Epstein–Barr virus BDLF2–BMRF2 complex affects cellular morphology

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Herpesvirus glycoproteins often form specific heterodimers that can fulfill functions that cannot be carried out by either of the partners acting alone. This study showed that interactions between the Epstein–Barr virus (EBV) multi-spanning transmembrane envelope protein BMRF2 and type II membrane protein BDLF2 influence the way in which these proteins are trafficked in the cell, and hence the subcellular compartment in which they accumulate. When expressed transiently in mammalian cells, BDLF2 accumulated in the endoplasmic reticulum (ER), whereas BMRF2 accumulated in the ER and Golgi apparatus. However, when the two proteins were co-expressed, BDLF2 was transported with BMRF2 to the Golgi apparatus and from there to the plasma membrane, where the proteins co-localized extensively. The distribution of the two proteins at the plasma membrane was reproducibly associated with dramatic changes in cellular morphology, including the formation of enlarged membrane protrusions and cellular processes whose adhesion extremities were organized by the actin cytoskeleton. A dominant-active form of the small GTPase RhoA was epistatic to this morphological phenotype, suggesting that RhoA is a central component of the signalling pathway that reorganizes the cytoskeleton in response to BDLF2–BMRF2. It was concluded that EBV produces a glycoprotein heterodimer that induces changes in cellular morphology through reorganization of the actin cytoskeleton and may facilitate virion spread between cells.

INTRODUCTION

The molecular mechanisms of Epstein–Barr virus (EBV) infection in vivo remain elusive, particularly primary infection of the oropharyngeal epithelium and cell-to-cell spread in permanently infected hosts (Hutt-Fletcher, 2007; Young & Rickinson, 2004). Recent research indicates that herpesvirus infections require the concerted action of several envelope glycoprotein complexes, some of which function as ligands for cellular receptors. Like other herpesviruses, EBV appears to have evolved sophisticated mechanisms in which these proteins are used to circumvent the host’s immune response or to steer the tropism of the virions towards specific cell types (Borza & Hutt-Fletcher, 2002). The number of known envelope glycoprotein ligands and corresponding host cell receptors has increased recently through the characterization of novel cooperative mechanisms (Borza & Hutt-Fletcher, 2002; Hutt-Fletcher, 2007). However, the EBV glycoproteins BMRF2 and BDLF2 remain poorly understood.

BMRF2 (Johannsen et al., 2004), encoded by rightward frame 2 located in the BamHI M region of the EBV genome (Cho et al., 1984), is an integral membrane glycoprotein with multiple predicted transmembrane domains (Modrow et al., 1992). BMRF2 is well conserved within the family Herpesviridae, but homologues in other gammaherpesviruses (e.g. ORF58 of Kaposi’s sarcoma-associated herpesvirus) have no known function (Majerciak et al., 2006). Tugizov et al. (2003) and Xiao et al. (2007a, b) recently showed that an arginine-glycine-aspartate (RGD) motif on the major extracellular domain of BMRF2 binds to cellular integrins, e.g. α5β1, at the basal membrane of polarized oropharyngeal epithelial cells. These authors concluded that BMRF2 is an important component of the protein machinery involved in the attachment of virions to the basolateral membrane of polarized epithelial cells, and
therefore probably plays an important role in primary infections.

BDLF2, encoded by leftward frame 2 located in the Banh1D region of the EBV genome (Cho et al., 1984), is described as a component of the EBV virion tegument (Johannsen et al., 2004). Hayes et al. (1999) reported strong BDLF2 expression in oral hairy leukoplakia but not in lymphoid malignancies. The authors proposed that bdlf2 is a late gene in the EBV lytic cycle in epithelial cells. May et al. (2005b) recently showed that ORF27, a distantly related BDLF2 homologue from murine herpesvirus 68 (MHV-68) is required for cell-to-cell spreading. However, the same authors revealed that ORF27 interacts with ORF58, the BMRF2 homologue, and this interaction influences their subcellular distribution (May et al., 2005b). It appears that the expression of ORF58 is sufficient to induce the export of ORF27 from the endoplasmic reticulum (ER) and transport of the non-covalent ORF58/ORF27 complex to the plasma membrane.

Here, we have described novel details of the interaction between EBV glycoproteins BMRF2 and BDLF2. We showed that their co-expression in different cell lines is sufficient to direct both proteins through the secretory pathway to the plasma membrane, and that the protein complex interacts with the actin cytoskeleton at the cell boundaries to alter the cell shape. The impact of our data on the mechanism of EBV infection and cell-to-cell spread is discussed.

**METHODS**

**Cell lines and cell culture.** We cultured 293T (ATCC CRL-11628), Cos7 (ATCC CRL-1651) and HeLa cells (DSMZ ACC 57) in Dulbecco’s modified Eagle’s medium/F12 without phenol red (Invitrogen) supplemented with 5 % fetal calf serum (Invitrogen) and 2 % penicillin/streptomycin (Invitrogen). The cells were cultured under standard conditions (37 °C and 2 % penicillin/streptomycin (Invitrogen). The cells were cultured under standard conditions (37 °C, 5 % CO₂, 90 % humidity) and subcultured a limited number of times to ensure reproducible cellular responsiveness and growth behaviour.

**Mammalian expression vectors.** The EBV bdlf2 open reading frame (genomic coordinates 131127–132389; GenBank accession no. V01555) was amplified by PCR (Hi-Fidelity kit; Roche Applied Science) using genomic DNA from the cell line B95-8 as template and specifically designed primers to engineer BanhI and Sall restrictions sites at the 5’ and 3’ ends, respectively. The amplified DNA product was inserted into pmCherry-C1 (Clontech) linearized with the same enzymes to produce expression vector pmCherry-BDLF2. Vector pEFGP-C2-BMRF2 was a kind gift from Dr Philip Stevenson (University of Cambridge, UK). Plasmids pCDNA3-EGFP-Cdc42-Q61L, -T17N and -wt; pCDNA3-EGFP-RhoA-Q63L, -T17N and -wt; and pCDNA3-EGFP-Rac1-Q61L, -T17N and -wt (Subauste et al., 2000), encoding dominant-active, dominant-negative and wild-type versions, respectively, of the three small GTPases RhoA, Cdc42 and Rac1, were purchased from Addgene. In the dominant-active mutants, the glutamine in position 61 or 63 was replaced by leucine (Q61L or Q63L) whereas in the dominant-negative mutants, the threonine in position 17 or 19 was replaced by asparagine (T17N or T19N).

**Cell transfections.** According to the cell line and doubling time, 6000–12 000 cells per well were seeded into μClear 96-well plates (Greiner BioOne) 1–2 days before transfection. Transfections were typically carried out 24 h after seeding, at 70–80 % cellular confluency. For each transfection, 100 ng vector was mixed with enough sheared salmon sperm DNA (Sigma-Aldrich) to obtain 2 μg total DNA in 100 μl medium without additives. This was mixed extensively with 3 μl Fugene HD (Roche Applied Science) in a 96-well plate and incubated for at least 20 min at room temperature to promote the formation of DNA–liposome complexes. Finally, 5 μl transfection mixture was added dropwise onto the cells in each well and swirled gently to distribute the transfection complexes homogeneously. The cells were incubated overnight under the cell culture conditions stated above, ensuring transfection efficiencies of 50–90 %.

293T cells attached weakly and were grown on supports coated with fibronectin. A stock solution of bovine fibronectin (1 mg ml⁻¹; Sigma-Aldrich) was diluted 1:100 in PBS, dispensed in aliquots of 50 or 200 μl per well into 96- or 24-well plates, respectively, and incubated for 1 h at room temperature to allow the fibronectin to bind to the plastic support. The solution was removed from the wells and replenished with 100 μl sterile PBS prior to seeding the cells.

**Fluorescence microscopy.** Fluorescence microscopy was carried out using a Zeiss LSM 510 inverted confocal microscope or the Opera system (PerkinElmer), a fully automated fluorescence confocal microscope for imaging cells grown in microtitre plates. With this system, the cells were imaged using a 10 × 0.42, 20 × 0.8 or 40 × 1.3 air objective at a focal height of ±3 μm with respect to the automatically selected focal plane above the bottom of each well. Images were acquired using exposure times of 250–2000 ms. In particular, multiplexing experiments were carried out exploiting the consecutive exposure modus of the Opera system, which eliminates ‘cross-talk’ effects at the different emission wavelengths. Live-cell imaging for end-point assays or kinetic time-lapse experiments was carried out under growth conditions controlled by the environmental control unit of the Opera system, which provided constant temperature, humidity and CO₂ supply. Image analysis was performed using Acapella, an integrated image analysis suite (PerkinElmer), or Image software (NIH), whereas image pseudo-colouring was carried out in Adobe Photoshop.

Immunofluorescence analysis was carried out on wild-type or transfected cells grown in sterile 96-well μClear plates (Greiner BioOne) using a standard protocol. Briefly, cells were fixed with 4 % paraformaldehyde in PBS prepared from a 16 % methanol-free stock solution (PolySciences) for 15 min at room temperature. The fixative was replaced with 0.1 % Triton X-100 in PBS. After extensive washing with PBS, the cells were blocked with 4 % paraformaldehyde in PBS and incubated with the cells at room temperature for 1 h followed by three PBS washes and 1 h incubation at room temperature with specific fluorescent-labelled secondary antibodies diluted 1:1000 in 3 % BSA in PBS. After a final washing step with PBS, the cells were imaged on the Opera system.

Filamentous actin (F-actin) was stained with Alexa Fluor 647-labelled phalloidin. Primary antibodies against the Golgi resident protein GM130 (BD Biosciences), the ER resident protein disulphide isomerase (PDJ) and β-tubulin (Sigma-Aldrich) were detected using pre-adsorbed specific secondary antibodies conjugated with Pacific Blue or Alexa Fluor 647 (Invitrogen).

**Fluorescent labelling of plasma membranes.** The cell plasma membrane was stained using the amphiphilic dye AM1-43 (Biotium), Blue or Alexa Fluor 647 (Invitrogen).
Fig. 1. Subcellular distribution of BDLF2 and BMRF2 in single transfection and co-transfection experiments. Cos7 cells were transfected with pEGFP-C2-BMRF2 (a) or pmCherry-BDLF2 (b) or co-transfected with both expression vectors (c). At 48 h p.t., the cells were fixed and stained by indirect immunofluorescence using primary antibodies specific for the Golgi marker GM130 and the ER marker PDI and fluorescently labelled secondary antibodies. In the merged images, co-localization is displayed in yellow (a, b, c), or in white or cyan (c). The images were acquired on a Zeiss LSM 510 inverted confocal microscope using a ×63 oil-immersion objective and were pseudo-coloured in Adobe Photoshop. For representation purposes, mCherry–BDLF2 in (a) is pseudo-coloured in green. Bars, 10 μm.
a fixable version of the FM1-43 dye whose green fluorescence increases in the hydrophobic environment of the plasma membrane. Briefly, the culture medium was replenished with 50 μl medium mixed with AM1-43 dye to a final concentration of 5 μg ml⁻¹. This solution was discarded after 10–30 s, depending on the cell line and cell density. After staining, the cells were washed with PBS and imaged directly.

Disruption of cytoskeleton components. The F-actin cytoskeleton was disrupted by incubating the cells for 1 h in pre-warmed medium supplemented with 1 μM latrunculin B (Sigma-Aldrich), whilst microtubules were disrupted by supplementing the culture medium with 50 μg nocodazole ml⁻¹ (Sigma-Aldrich). After incubation with the toxins, the cells were fixed and further processed for microscopic analysis as described above.

RESULTS

Analysis of the subcellular distribution of BDLF2 and BMRF2 expressed individually and together

The subcellular distribution of BMRF2 and BDLF2 was analysed by the transient expression of enhanced green fluorescent protein (EGFP)–BMRF2 and mCherry–BDLF2 in three mammalian cell lines (293T, Cos7 and HeLa). As shown for Cos7 cells in Fig. 1(a), confocal fluorescence microscopy carried out 24 and/or 48 h post-transfection (p.t.) revealed that BMRF2 expressed alone co-localized extensively with PDI, a disulphide isomerase chaperone resident in the ER lumen, and GM130, a peripheral membrane protein of the cis-Golgi network. This indicated that BMRF2 was trafficked from the ER to the Golgi apparatus. However, in some transfected HeLa cells, faint BMRF2 fluorescence was detected on very fine membrane protrusions (data not shown), suggesting differential trafficking and localization according to the cell line used for transfection. Transient expression of mCherry–BDLF2 showed that BDLF2 expressed alone localized to a fine intracellular meshwork reminiscent of the ER, largely overlapping with the distribution of PDI, as shown for Cos7 cells in Fig. 1(b). Co-staining the transfected Cos7 cells with antibodies against GM130 showed only minimal co-localization of BDLF2 with the Golgi marker (Fig. 1b). Cos7, 293T and HeLa cells were then co-transfected with pmCherry-BDLF2 and pEGFP-C2-BMRF2, and analysed as described above. The transfected cells showed anterograde transport of BDLF2 from the ER to the Golgi system, such that BDLF2 co-localized extensively with GM130 (Fig. 1c). There was also clear co-localization of BMRF2 and BDLF2 at the plasma membrane (Figs 2 and 3a). Fig. 2 shows that the two viral proteins co-localized extensively within vesicles that were found abundantly beneath the cell membrane and within extended cellular protrusions.

Co-expression of BDLF2 and BMRF2 leads to striking changes in cell morphology

A large proportion of the cells co-expressing BDLF2 and BMRF2 underwent dramatic morphological changes, manifesting as the appearance of multiple cytoplasmic protrusions, a significant increase in the number of lamellipodia and increased cell spread. The strongest effect was observed in Cos7 cells, where the morphological changes gave the cells a fissured appearance; the weakest effect was seen in HeLa cells. Fig. 3(a) shows representative morphological changes induced by the co-expression of BDLF2 and BMRF2 in the three cell lines. The same changes were not seen in Cos7, 293T and HeLa cells.
Fig. 3. Co-expression of BDLF2 and BMRF2 leads to striking changes in cell morphology. Cos7, 293T and HeLa cells were co-transfected with pEGFP-C2-BMRF2 and pmCherry-BDLF2, and 48 h later the cells were fixed and imaged. (a) BDLF2 (red) co-localizes with BMRF2 (green) at the plasma membrane. In the merged image, co-localization is shown in yellow. The white squares indicate the borders of the detailed images. The three cell lines were also transfected with pmCherry-C1 (red) and stained 24 h later with the AM1-43 membrane dye (green), washed twice and then imaged. (b) Time-lapse study of doubly transfected Cos7 cells between 24 and 48 h p.t. The nuclei of transfected and non-transfected cells were stained with Hoechst and pseudo-coloured in blue. Images were acquired by confocal microscopy on the Opera system using a ×40 air objective and were pseudo-coloured in Adobe Photoshop. Bars, 20 μm (main panels); 10 μm (detailed panels).
transfected with pmCherry-C1, a control plasmid encoding mCherry alone using the same promoter (Fig. 3a). Time-lapse studies were carried out to show how the cell morphology developed over time (Fig. 3b). The shape of the doubly transfected cells had already changed significantly by 24 h p.t. and the phenotype became more extreme over the subsequent 24 h, resulting from the uncontrolled extension of lamellipodia that could not be retracted and the formation of narrow processes where cells contacted each other, which tethered cells together irreversibly and became stretched and distorted when tethered cells moved apart (Fig. 3b).

**BDLF2–BMRF2-induced cell morphology depends on a viable actin skeleton**

The altered cell morphology induced by the co-expression of BDLF2 and BMRF2 bore a strong resemblance to rearrangements of the cytoskeleton. Therefore, we compared the organization of the two major cytoskeleton components, actin and tubulin, in co-transfected and control cells. F-actin was stained with fluorescent phalloidin, whereas tubulin was detected using an antibody against α-tubulin. Both F-actin and tubulin were found in the BDLF2–BMRF2-induced processes, F-actin at the plasma membrane and tubulin in the cytosolic interior (Fig. 4). Comparable structures were not observed in the plasma membrane of control cells. The disruption of F-actin with the depolymerizing agent latrunculin B (Fig. 5) extensively destroyed the sites of adhesion in wild-type as well as in BDLF2 and BMRF2 co-expressing cells. The latter showed a striking collapse of the elongated processes at the plasma membrane where actin and the two viral proteins co-localized. In contrast, the microtubule-depolymerizing drug nocodazole had only a limited effect on the phenotype of cells co-expressing the two viral genes (Fig. 5).

**Fig. 4.** F-actin but not tubulin localizes to cellular protrusions induced by BDLF2–BMRF2. Cos7 cells were co-transfected with pEGFP-C2-BMRF2 and pmCherry-BDLF2, and 48 h later the cells were fixed and then stained with Alexa Fluor 647-labelled phalloidin (pseudo-coloured in blue) or using an anti-α-tubulin antibody (blue) and imaged by confocal microscopy on the Opera system using a ×40 air objective. The images were pseudo-coloured in Adobe Photoshop. Bars, 20 μm (upper panels); 5 μm (lower panels).

**Dominant-active RhoA shows inhibitory effects on BDLF2–BMRF2-induced morphological changes**

Several families of cellular effectors regulate changes in the actin skeleton. To date, the best-characterized effectors are the small GTPases RhoA, Rac1 and Cdc42, which are key regulators in the formation of stress fibres, lamellipodia and filopodia, respectively. To investigate a possible involvement of these GTPases in the BDLF2–BMRF2-induced phenotype, expression plasmids encoding EGFP fusions to wild-type, dominant-active or dominant-negative versions of these effectors were co-transfected with the plasmids encoding BDLF2 and BMRF2. Transfected cells were analysed by confocal fluorescence microscopy as described above, to determine whether manipulating the activity of any of the small GTPases had any impact on the
Although a large number of filopodia-like protrusions were still observed in some cells, the co-expression of dominant-active RhoA (Q61L) with BDLF2 and BMRF2 effectively reversed the phenotype, causing the cell to contract (Fig. 6). Phalloidin staining of F-actin in these cells revealed a pronounced increase in the formation of stress fibres compared with control cells. Dominant-negative RhoA (T17N) did not alter the BDLF2–BMRF2-induced phenotype and phalloidin staining showed only a limited effect on the formation of stress fibres in these cells compared with wild-type cells (Fig. 6). The wild-type and mutant forms of the other GTPases had no significant effect on the phenotype induced by BDLF2 and BMRF2 (data not shown).

DISCUSSION

Recent research has shown that EBV primary infection and cell-to-cell spread involves a number of relatively uncharacterized viral glycoproteins in addition to those that have been studied in detail (Borza & Hutt-Fletcher, 2002; Hutt-Fletcher, 2007). To further our understanding of such functions, we co-expressed the EBV glycoproteins BMRF2 and BDLF2 and showed that co-expression influenced the trafficking of the proteins, allowing both to transit the secretory pathway and ultimately reach the plasma membrane. Thus, we have extended the findings of May et al. (2005a) and Gill et al. (2008), who demonstrated similar interactions between ORF27 and ORF58, the homologues of BMRF2 and BDLF2, in a murine gammaherpesvirus. Gill et al. (2008) reported the formation of ‘membrane fronds’ following interactions between ORF27 and ORF58. These fine protrusions are important for MHV-68 cell-to-cell spread, and ORF27 is thought to play a major role in this process (May et al., 2005b). Gill et al. (2008) also presented preliminary evidence for an interaction between BMRF2 and BDLF2, based on the observation that co-expression of the proteins generated a cellular phenotype similar to the one induced by co-expressing ORF27 and ORF58. However, they did not show the mutual transport of the two EBV proteins through the secretory pathway because only BMRF2 was labelled in their study – the homologous interactions between the pairs of proteins in the distantly related murine and human gammaherpesviruses were therefore inferred from the cellular phenotype alone. Recently, Gore & Hutt-Fletcher (2008) showed that BDLF2 is bound directly to and processed by BMRF2, resulting in co-localization at the plasma membrane.

In agreement with the inferences made by Gill et al. (2008) and the results presented by Gore & Hutt-Fletcher (2008), we demonstrated that, in a cellular context, the two EBV proteins interact to determine their trafficking and subcellular localization. As reported by Xiao et al. (2007b), we confirmed that BMRF2 is distributed within the ER and Golgi system when expressed alone and that, under the same circumstances, BDLF2 is restricted to the ER endomembrane system like its MHV-68 homologue, ORF27. Co-expression of the two proteins provided the first clear evidence for anterograde trafficking and co-transport of the two proteins within the same vesicles, and their strict co-localization within the Golgi system and at the cell membrane. However, in contrast with Gill et al.
(2008), we found that co-expression did not induce tiny membrane fronds but dramatic changes in cellular morphology involving the formation of thick cytoplasmic protrusions that were not seen in control transfections or non-transfected cells. Such morphological changes were not reported by Gore & Hutt-Fletcher (2008), perhaps because in their study a different sample preparation protocol was used which reduced preservation of the cellular morphology.

We also investigated the role of actin and tubulin in the formation of the cytoplasmic protrusions. Actin was found at the boundaries of the protrusions, whereas the cytosol within them was filled with tubulin. Similar structures were formed following ectopic expression of the pseudorabies virus US3 protein (Favoreel et al., 2005). By chemical perturbation, we confirmed the involvement of actin and showed that BMRF2 and BDLF2 strongly co-localized with actin.

Time-lapse microscopy of cells co-expressing BMRF2 and BDLF2 showed that the anomalous phenotype predominantly reflected uncoordinated spreading and detachment during cell migration, suggesting that adhesion and migration might be perturbed in these cells. As the three major Rho GTPases (RhoA, Rac1 and Cdc42) are central players in the regulation of cellular adhesion and migration (Nobes & Hall, 1995), we tested the involvement of these proteins in the altered phenotype. We found that dominant-active RhoA could reverse the morphological changes, whilst the wild-type and dominant-negative versions had no impact. This contrasts with the results of similar experiments on the homologous MHV-68 proteins, where the dominant-negative version of RhoA inhibited the appearance of membrane fronds (Gill et al., 2008). This discrepancy may reflect the evolutionary distance between BMRF2 and BDLF2 and their MHV-68 homologues, ORF58 and ORF27. The engagement of cellular integrin receptors with ligands displaying RGD motifs, such as extracellular matrix proteins, can repress RhoA activity locally (Arthur & Burridge, 2001; Arthur et al., 2000; Bass et al., 2008). Interestingly, BMRF2 exposes an RGD motif on the major extracellular loop, which is thought to mediate interactions with integrin receptors (Tugizov et al., 2003; Xiao et al., 2007a). It has been shown that abnormal integrin-mediated perturbation of RhoA function affects

**Fig. 6.** A dominant-active RhoA (Q61N) mutant eliminates the BDLF2–BMRF2-induced phenotype. Cos7 cells were co-transfected with pEGFP-C2–BMRF2, pmCherry–BDLF2 and a dominant-active (RhoA-DA; pcDNA3-EGFP–RhoA-Q63L) or dominant-negative (RhoA-DN; pcDNA3-EGFP–RhoA-T17N) mutant of RhoA. At 24 h p.t., the cells were fixed and stained with the F-actin-specific dye Alexa Fluor 647-labelled phalloidin and imaged by confocal microscopy. In control cells, the RhoA mutants are pseudo-coloured in red, whereas in transfected cells, the RhoA mutants and BMRF2 are pseudo-coloured in blue. BDLF2 is pseudo-coloured in red. Co-localization of the proteins is shown in magenta. In all panels, F-actin is pseudo-coloured in green. Images were acquired by confocal microscopy on the Opera system using a ×40 air objective and were pseudo-coloured in Adobe Photoshop. Bars, 20 μm.
cell polarity and promotes the formation of protrusions, with cells assuming a star-like branched phenotype similar to the one we observed for Cos7 cells (Worthylake & Burridge, 2003; Cox et al., 2001). Based on these findings, we believe that the morphology of cells co-expressing BMRF2 and BDLF2 reflects changes to the signalling machinery that coordinates RhoA activity during the processes of cell attachment and migration. Reduced RhoA activity stops focal adhesions at the rear of the cell being dismantled during migration (Worthylake et al., 2001), resulting in cellular projections that are occasionally left behind and tear from the cell body as we observed (data not shown). Interestingly, the morphology of our epithelial cell lines co-expressing BDLF2 and BMRF2 was very similar to that described by Sanderson et al. (1998) for cells infected with vaccinia virus (VV). As VV infection progresses, the coordinated expression of early and late viral genes induces the formation of elongated cellular processes as a result of uncoordinated dismantling of focal adhesions at the leading edge of several lamellipodia (Sanderson et al., 1998). Recently, using disarmed VV mutants, Valderrama et al. (2006) proposed that the morphology described by Sanderson and colleagues could largely be explained by the altered activity of RhoA signalling. A similar phenotype has been described for the swine pseudorabies virus by ectopic expression of the US3 ORF or by chemical inhibition of Rho kinase (Favoreel et al., 2005). Thus, distantly related viruses appear to exploit the interference with RhoA function as an ancestral strategy to affect the shape and migration of host cells.

Taken together, our results demonstrate the existence of an apparently well-conserved interaction among glycoproteins within the gammaherpesvirus family. We propose that BMRF2 and BDLF2 mutually influence their subcellular location by forming heteromeric complexes that travel through the secretory pathway to reach the plasma membrane, as implied by the biochemical characterization of the two proteins described by Gore & Hutt-Fletcher (2008). Furthermore, we speculate that the RGD motif exposed by BMRF2 engages integrin receptors (e.g. α5β1; Tugizov et al., 2003) en route to or at the membrane of the host or neighbouring cells, causing local inhibition of RhoA and unbalancing the signalling pathway that regulates cellular adhesion and migration, thereby inducing formation of extended cellular processes. As such cellular processes in alphaherpesviruses have often been reported to gate viral particles to surrounding cells (Favoreel et al., 2005, 2007; La Boissière et al., 2004; van Leeuwen et al., 2002), we believe that the BDLF2–BMRF2 complex is an as yet uncharacterized component of the EBV machinery that promotes cell-to-cell virus spread. However, the ectopic expression studies presented here allow only indirect conclusions regarding the role of BDLF2–BMRF2 in EBV-infected cells. Thus, to further our understanding of the infection process, it will be important to study this protein interaction as well as the interaction with host proteins in the context of virus infection.

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