel (data not shown).

BMRF-2 C-terminus is localized in the cytoplasm

Because we tagged the BMRF-2 C-terminus with HA epitope, it may be possible to determine its localization within the cell membrane. To examine this possibility, 293T cells were transfected with a DNA plasmid containing the BMRF-2 gene tagged with HA at its C-terminus. Flow cytometry analysis of BMRF-2-HA in 293T cells using EBV-specific human sera confirmed surface transport of BMRF-2 in 293 cells (Fig. 7D). However, flow cytometry assay using anti-HA antibodies did not detect BMRF-2 protein on the cell surface. We showed in Fig. 7B that the same anti-HA antibody recognized BMRF-2 protein in 293T cells, which was fixed with methanol/acetone (i.e., permeabilized), indicating that anti-HA antibodies recognize intracellular BMRF-2. The absence of BMRF-2 on the cell surface using the same antibody by flow cytometry of live cells indicates that the BMRF-2 C-terminal HA epitope for anti-HA antibody is not accessible on the plasma membranes. These data suggest that the C-terminus of BMRF-2 could be in the cytoplasm, or it could be embedded in the plasma membrane. Since it is hydrophilic, the more likely localization is the cytoplasm.

BMRF-2 protein is glycosylated by O-linked oligosaccharides

To study intracellular localization and glycosylation of BMRF-2 protein, HSC-3^{sort} oral epithelial cells were transduced with a retrovirus vector expressing BMRF-2-GFP. Co-immunofluorescence analysis of BMRF-2 with the ER marker

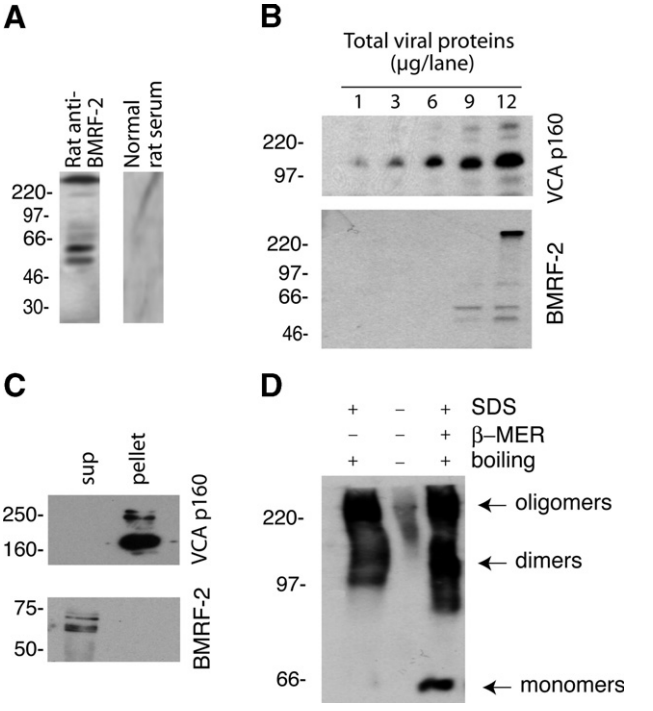

Fig. 6. Detection of EBV BMRF-2 in gradient-purified EBV virions. (A) Purified EBV viral proteins (10 μg/lane) were separated on a 4–20% SDS-PAGE gradient gel and BMRF-2 was detected using rat anti-BMRF-2 antiserum. Normal rat serum was used as a control. (B) Purified EBV viral proteins were loaded at 1, 3, 6, 9 and 12 μg/lane and subjected to Western blot analysis using mouse mAb to VCA p160 and rat anti-BMRF-2 immune serum. (C) Double-purified virions from Nycodenz gradient were extracted with Triton X-100 and 0.5% deoxycholate and tegument/capsid proteins were separated from the envelope by high-speed centrifugation. Proteins were separated in 4–20% SDS-PAGE gradient gel, and BMRF-2 and VCA p160 proteins were detected using a Western blot assay using rat antiserum and mouse monoclonal antibody. Sup, supernatant. (D) Total EBV proteins from purified virions were treated for non-reducing (SDS and boiling), native (not treated with SDS or beta-mercaptoethanol and heat) and reducing (SDS, β-MER/beta-mercaptoethanol and boiling) conditions, and were separated on a 7.5% native polyacrylamide gel. BMRF-2 was detected using rat antiserum.

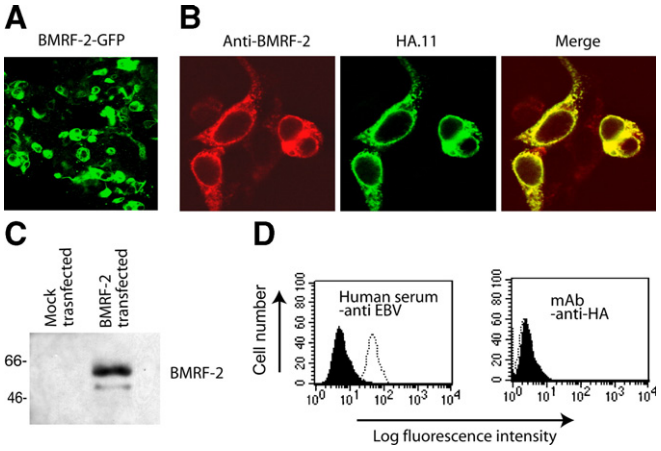

Fig. 7. Analysis of BMRF-2 protein expression in 293T human embryonic kidney cells transfected with BMRF-2 gene. (A) 293T cells were transfected with BMRF-2-GFP construct and at 48 h later cells were analyzed by confocal microscopy. Magnifications: ×200. (B) 293T cells were transfected with BMRF-2-HA DNA construct. At 48 h after transfection, cells were fixed with methanol/acetone and co-immunostained with mAb to HA and rat polyclonal antibody to BMRF-2. Green represents HA staining, red represents BMRF-2 staining, and yellow indicates the merging of the two colors. Magnifications: ×1200. (C) Western blot analysis of BMRF-2 in 293T cells. 293T cells were transfected with BMRF-2-HA DNA construct, at 48 h post-transfection, membrane-enriched cellular proteins were isolated and separated on a 7 M denaturing urea gel. BMRF-2 protein was detected using rat anti-BMRF-2 antibody. Untransfected 293T cells served as a negative control. (D) Surface transport of BMRF-2 in 293T cells transfected with BMRF-2 gene. 293T cells were transfected with BMRF-2-HA DNA construct, 48 h later, cells were analyzed by flow cytometry using human anti-EBV sera and mAb to HA.

GRP94 and the Golgi marker Rhodamine-lens Culinaris Agglutinin showed that BMRF-2 co-localized with ER and Golgi markers (Fig. 8A), indicating that BMRF-2 is present in ER and Golgi compartments. Co-localization of BMRF-2 with ER and Golgi markers was detected also in 293T cells transfected with the BMRF-2 gene (data not shown). These data show that the intracellular sorting and biosynthetic pathways of BMRF-2 protein are typical for viral glycoproteins, which may undergo post-translational modifications by oligosaccharides. Analysis of the amino acid sequence of BMRF-2, however, showed that it does not have consensus Asn-X-Ser/Thr (X, any amino acid except Pro) motif for potential *N*-glycosylation. The glycoprotein could be post-translationally modified by *O*-linked oligosaccharides; therefore, we examined BMRF-2 glycosylation by *O*-linked oligosaccharides in gradient-purified virions and in 293T cells transfected only with the BMRF-2 encoding sequence. Proteins from purified EBV virions and the membrane fractions of 293T cells expressing BMRF-2-HA were treated with neuraminidase and *O*-glycosydase independently or in combination. The protein was separated on urea gel and detected using a Western blot assay. As shown in Fig. 8B, neuraminidase treatment alone resulted in a significant reduction of molecular mass, indicating a high degree of peripheral sialylation of the protein. Treatment with *O*-glycanase resulted in a significant reduction of the upper band and increased the intensity and mobility shift of the lower band, suggesting that the heavier isoform of the protein is highly glycosylated through *O*-linked oligosaccharides. Treatment of proteins with the combination of *O*-glycanase and neuraminidase further decreased the size of the protein, indicating full deglycosylation. These data demonstrate that BMRF-2 protein in transfected cells and in the virion envelope was extensively glycosylated by *O*-linked oligosaccharides.

Purified EBV virions and BMRF-2 protein are associated with integrins

We have previously shown that purified, biotinylated EBV and the GST-BMRF-2 RGD fusion protein can bind β1 and α5 integrins in vitro (Tugizov et al., 2003). Here we show that the BMRF-2 glycoprotein is associated with mature extracellular virions and that its RGD motif is exposed on the surface of the viral envelope, suggesting that progeny EBV virions during their intracellular transport and/or release may bind to integrins. To examine the association of integrins and released progeny virions, we determined if integrins were present in gradient-purified EBV from B95-8 cells using a Western blot assay. Detection of VCAp160 served as a control in this experiment. The results showed that β1 integrin was present in purified EBV

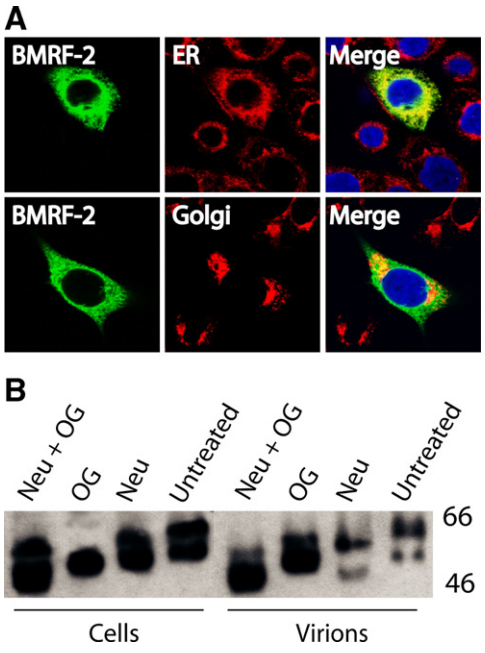

Fig. 8. Glycosylation of BMRF-2. (A) HSC3^{sort} cells were transduced with a retroviral vector expressing BMRF-2-GFP. After 48 h, cells were fixed and co-immunostained for BMRF-2 with ER or Golgi markers. ER and Golgi were detected with staining for GRP94 and Rhodamine-Lens Culinaris Agglutinin, respectively. BMRF-2 is shown in green fluorescence as a GFP fusion protein and the ER and Golgi markers are shown in red fluorescence. Yellow indicates colocalization of BMRF-2 with ER or Golgi markers. (B) The 293T cells were transfected with the BMRF-2 gene for 48 h and the membrane fractions of these cells were isolated. In a parallel experiment, total proteins from purified EBV virions were extracted. Transfected cells and virion samples were treated with a combination of neuraminidase and *O*-glycosidase, and with *O*-glycosidase and neuraminidase independently. Untreated samples served as a control. Treated and untreated samples were subjected to Western blot analysis using rat anti-BMRF-2 immune serum and proteins were separated in 7 M urea gels. Neu, neuraminidase; OG, *O*-glycosydase. Cells, 293T cells transfected with BMRF-2. Virions, gradient-purified EBV virions.

virions, although it was detected only if a high concentration of viral proteins was loaded (i.e., 6 μg/lane and above) (Fig. 9A). Analysis of purified EBV samples from two independent lots showed that both purified virions contained β1 integrin (data not shown), indicating that the EBV-β1 integrin association is consistent. Western blotting of double gradient-purified EBV B95-8 also revealed β1 integrin in virions (data not shown), indicating that the EBV-integrin association is not due to contamination with integrin.

It has been shown that EBV may activate expression of αv integrin in B cells (Huang et al., 2000). To further study the relation between EBV and integrins, we searched for αv, β1, α3, and α5 integrins in purified EBV virions and B95-8 cells with and without PMA treatment. Our data showed that purified EBV virions and in B95-8 cells contain α3, α5 and β1 integrins, indicating that the mature EBV virions acquired these integrins from host cells (Fig. 9B). In contrast, αv integrins were detected only in host cell samples and not in virions; thus, the EBV-integrin association is not because of contamination of purified virions with these integrins. PMA treatment of B95-8 cells resulted in a decrease, not an increase, of integrin expression. Western blot analysis showed that B95-8 cells express β1, α3 and α5 integrins in their fully processed mature form and under-processed precursor form (Fig. 9B), but only the fully processed forms were detected in the virions. The size of virion-associated β1 integrin was 180 kDa, which was larger than that of B95-8 cells (160/125/116 kDa), indicating that highly processed 180 kDa integrin was not detectable in B95-8 cells by Western blot assay, i.e., only very low level of this highly processed form of β1 integrin is expressed in these cells. The presence of highly processed integrins in EBV virions suggests that EBV preferentially binds fully processed integrins. To determine whether the virion binding with integrins is mediated by the BMRF-2 RGD domain, we performed a GST-BMRF-2 RGD pull-down assay in B95-8 cell extracts, which showed that the BMRF-2 RGD domain binds to fully processed β1, α5, and α3 and αv integrins (Fig. 9C).

Discussion

Using antibodies to BMRF-2 and integrins, BMRF-2 RGD peptides, soluble forms of integrins and integrin ligands, we have shown that interaction between the EBV BMRF-2 glycoprotein and β1 and/or α5β1 integrins plays a significant role in EBV infection at the basolateral membrane of polarized oral epithelial cells (Tugizov et al., 2003). We have observed that α3β1, αvβ3, and αvβ5 integrins also are involved in the infection with EBV of polarized oral epithelial cells, but to a lesser extent than are β1 and/or α5β1 integrins. In the present work, we characterize EBV BMRF-2 protein expression in EBV-infected B lymphoblastoid and polarized oral epithelial cells and in HL. Analysis of BMRF-2 in EBV-infected B lymphoblastoid cells and polarized tongue and pharyngeal epithelial cells showed that this protein is expressed in both B cells and epithelial cells. In EBV-infected B cells, BMRF-2 expression was dependent on PMA treatment, indicating that BMRF-2 expression occurs during the lytic cycle of EBV replication. The highest level of BMRF-2 expression in B cells was at 5 days post-induction, and it declined after 5 days of protein expression. In contrast, BMRF-2 expression reached its highest level in polarized epithelial cells at 5 days post-infection, but this level of expression was stable for 9 days in HSC-3^{sort} and 3–4 weeks in Detroit^{sort} cells. EBV-infected polarized cells that was dissociated from the filter inserts failed to grow into established EBV-infected epithelial cell lines, indicating that polarization of oral epithelial cells may be critical for EBV replication. Development of epithelial cell polarity leads to acquisition of a cuboidal cell shape, which occurs prior to formation of epithelial sheets and terminal differentiation of epithelium (Fujita and Braga, 2005; Pilot and Lecuit, 2005; Sourisseau et al., 2006). Since EBV replication in HL is restricted to the terminally differentiated stratum spinosum and granulosum layers (Niedobitek et al., 1991; Sandvej et al., 1992; Young et al., 1991), in vitro polarization and differentiation of tongue epithelial cells may generate a favorable environment for EBV replication.

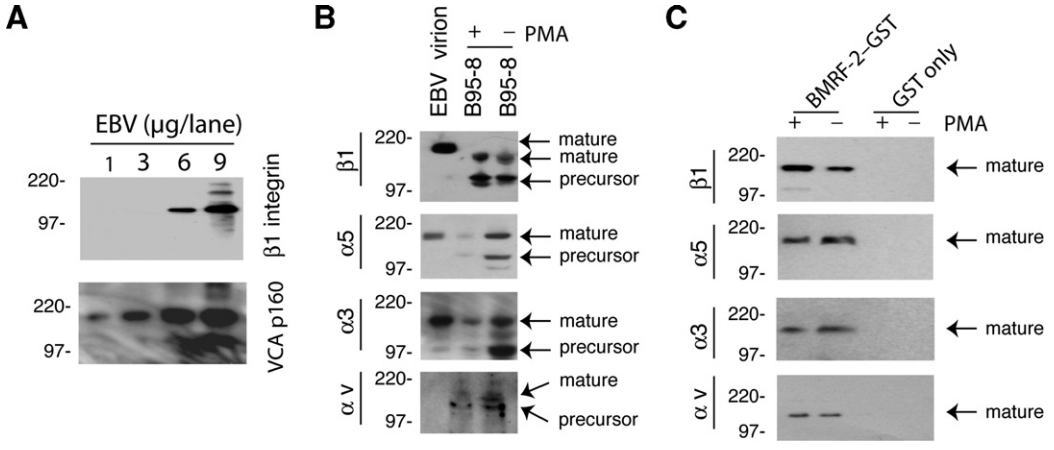

Fig. 9. Association of purified EBV virions and BMRF-2 protein with β1, α3 and α5 integrins. (A) Purified EBV virions were extracted and loaded at 1, 3, 6 and 9 μg/lane, separated on a 10% SDS-PAGE gradient gel and examined for β1 integrin and VCAp160 using appropriate antibodies. (B) Gradient-purified EBV virions (10 μg/lane) and PMA-treated or -untreated B95-8 cells were analyzed for β1, α3 and α5 integrins using a Western blot assay. (C) Association of β1, α3, α5 and αv integrins with GST-BMRF-2 RGD fusion protein. Total cellular proteins from PMA-treated and-untreated B95-8 cells were pulled down with the GST-BMRF-2 RGD fusion protein and separated on a 10% SDS-PAGE gel. Pull-down with GST alone was performed as a control.

Confocal immunofluorescence analysis of BMRF-2 expression in HL sections showed that BMRF-2 protein was present within the spinosum and granulosum layers, where high-level EBV replication occurs. These data support our earlier finding that abundant mRNA transcription of the BMRF-2 gene was detected in the more differentiated stratum spinosum cell layer of HL (Palefsky et al., 1997; Penaranda et al., 1997). Accumulation of BMRF-2 protein within the lateral borders of oral keratinocytes suggests that it may be transported to the lateral surface of oral epithelial cells. Indeed, these data were well supported by the basolateral transport of BMRF-1 in EBV-infected polarized Detroit^{sort} cells.

BMRF-2 has a di-leucine and tyrosine motif YLLVTFIKS at the 349–357 amino acid position of its tail that may serve as a potential basolateral sorting signal. The C-terminus of the protein is oriented to the cytoplasm, since it is not detectable on the cell surface. It has been well documented that such motifs at the cytoplasmic tail of cellular and viral glycoproteins may serve as basolateral sorting signals that mediate transport of viral and cellular glycoproteins from the *trans*-Golgi-network (TGN) to the basolateral membranes of epithelial cells (Rodriguez-Boulan et al., 2005; Rodriguez-Boulan and Musch, 2005). Accumulation of BMRF-2 at the lateral borders of the HL epithelium and its transport to the basolateral membranes of EBV-infected polarized Detroit^{sort} cells may be due to basolateral sorting of BMRF-2 that may, in turn, be critical for the cell-to-cell spread of progeny virions. Co-localization of BMRF-2 with β1 integrin at the basolateral membranes of EBV-infected epithelial cells suggests that the BMRF-2–integrin interaction may play an important role in the lateral spread of virus within the oral mucosal epithelium.

We have shown previously and in this report that BMRF-2 is detectable in gradient-purified EBV virions (Tugizov et al., 2003). Johannsen et al. (2004) also revealed BMRF-2 in gradient-purified EBV virions; however, it was at very low level and was detected at between 225 and 350 kDa. Complete separation of BMRF-2 was not possible on a SDS-PAGE gel and in urea gel systems. EBV BMRF-2 is a highly hydrophobic protein with multiple potential transmembrane domains; consequently, its full separation may not be possible. The actual amount of BMRF-2 protein in mature virions could be much greater than that detected in our studies and those of others (Johannsen et al., 2004). BMRF-2 was detected in Triton X-100 soluble fraction of purified virions, showing its association with the virion envelope. BMRF-2 also was detected by immunoelectron microscopy in the envelope of extracellular EBV in HL tissue (manuscript in preparation). Analysis of BMRF-2 in purified EBV virions under native, reducing and non-reducing conditions showed that it might be present in monomeric, dimeric and oligomeric forms in mature virions.

It has been shown that BMRF-2 is glycosylated by *O*-linked oligosaccharides in EBV-infected lymphoblastoid cell lines (Modrow et al., 1992). In this report, we also found that BMRF-2 is glycosylated by *O*-linked oligosaccharides in transfected cells as well as in purified EBV virions. The mobility shift after treatment with *O*-glycosidase was much greater than previously reported by Modrow et al. (1992). We also found substantial reductions in size after neuraminidase treatment alone and with a combination of neuraminidase and *O*-glycosydase. Treatment with neuraminidase alone resulted in a decrease in size of 5–10 kDa in both isoforms of BMRF-2, suggesting that sialylation contributes significantly to its post-translation processing and molecular weight. It is unclear whether sialylation modulates the functions of BMRF-2, as others have reported that it may or may not affect protein function (Arao et al., 1997; Pirie-Shepherd et al., 1995, 1997). Treatment with *O*-glycosydase alone primarily reduced the molecular size of the upper protein band, indicating that the heavier isoform is more glycosylated than the smaller one or carries no sialic acid. Treatment with both neuraminidase and *O*-glycosydase reduced the molecular mass to about 45 kDa, which is close to its predicted monomer size of 39 kDa. The reason for the differences between our findings and those of Modrow et al. (1992) is probably due to the different techniques used for sample preparation and protein separation. Detection of the fully glycosylated form of BMRF-2 in purified EBV virions suggests that only the fully processed form may be associated with the virion envelope.

BMRF-2 was efficiently transported to the cell surface of EBV-infected epithelial cell lines and lymphoblastoid cell lines, and its RGD motif was exposed on the surface of the plasma membranes. Recognition of BMRF-2 on the cell surface by EBV-specific human serum from nasopharyngeal cancer patients indicates that BMRF-2 may be a highly immunogenic target for the humoral immune response. Localization of the RGD domain of BMRF-2 on the surface of the virus envelope indicates its accessibility for interaction with the integrins of target cells. Because BMRF-2 in the virion envelope may be present as oligomers, their multiple RGD motifs may bind to integrins, leading to a strong binding of virions to the cell surface.

Previously we have shown that BMRF-2 RGD and purified EBV virions may bind to α5 and β1 integrins from polarized oral epithelial cells (Tugizov et al., 2003). Immunoelectron microscopy analysis of EBV virions in HL showed that the virion envelope contains BMRF-2 and β1 integrin (manuscript in preparation). Here, we have shown that purified EBV virions released from B95-8 cells contain β1, α5 and α3 integrins of host cells. Virions released from B95-8 cells were associated exclusively with the fully processed β1, α3 and α5 integrins, which are present only in the sorting compartments of the TGN and on the cell surface (Akiyama and Yamada, 1987; Hotchin and Watt, 1992; Kazui et al., 2000; Kim et al., 1992; Wang and Howell, 2000). This suggests that EBV association with integrins may occur within the TGN and/or at plasma membranes of B95-8 cells. Binding of GST-BMRF-2 RGD with β1, α3, α5, and αv integrins indicates that the virion association with integrins may be mediated via the BMRF-2 RGD motif. However, we cannot exclude a possible interaction of other EBV glycoproteins such as gB and gH with integrins. It has been shown that HCMV gB and gH bind to α2β1, α6β1 and αvβ3 integrins via non-RGD motifs (Feire et al., 2004; Wang et al., 2005). We have shown previously that antibodies to β1 and α5 integrins and soluble forms of α5β1 and α3β1 integrins inhibit EBV infection of polarized oral epithelial cells (Tugizov

et al., 2003), indicating that virion-associated β1, α3 and α5 integrins did not interfere virus binding to cell surface integrins.

The functional role of integrin when bound to EBV virions is not understood. It has been shown that integrins may directly interact with Fc receptors (FcR) (Kindzelskii et al., 2000; Lecoanet-Henchoz et al., 1995; Ortiz-Stern and Rosales, 2005; Petty et al., 2002; Van Spriel et al., 2002), and this interaction may lead to modulation of the immune response, such as lymphocyte proliferation, antibody and chemokine/cytokine secretion, antigen presentation and phagocytosis (Ortiz-Stern and Rosales, 2003). Thus, EBV-associated integrins may interact with the FcR of immune cells and thereby play a role in EBV pathogenesis in vivo.

In summary, our data show that EBV BMRF-2 is a virion-associated, highly hydrophobic viral glycoprotein with multiple potential functional properties and that BMRF-2 is the ligand for β1, α5, α3, and αv integrins. BMRF-2 interaction with these integrins may activate integrin and integrin-associated signaling pathways that may lead to regulation of virus attachment, entry and cell-to-cell spread.

Materials and methods

Cells and virus

EBV-permissive polarized human squamous tongue HSC-3^{sort} and pharyngeal Detroit^{sort} epithelial cell lines were established using a fluorescence-activated cell-sorting approach with UV-inactivated EBV virions (Tugizov et al., 2003). Cells were grown in Dulbecco's Modified Eagle's medium (DME) containing 10% fetal bovine serum (FBS) (HyClone), 200 mM L-glutamine, 0.1 mg/ml streptomycin and 100 μg/ml penicillin. A human embryonic kidney cell line (293T) and a retroviral packaging cell line (ProPak A.52) were purchased from American Type Culture Collection (ATCC, Manassas, Virginia). Cells were grown in DME containing 10% FBS (HyClone), 200 mM L-glutamine, 0.1 mg/ml streptomycin and 100 μg/ml penicillin.

The B95-8 EBV-producing marmoset B-lymphoblastoid cell line, the P3HR-1 human Burkitt's lymphoma cell line and the human EBV-negative BJAB Burkitt's lymphoma cell line were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. EBV replication was induced in B95-8 cells by adding 30 ng/ml 12-*O*-tetradecanoyl phorbol-13 acetate (PMA) and 4 mM butyric acid (both from Sigma) to the growth medium for 10 days. Virus-containing medium was clarified of cell debris by centrifugation at 5000 g for 15 min and supernatant filtered first through 0.8-μm pore-size and then through 0.45-μm pore-size filters (Millipore). Virus was concentrated by high-speed centrifugation at 16,000 round per minute for 90 min. The viral titer was determined by quantitative real-time PCR assay using EBV BZLF-1 gene-specific primers (forward 5′-AAA TTT AAG AGA TCC TCG TGT AA ACA TC-3′ reverse 5′-CGC CTC CTG TTG CCG CAG AT-3′) and fluorigenic probe (5′(6FAM)-ATA ATG GAG TCA ACA TCC AGG CTT GGG C (TAMRA)-3′) (Ryan et al., 2004). The beta-globin gene was used as a positive control (forward 5′-TGG CCA ATC TAC TCC CAG GA-3′, reverse 5′-CAT GGT GTC TGT TTG AGG TTG C-3′; fluorigenic probe 5′(6FAM)-CAG GGC TGG GCA TAA AAG TCA GGG C-(TAMRA)-3′). Q-PCR analysis was performed on an AB Prism 7900 detection system (Applied Biosystems). Thermocycling conditions were: 50 °C for 2 min, 95 °C for 10 min; 95 °C for 15 s and 60 °C for 1 min for 40 cycles. Each reaction contained 1× TaqMan® Universal Master Mix and with final concentrations of 5.5 mM $MgCl_2$, 200 μM dNTPs, forward and reverse primer (20 pmol each), TaqMan® probe (10 pmol) and 0.5 units Hotstart AmpliTaq® Gold (Applied Biosystems) in a 20-μl volume in a 384-well plate. Experimental samples were run in triplicate and the mean viral load was calculated. Every run had at least two "no template" controls to check for amplicon contamination. DNA from the Namalwa cell line (American Type Culture Collection) containing two copies of EBV per cell (Ryan et al., 2004) was used to generate standard curves.

Sucrose gradient-purified EBV virions (lot numbers: 106-099, 13B-0008) were purchased from ABI Biotechnologies (Columbia, Maryland). Released virus from B95-8 cells treated with 30 ng/ml PMA and 4 mM butyric acid was purified on a 3%–60% sucrose gradient. Virions were collected between 41% and 48% of the gradient and the purity of the virions was examined by electron microscopy.

EBV infection of polarized cells on permeable filter supports

To establish a polarized monolayer, the cells were cultured on 12-mm-diameter, 0.45-μm pore size, polycarbonate membrane Transwell filters (Costar Corp., Cambridge, Massachusetts), which were coated with mouse laminin (10 μg/cm^2) (Sigma) (Tugizov et al., 2003). Polarized HSC-3^{sort} tongue (between passages 15–20) and Detroit^{sort} pharyngeal (between passages 12–18) cells were infected with cell-free EBV at 1000 multiplicity of infection (MOI)/cell through their basolateral membranes. As only10% of virions may traverse filter pores (Tugizov et al., 2003), the real MOI will be about 100 virions/cell. The volume of virus inoculum was 250 μl. To allow virions to traverse the filter pores, cells were incubated at 37 °C for 1 h on a shaker, then washed with medium and maintained in DME containing 10% FBS.

HL tissue biopsies

Biopsies of HL tongue tissue containing epithelium and connective tissue were obtained using 4-mm-diameter biopsy punches from 3 HIV-positive individuals. Biopsies were done before highly active anti-retroviral therapy (HAART) was available, and the tissues were frozen and stored in the tissue bank of the Oral AIDS Center Clinic of the Department of Orofacial Sciences, University of California, San Francisco. The biopsy tissues were sectioned in slices 7 μm thick.

Antibodies

Polyclonal rat anti-BMRF-2 antibody designated as C2 was previously generated in our laboratory by immunizing rats with

a GST-BMRF-2 RGD fusion protein containing the RGD domain of the BMRF-2 protein (aa 171–218) (Tugizov et al., 2003). Monoclonal antibodies (mAbs) to EBV gp350/220 and VCA p160 were purchased from ABI. mAb to EBV BZLF-1 was obtained from Dako (Carpinteria, California). mAb HA.11 against influenza hemmaggutinin (HA) was purchased from Covance Research Products (Berkeley, California). EBV-specific human serum was a gift from Dr. Evelyn Lenett (Virolab Inc., Berkeley, California). Use of human sera was approved by the Committee on Human Research Review Board of the University of California, San Francisco (UCSF) (CHR approval #RS00908). GRP94, a rat mAb for staining the endoplasmic reticulum was purchased from NeoMarkers (Fremont, California). Rhodamine-Lens Culinaris Agglutinin for Golgi staining was purchased from Vector Laboratories Inc (Burlingame, California). Antibodies to β1 (MAB2000), α5 (AB1949), α3 (AB1920) and αv (AB1930) integrins were purchased from Chemicon (Temecula, California). All secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc (West Grove, Pennsylvania).

PCR cloning

The expression vector plasmid, LNCX, is a retroviral vector kindly provided by Dr. Michael Bishop at UCSF (Haddad and Hutt-Fletcher, 1989). The BMRF-2 gene of EBV was PCR-cloned from EBV genomic DNA of the B95-8 strain, either tagged with the influenza hemagglutin (HA) epitope (YPYDVPDYA) or with the enhanced green fluorescence protein (GFP). The following primers were synthesized by Qiagen/Operon Inc. (Valencia, California): BMRF-2–5′: ATGGAATTCTTTAACCACGCCTCCGAAGAG; BMRF-2–3′ha: AAGGGAGGCATAATCCGGCACATCATAAGGGTAACAGGATTTAATGAATGTTCACCAAC; BMRF-2–3′Bam: ATGGGATCCGGATTTAATGAATGTTCACCAAC; Gfp5′ bam: ATGGGATCCGGTGGAGGTATGGTGAGCAAGGGCGAGGAG); Gfp3′: ATGGAATTCTTACTTGTACAGCTCGTCCATG). To clone the HA-tagged BMRF-2, primers BMRF-2–5′ and BMRF-2–3′ha were used to amplify a product corresponding to nucleotides 80701–82188 of the EBV genome, which includes the coding sequence of BMRF-2 and its upstream promoter region. To clone BMRF-2 tagged with GFP, primers BMRF-2–5′ and BMRF-2–3′Bam were used to amplify the BMRF-2 gene from EBV and primers Gfp5′bam and Gfp3′ were used to amplify the GFP gene from plasmid pIRES2EGFP (Clonetech, Palo Alto, California). The orientation of insertions in these two constructs was determined by restriction digestion; two of each with the correct orientation were sequenced by MClab (South San Francisco, California) and the sequences were found to be identical to the published sequence. Expression of the BMRF-2 protein was confirmed by transient transfection followed by direct fluorescence microscopy for the BMRF-2 GFP fusion protein or by immunofluorescence staining using HA.11 (Convance) for the HA-tagged protein. To express the RGD domain of BMRF-2 (aa 172–218) as a GST-fusion protein, the RGD fragment was amplified by PCR using the following primers: BMRF-2RGD5′, ATGGGATCCTCTGGAAGCCTGGCCGGTGC; BMRF-2RGD3′, GTCTTCCCACGGGCATTTATG. The PCR products were cloned into pCR2.1 vector by Topo-cloning (Invitrogen) to give pCR2.1/BMRF-2 RGD. The cloned fragment was cleaved from pCR2.1/BMRF-2 RGD by restriction digestion with *Bam*HI and *Eco*RI and was gel-purified. The DNA fragment was ligated with pGEX2-T (Amersham) cut with the same restriction enzymes to create the GST-BMRF-2 RGD fusion plasmid.

DNA transfection and retroviral transduction

For transient expression of the BMRF-2 protein, plasmid DNA carrying the BMRF-2 gene was transfected into 293T human embryonic kidney cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. Twelve to 16 h before transfection, cells were seeded in 6-well plates or 10-cm Petri dishes to reach 80–90% confluence at the time of transfection. Twenty-four to 36 h post-transfection, cells were harvested for protein extraction or used for immunofluorescence staining or flow cytometry analysis. For stable expression of the BMRF-2 protein, the BMRF-2 gene was transfected into the retroviral packaging cell line ProPak A.52 as above for 48 h. Supernatant was collected, clarified by centrifugation and filtered with a 0.8-μM filter. The supernatant then was added to the cell monolayer (5 ml/T-25 flask) and the cells were incubated at 30 °C for 16–20 h. Cells were grown in normal growth medium for another 24 h before G418 selection (500 μg/ml) for 2–3 weeks. Neo-resistant colonies were then screened for BMRF-2 expression either by GFP expression (for the BMRF-2 fusion protein) or by immunostaining (for BMRF-2-HA tagged protein).

Confocal immunofluorescence and flow cytometry assays

For immunofluorescence assays, EBV-infected polarized HSC-3^{sort}, Detroit^{sort} cells, EBV-positive lymphoblastoid cells P3HR-1 and B95-8, and EBV-negative BJAB cells were washed with phosphate-buffered saline (PBS, pH 7.2), fixed in freshly prepared 4% paraformaldehyde and 2% sucrose for 5 min, and permeabilized with 0.01% Triton X-100 in 4% paraformaldehyde for 5 min. HL sections also were fixed as described above. To reduce background signals, they were incubated overnight with blocking buffer (3% bovine serum albumin (BSA) in PBS. Before incubation with primary antibodies, tissue sections were incubated with normal rat and mouse sera (Jackson Immunochemicals) for 1 h. The 293T cells transfected with BMRF-2 encoding sequence were fixed with methonal/acetone (50:50) for 30 min at −20 °C.

EBV-infected B lymphoblastoid cells and oral epithelial cells were immunostained with mAb to EBV BZLF-1, gp350/220 and rat antiserum (C2) to EBV BMRF-2. To examine BMRF-2 and BZLF-1 expression in HL tissue, sections were immunostained with rat anti-BMRF-2 and mouse mAb to BZLF-1. To detect HA-tagged BMRF-2 in 293T cells transfected with the BMRF-2 ORF, cells were immunostained with mAb HA.11. Cells were washed twice with PBS and incubated with FITC-labeled

appropriate anti-mouse secondary antibody (Jackson Immunoresearch) at 37 °C for 30 min. Cells were analyzed using a krypton–argon laser coupled with a Bio-Rad MRC2400 confocal head. The data were analyzed using Laser Sharp software.

For surface immunofluorescence assays, Detroit^{sort} cells infected with EBV on permeable filters were washed with cold PBS (pH 7.2), and fixed with fresh 3% paraformaldehyde on ice for 5 min (Tugizov et al., 1998). Fixed cells were incubated on ice with rat anti-BMRF-2 and mouse anti-β1 integrin antibodies applied from the apical or basolateral surface for 30 min. Cells were washed with cold PBS and permeabilized with 0.5% Triton X-100 (5 min), and incubated with secondary anti-rat and anti-mouse antibodies conjugated to FITC and Texas Red, respectively applied to both surfaces. To examine apical and basolateral staining, the polarized cells were scanned by confocal microscopy by obtaining *z*-sections in an *x*–*z* horizontal plane or in an *x*–*z* vertical plane.

For flow cytometry assays, EBV-infected or BMRF-2 transfected cells were dissociated with enzyme-free cell dissociation buffer (Difco), and incubated with primary antibodies in PBS (pH 7.4) containing 1% BSA for 1 h on ice. Cells were washed three times in PBS by centrifugation at 500×*g* for 3 min each and reacted with appropriate FITC-labeled secondary antibodies for 30 min. Cells were analyzed in a FACSCAN (Decton-Dickinson, San Jose, California).

Membrane protein extraction of BMRF-2-transfected 293T cells

The method used to extract membrane-enriched proteins was modified from Obermann et al. (2003). Confluent monolayers of BMRF-2 transfected or mock-transfected 293T cells in 10-cm dishes were washed once with PBS and harvested in PBS (1 ml/dish) by pipetting up and down several times. One-twentieth volume of a cocktail of protease inhibitors (Sigma-Aldrich, St. Louis, Missouri) was added to the cell suspension. Cells were homogenized 3 times for 30 s at 28,000 rpm using a Brinkmann Polytron PT3000 homogenizer (Brinkmann Instruments, Westbury, New York). The homogenates were centrifuged at 1000×*g* (IEC Centra GP8R centrifuge, International Equipment Company, Needham Heights, Massachusetts) for 10 min at 20 °C to remove nuclei and debris. Supernatants were centrifuged at 16,000×*g* in an Eppendorf centrifuge at 4 °C for 60 min and the supernatants were discarded. The membrane-enriched pellets were resuspended in buffer (50 μl/10^7 cells) containing 50 mM Tris–HCl, 0.15 M NaCl, 2 mM $CaCl_2$, 5 mM KCl, 5 mM $MgCl_2$, 4 mM EDTA, pH 7.5, with the same protease inhibitors and stored at −80 °C in 50-μl aliquots.

Western blot assays

For detection of BMRF-2, sucrose gradient-purified EBV virions (ABI Biotechnologies, Columbia, Maryland) were extracted with radioimmunoprecipitation assay (RIPA) lysis buffer containing nonionic detergents 1% NP-40, 1% NaDOC (sodium deoxycholate), 0.1% sodium dodecyl sulfate (SDS), Tris, pH 8.0, and a cocktail of protease inhibitors: phenylmethylsulfonylfluoride (PMSF) (1 mM), aprotinin (10 μg/ml), leupeptin (10 μg/ml) and pepstatin A (10 μg/ml). Viral proteins were separated on a 4–20% gradient Tris–glycine gel (Invitrogen) and transferred to polyvinyliden-difluoride (PVDF) membranes (Millipore, Eschborn, Germany). BMRF-2 and VCA p160 in virions were detected using rat antiserum to BMRF-2 and mouse mAb to p160, respectively. Protein bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce, Rockford, Illinois) according to the protocols provided by the manufacturer. To detect EBV virion-associated integrins, viral protein from gradient-purified virions was extracted using RIPA buffer and separated by 10% Tris–glycine gels (Invitrogen), and integrins were detected using appropriate anti-integrin antibodies.

For native gel electrophoresis, EBV proteins from gradient purified virus (ABI) were extracted with RIPA lysis buffer. Viral proteins then were divided into three tubes and treated as follows: with sample buffer without SDS, beta-mercaptoethanol and boiling (native); with sample buffer containing 2% SDS without beta-mercaptoethanol (non-reduced); and with sample buffer containing 2% SDS and 0.5% beta-mercaptoethanol (reduced). Each sample was loaded at 10 μg/lane onto a 7.5% native Tris–glycine gel. Running buffer consisted of 40 mM boric acid, pH-adjusted to 8.65 by adding solid Tris base; stacking gel containing 5% acrylamide/bis-acrylamide (16/1), 0.1% SDS and 50 mM Tris–HCl, pH 6.1; and resolving gel containing 7.5% acrylamide/bis-acrylamide (10/1), 0.1% SDS and 50 mM Tris–HCl, pH 9.18. Proteins were transferred to PVDF and BMRF-2 was detected with rat anti-BMRF-2 serum.

For urea gel electrophoresis, the membrane fraction of 293T cells transfected with the BMRF-2 gene was solubilized in urea sample buffer (7 M urea, 2 M thiourea, 1% TX100, 1% DTT, 4% chaps and 10 mM Tris, pH 9.5) at room temperature for 1 h. Samples were mixed with one-tenth volume of 1 M DTT and denatured at 70 °C for 10 min. Proteins were separated with 7 M of urea-SDS PAGE (Resolving gel: 10% polyacrylamide, 7 M urea, 0.2% SDS, 250 mM Tris, pH 9.0. Stacking gel: 4% polyacrylamide, 7 M urea, 0.2% SDS, 250 mM Tris, pH 6.8). Proteins were transferred to the PVDF membranes and BMRF-2 was detected with rat anti-BMRF-2 antibody.

Detection of BMRF-2 in the virion envelope

To separate EBV-envelope proteins from tegument/capsid proteins, extracellular virions released from B95-8 cells were double purified in Nycodenz (Sigma) gradient. B95-8 cells were treated with 30 ng/ml PMA and 4 mM butyric acid for 10 days, and media were collected and cleared from cell debris by centrifugation three times at 8000×*g* for 15 min. Supernatant was filtered through 0.45-μm pore-size filters. Virions were pelleted at 16,000 rpm for 1 h and resuspended in 1× TNB buffer (0.01 M Tris–HCl, pH 7.2, 0.15 M NaCl) with 100 μg/ml bacitracin (Sigma) in 1/100 of the original volume. The concentrated virus particles were centrifuged through a 24–42% Nycodenz (Sigma) step gradient at 21,000 rpm (70,000×*g*) for 1 h using a Sorvall TH641 rotor (Lake and Hutt-Fletcher,

2000). The virus band at the 24–26% interface was collected. The virions were then diluted with 1× PBS and pelleted at 19,000 rpm (60,000×*g*) for 2 h. The pellet was resuspended in 1× PBS and further purified through a 24–42% continuous Nycodenz gradient. After the second purification, virions were pelleted and extracted with RIPA buffer containing 1% Triton X-100, 0.5% deoxycholate (DOC) and a cocktail of protease inhibitors for 30 min at 37 °C. The viral extract was separated into supernatant containing envelope proteins and pellet containing tegument/capsid proteins by centrifugation at 100,000×*g* for 1 h (Zhu et al., 2005). Equal amounts of protein from the supernatant and pellet were separated in 4–20% Tris–glycine gel, and BMRF-2 and VCA p160 were detected using rat antiserum and mouse monoclonal antibody, respectively.

EBV BMRF-2 endoglycosidase treatment assay

Membrane proteins or gradient-purified EBV virions were treated with glycosidases as described by Tarentino et al. (Tarentino and Plummer, 1994). Briefly, 40–50 μg of proteins was denatured at 95 °C for 5 min in 0.5% SDS, 0.5% β-mercaptoethanol in 25 μl volume with 2.5 μl of protease inhibitor cocktail (Sigma-Aldrich). Samples were digested overnight in a 50-μl reaction with 3.3% (v/v) nonidet P-40, 2 mU (5 μl) of *O*-glycosidase or neuraminidase, or both, in 50 mM sodium phosphate, pH 5.0. Enzymes were inactivated before loading onto SDS-PAGE by mixing with equal volumes of 2× urea sample buffer (12 M urea, 4 M thiourea, 8% Chaps, 2% Triton X-100, 2% DTT, 40 mM Tris, pH9.5) and heating at 70 °C for 10 min.

GST pull-down assays

GST fusion proteins were purified using glutathione Sepharose 4B columns according to the protocol provided by the manufacturer (Amersham). Total cellular proteins were extracted with RIPA buffer and centrifuged at 14,000×*g* for 30 min at 4 °C. The supernatant was incubated with 20 μg/ml of GST-BMRF-2 RGD (amino acid 171–218) pre-bound to glutathione-sepharose beads at 4 °C and gently rocked for 1 h. The beads were washed three times with RIPA buffer, treated at 95 °C for 5 min with SDS sample buffer containing 5% β-mercaptoethanol and used for Western blot analysis.

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