K1 and K15 of Kaposi’s Sarcoma-Associated Herpesvirus Are Partial Functional Homologues of Latent Membrane Protein 2A of Epstein-Barr Virus

Lisa Steinbrück, Montse Gustems, Stephanie Medele, Thomas F. Schulz, Dominik Lutter, Wolfgang Hammerschmidt

Research Unit Gene Vectors, Helmholtz Zentrum München, German Research Center for Environmental Health and German Centre for Infection Research (DZIF) Partner Site, Munich, Germany; Institute of Virology, Hanover Medical School, Hanover, Germany; Institute of Diabetes and Obesity, Helmholtz Zentrum München, German Research Center for Environmental Health, Garching, Germany

ABSTRACT

The human herpesviruses Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV) are associated with Hodgkin’s lymphoma (HL) and Primary effusion lymphomas (PEL), respectively, which are B cell malignancies that originate from germinatal center B cells. PEL cells but also a quarter of EBV-positive HL tumor cells do not express the genuine B cell receptor (BCR), a situation incompatible with survival of normal B cells. EBV encodes LMP2A, one of EBV’s viral latent membrane proteins, which likely replaces the BCR’s survival signaling in HL. Whether KSHV encodes a viral BCR mimic that contributes to oncogenesis is not known because an experimental model of KSHV-mediated B cell transformation is lacking. We addressed this uncertainty with mutant EBVs encoding the KSHV genes K1 or K15 in lieu of LMP2A and infected primary BCR-negative (BCR−) human B cells with them. We confirmed that the survival of BCR− cells and their proliferation depended on an active LMP2A signal. Like LMP2A, the expression of K1 and K15 led to the survival of BCR− cells prone to apoptosis, supported their proliferation, and regulated a similar set of cellular target genes. K1 and K15 encoded proteins appear to have noncomplementing, redundant functions in this model, but our findings suggest that both KSHV proteins can replace LMP2A’s key activities contributing to the survival, activation and proliferation of BCR− PEL cells in vivo.

IMPORTANCE

Several herpesviruses encode oncogenes that are receptor-like proteins. Often, they are constitutively active providing important functions to the latently infected cells. LMP2A of Epstein-Barr virus (EBV) is such a receptor that mimics an activated B cell receptor, BCR. K1 and K15, related receptors of Kaposi’s sarcoma-associated herpesvirus (KSHV) expressed in virus-associated tumors, have less obvious functions. We found in infection experiments that both viral receptors of KSHV can replace LMP2A and deliver functions similar to the endogenous BCR. K1, K15, and LMP2A also control the expression of a related set of cellular genes in primary human B cells, the target cells of EBV and KSHV. The observed phenotypes, as well as the known characteristics of these genes, argue for their contributions to cellular survival, B cell activation, and proliferation. Our findings provide one possible explanation for the tumorigenicity of KSHV, which poses a severe problem in immunocompromised patients.
KSHV itself encodes a BCR mimic. The K1 and K15 KSHV proteins are likely candidates because they are transmembrane proteins with cytoplasmic domains, which could activate certain signaling pathways similar to EBV’s latent membrane proteins. For example, K1 encodes an ITAM similar to LMP2A but has a genomic location homologous with EBV’s LMP1 (see reference 16 for a recent review). K15, in turn, has a genomic location like that of LMP2A but lacks an ITAM and recruits signaling mediators such as LMP1 (17). In a recombinant herpesvirus saimiri chimera and in transgenic mice, K1 is oncogenic in vivo (18, 19). In addition, K1 protein downregulates BCR surface expression (20), whereas K15 blocks BCR-induced Ca2+-influx antagonizing BCR signaling (21) similar to LMP2A (22).

EBV infects quiescent primary human B cells, induces their proliferation, and establishes a latent infection in them, which emerge as growth-transformed lymphoblastoid cell lines (LCLs) in vitro. A comparable model is not available for the study of KSHV, which has been an obstacle to our understanding of KSHV-induced B cell lymphomas (23; see reference 24 for a recent review). We therefore introduced the K1 or K15 genes in lieu of LMP2A into mutant EBV strains and tested their phenotypes in infected primary human B cells in order to analyze the contribution of the KSHV genes to B cell growth transformation in a tractable experimental setting.

MATERIALS AND METHODS

Ethics statement. The human material used in the present study has been obtained in accordance with the Declaration of Helsinki, stems from anonymous healthy donors, and therefore does not require the approval of the board of the local ethics committee.

Isolation and separation of human primary B lymphocytes. Anonymous adenoid tissue samples from routine adenoidectomies were provided by the Department of Otorhinolaryngology, Klinikum Grosshadern, Ludwig Maximilians University of Munich, and Dritter Orden Clinic, Munich-Nymphenburg, Germany. Human primary B cells from adenoids were prepared as described previously (25). To isolate BCR- and BCR+ B cells, the cells were labeled with α-CD3-PE (ImmunoTools), α-α-FITC, and α-κ-APC light chain antibodies (Invitrogen) and sorted with a fluorescence-activated cell sorter (FACS) Aria III instrument (Becton Dickinson). BCR- B cells were defined as CD3+ and α- or κ- lymphocytes, and BCR+ B cells were defined as CD3+ and both λ- and κ- lymphocytes. BCR+ and BCR- lymphocytes are term ed λ/κ and λ/κ+, respectively, throughout the manuscript.

Cell lines and culture conditions. The B-cell line Raji and the EBV-infected patient and does not express a functional BCR. Primary B cells infected with EBV stocks were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 μg of streptomycin/ml, 100 U of penicillin/ml, 1 mM sodium pyruvate, 100 mM sodium selenite, 50 μM β-mercaptoethanol, 250 μM α-tocopherol, 10 μg of ciprofloxacin/ml, and 1 μg of cyclosporine/ml. Primary B cells infected with EBV were kept at a reduced oxygen level adjusted to 5%.

Construction of mutant EBV strains. EBV mutants were derived from p2089, which comprises the B95.8 EBV genome cloned onto an F-factor plasmid in Escherichia coli (29). p2089 was genetically modified in E. coli by homologous recombination with the galK-based recombineer system (30). The ΔLMP2A EBV (p2525) was described previously (4). The mutant EBVs that express the KSHV genes K1 and K15 were constructed essentially as described in detail recently (31, 32). In p4082 and p3998, the cDNAs of KSHV K1 and K15 P type were inserted in between nucleotide coordinates 166100 to 166458 and coordinates 166103 to 166458 of the B95.8 reference EBV genome, respectively, replacing the first exon of LMP2A in the p2089 maxi-EBV plasmid (Fig. 1A). The EBV plasmid DNAs were prepared from E. coli by two sequential rounds of CsCl-ethidium bromide density ultracentrifugation and carefully analyzed on agarose gels after cleavage with several restriction enzymes (Agel, BamHI, MluI, and XhoI). The modified loci and flanking regions were confirmed by extensive DNA sequencing in the E. coli derived EBV DNAs covering >6 kbp in each of the two maxi-EBV plasmids p3998 and p4082.

Generation and quantification of viral stocks. Viral stocks were generated in stably transfected single cell clones of HEE293 producer cells and quantified as described in detail (25, 31–33). Briefly, we scrutinized the virus concentrations of the four different virus stocks (Fig. 1B). All recombinant EBV’s used here were engineered to carry the egfp gene. Infectious units were defined with the aid of the Raji cell line, which is our model B cell for virus quantitation. Raji cells turn green fluorescent protein (GFP)-positive upon infection and allow the direct assessment of the concentration of infectious EBV virions as green Raji units (GRU) per milliliter of virus stock, as described earlier (34).

Quantitative reverse transcription-PCR (RT-PCR). RNA was isolated using the RNeasy Mini Kit (Qiagen) and digested with Dnase I (Invitrogen) prior to cDNA synthesis with the SuperScript III first-strand synthesis System (Invitrogen) and oligo(dT)20 as primers. cDNAs were quantified with a LightCycler 480 instrument and SYBR green I (Roche Diagnostics). The crossing point (Cp) values were corrected for primer efficiencies. Relative expression levels were assessed with CDC-like kinase 2 gene (Clk2) as housekeeping gene to calculate Cp(target)/Cp(refERENCE) ratios. The following primer pairs were used: LMP2A (5'-CGTCATCGACATGTAATCCAAA-3' and 5'-TACAGGCCGCACTGGCTGATG-3'), K1 (5'-GAGGATTTCCAAGGCGCTTA-3' and 5'-GGCATGATATCTCCAAAGTCGT-3'), K15 (5'-GGGCTCTAGGTGATGTTT-3' and 5'-GGATGAAGGCGATTTAGGAT-3'), and Clk2 (5'-CTGCAACATACGACCTCAACGCTG-3' and 5'-ACCAGCAACAATCAAGGATC-3').

Phospho-specific flow cytometry. 5 × 106 cells were washed in phosphate-buffered saline (PBS), pelleted, and recovered in 160 μl of PBS and 25 μg of goat α-hlgG/M/Antibody (Jackson Research)/ml for 15 min on ice. Signaling was induced at 37°C in a water bath for 10 min. A total of 160 μl of prewarmed BD Cytofix fixation buffer was added to the cell solution for 10 min at 37°C. The cells were pelleted, diluted in 30 μl of supernatant, permeabilized in 1 ml of ice-cold methanol for 30 min on ice, washed twice in Phosflow buffer (BD Biosciences), and stained with phospho-specific α-pSylk-Alexa 647 or α-pPLcy2-Alexa 647 antibodies (BD Biosciences) for 30 min at room temperature.

Western blot immunodetection. Cells were lysed in lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 2 mM EDTA, complete protease inhibitor [Roche]). Protein concentration was determined by Bradford analysis. All Western blots were performed according to standard protocols, using SDS-PAGE, nitrocellulose membranes (Hybond ECL; GE Healthcare, catalog no. RPNO3020D), 5% nonfat milk in PBS-Tween for blocking, and antibody dilutions and ECL Western blotting detection reagents (GE Healthcare, catalog no. RPNO2106). The following primary antibodies were used: rabbit anti-Syk (Cell Signaling, catalog no. 2712), rabbit anti-PLCγ2 (Cell Signaling, catalog no. 3872), and mouse anti-α-tubulin (Santa-Cruz, catalog no. 2304). The following secondary antibodies were used: anti-rabbit HRP-conjugated antibody (Cell Signaling, catalog no. 7074) and anti-mouse HRP-conjugated antibody (Cell Signaling, catalog no. 7076).

Calcium flux analysis. Cells were loaded with 3 μM Indo-1 AM (Invitrogen) at 37°C for 30 min, washed once, and diluted in 2 ml of RPMI 1640 on ice. A total of 106 cells in 200 μl were prewarmed at 37°C for 20 min, and the baseline violet/blue ratio of Indo-1-loaded cells was measured for 1 min on a FACS LSR II flow cytometer (Becton Dickinson). Subsequently, the BCR was cross-linked with 25 μg of goat α-hlgG/M Antibody (Jackson Research)/ml, the cells were quickly mixed, and the Ca2+-influx was measured based on the change in the violet/blue ratio over 5 min.
Cellular targets of LMP2A, K1, or K15. A total of 4 × 10⁶ B cells each were infected with the four virus stocks (wt EBV, K1 EBV, K15 EBV and ΔLMP2A EBV). On day 8 postinfection (p.i.), cells with characteristics of lymphoblastoid cells were identified by forward and side-ward scatter criteria and physically sorted on a FACS Aria III instrument (Becton Dickinson). Four biological replicates were conducted with B cells from four different donors. Cellular RNA samples were prepared with the RNeasy Mini Kit (Qiagen), and their quality was analyzed on an Agilent 2000 Bioanalyzer. RNA was converted to cDNA by using an Ambion WT expression kit, and the DNA was fragmented and labeled with an Affymetrix GeneChip WT terminal labeling and hybridization kit. Hybridization to GeneChip Human Gene 1.0 ST arrays (Affymetrix) and scanning of the arrays were performed according to the manufacturer’s protocol.

Microarray data preprocessing and bioinformatic analysis. Affymetrix CEL files were preprocessed using the R packages affy, huggingene10stranscriptcluster.db, and genefilter provided by bioconductor.org (35, 36). Microarray data sets were normalized using the robust multiarray average (RMA). Probe sets were filtered using the nsFilter (available with the R genefilter package). We performed a principal component analysis (PCA) for quality control and outlier detection (37). A PCA plot (see Fig. 6A) shows that the first two principal components cluster the data into biological interpretable groups. No experimental or technical outlier could be detected. To decompose...
EBV defined with the aid of these cells, which turn GFP-positive upon infection. Two EBV strains were engineered to carry the BCR in primary human B cells. LMP2A is encoded by nine exons and shares exons two to nine with B95.8 B cells. Among the 2089 EBV plasmid p2089 (29), which encompasses the entire genome of the B95.8 prototype EBV strain. To express K1 or K15 under the control of the LMP2A promoter (Fig. 1A), the first exon of LMP2A was replaced by the cDNAs coding for K1 or K15 P-type (45-kDa isoform) to yield K1 EBV (p4082) or K15 EBV (p3998), respectively.

Two EBV plasmids p4082 and p3998 were engineered in E. coli, and plasmid DNAs were purified, carefully scrutinized by restriction fragment analysis and extensive DNA sequencing, and stably introduced into HEK293 cells. Successfully transfected cells were selected, and clonal cell lines were established as described previously (31). The genetic composition of the EBV genomes in the different producer cell lines was carefully evaluated and confirmed by Southern blotting hybridization with total cellular DNA and appropriate EBV-specific probes. DNA sequencing of PCR-amplified EBV DNA from the producer cell lines covering all critical regions of the mutant EBVs confirmed the integrity of the mutated loci in EBV. The potential producer cell lines were tested for virus release upon transient transfection with two expression plasmids encoding the viral genes BZLF1 and BALF4 essentially as described previously (31).

Analysis of the concentration of viral stocks. We routinely analyzed the concentration of infectious virus particles in our viral stocks with the aid of Raji cells. All recombinant EBVs used here are engineered to carry the gfp gene, and infectious units are defined with the aid of these cells, which turn GFP-positive upon infection, allowing the direct assessment of the concentration of infectious EBV virions as green Raji units (GRU) per milliliter by FACS analysis as described previously (33, 34, 42). As shown in Fig. 1B, the concentration of infectious recombinant EBVs was determined by regression assays measuring the fraction of GFP-positive cells 3 days after infection. The Raji cell line is our preferred target cell to determine the concentration of recombinant EBV stocks, because Raji cells deliver consistent and very reproducible results in infection experiments with EBV. The definition of GRU was introduced about 15 years ago and has evolved as a standard approach in the field, but it underestimates the concentration of biologically active, infectious Epstein-Barr virions by at least a factor of 10 (see below).

K1 and K15 are expressed by the K1 EBV or K15 EBV mutants. We established lymphoblastoid cell lines (LCLs) with BCR+ B cells infected with the different viruses and measured the transcripts of LMP2A, K1, and K15 in these cell lines by quantitative RT-PCR. The K1 and K15 genes are expressed from the LMP2A promoter (Fig. 1A) and showed steady-state transcript levels, which were similar (K1 LCL) or lower (K15 LCL) compared to transcripts of LMP2A in LCLs infected with wt EBV (wt LCL) (Fig. 1C). We failed to detect K1 or K15 protein in the LCLs infected with either K1 EBV or K15 EBV in several attempts at immunoprecipitation and/or Western blotting, presumably because the available K1 and K15 specific antibodies are not sensitive enough to detect the two KSHV proteins. We therefore assumed that both KSHV proteins are expressed at low levels in our LCLs compared to KSHV-infected endothelial cells, which we used as positive controls.

Assessment of optimal B cell infections. In order to optimize the success of EBV infection in the experiments throughout the manuscript, we assessed the optimal ratio of EBV and human primary B lymphocytes. We infected primary B lymphocytes purified from different multiplicities of infection (MOIs) of a wt 2089 EBV stock and measured the absolute number of emerging B cells infected with the different viruses and measured the transcripts of LMP2A, K1, and K15 in these cell lines by quantitative RT-PCR. The K1 and K15 genes are expressed from the LMP2A promoter (Fig. 1A) and showed steady-state transcript levels, which were similar (K1 LCL) or lower (K15 LCL) compared to transcripts of LMP2A in LCLs infected with wt EBV (wt LCL) (Fig. 1C). We failed to detect K1 or K15 protein in the LCLs infected with either K1 EBV or K15 EBV in several attempts at immunoprecipitation and/or Western blotting, presumably because the available K1 and K15 specific antibodies are not sensitive enough to detect the two KSHV proteins. We therefore assumed that both KSHV proteins are expressed at low levels in our LCLs compared to KSHV-infected endothelial cells, which we used as positive controls.

K1 and K15 can partially replace LMP2A. BCR- B cells are proapoptotic because they lack the survival signals normally provided by the BCR. Upon infection, the wt 2089 EBV strain encoding LMP2A, which can substitute BCR’s functions, can rescue these cells from apoptosis, as reported previously by us (28) and with B95.8 EBV by others (39, 40). We postulated that the KSHV proteins K1 and K15 might also functionally replace LMP2A. In order to challenge this hypothesis, we established mutant EBVs encoding K1 or K15 in lieu of LMP2A (Fig. 1A).

Unsorted, BCR+ (CD3+ λ/κ/κ), or BCR- (CD3-, λ/κ/κ) primary B cells were infected with wt 2089 EBV, ΔLMP2A EBV, K1 EBV, or K15 EBV. The proliferation of unsorted or BCR+ B cells did not depend on LMP2A, K1, or K15 because the endogenous BCR provides the necessary survival signals in contrast to BCR- B cells (Fig. 3A). As expected, ΔLMP2A EBV-infected BCR- B cells did not proliferate (Fig. 3A, bottom panel) but presumably went into apoptosis, as reflected by a negative doubling time of 3.65 days (Fig. 3B). K1 EBV or K15 EBV-infected BCR- B cells were rescued from apoptosis with doubling times of 5.2 and 4.65 days, respectively, and longer than 2089 wt EBV-infected BCR- B cells (3.25 days). We concluded from this set of experiments that K1 and K15 could partially replace LMP2A’s function in this infection model.

Dual infection with K1 EBV and K15 EBV revealed no cooperativity of the two KSHV genes. The KSHV proteins K1 and K15 differ in their molecular design and in the cellular signaling mediators they recruit. It thus appears that the two proteins might serve different and yet additive or synergistic roles in B cells or in other cells infected with KSHV. We therefore sought to determine whether BCR- B cells dually infected with K1 EBV and K15 EBV might benefit from possible cooperative functions of K1 and K15.
FIG 2 Parameters and conditions optimally supporting EBV infection of primary B lymphocytes. (A) Determination of the number of proliferating, growth transformed cells after EBV infection. EBV-infected primary B lymphocytes increase in size and granularity to become B blasts, which can be detected in flow cytometry. The FSC (forward scatter) represents the size and the SSC (sideward scatter) represents the granularity of a cell. In contrast to noninfected, primary B cells, which exhibit low FSC/SSC signals, EBV-infected lymphoblasts locate to the “LCL gate” as indicated as early as 5 days p.i. In the experimental results shown, 6 × 10^5 adenoid B cells were infected with wt EBV (MOI = 0.1 GRU). At 1 day p.i., infected cells were supplied with fresh medium and divided into four fractions at a concentration of 1.5 × 10^5 cells/ml each. At 1, 5, 9, and 21 days p.i., one fraction was quantitatively analyzed by flow cytometry as indicated, and the total number of growth-transformed cells was calculated with the aid of a known number of APC beads, which provide a volume standard for sample normalization. The figure shows an example of a typical experiment with BCR^+ B cells infected with wt EBV. (B) Infection of primary B lymphocytes with different MOIs and cellular outgrowth over time. Unsorted B cells were infected with the indicated wt 2089 EBV. The different MOI values are designated in the figure. At the given time points, the absolute numbers of activated and proliferating cells in the LCL gate were determined by flow cytometry as in panel A. Three independent experiments are shown. (C) Titration of a wt EBV stock sample comparing the infectivity of Epstein-Barr virions in Raji versus EBV-negative Daudi cells. A total of 5 × 10^4 Raji cells or 2 × 10^5 Daudi cells were infected with the indicated volumes of a wt 2089 virus stock. Percentage of GFP-positive cells was assessed by FACS analysis at 3 days p.i. For comparison, the GFP values were adjusted to 10^5 cells total. Shown are the linear regressions and the coefficient of determination (R^2) derived from three independent experiments.
We chose to infect the primary B cells with virus stocks at concentrations that ensured doubly infected cells under optimal conditions. Two additional findings support the settings, which we chose for these experiments.

(i) We learned from quantitative PCR analysis that the experimental definition of GRU underestimates the concentration of physical particles by several logs (33). In addition, Raji cells are readily infected with EBV but less so than other cells. For example, EBV-negative Daudi cells (27) are far more permissive and better targets than are Raji cells. A careful titration of a wt 2089 EBV stock on both cell lines revealed that an infection of Raji cells underestimates the number of infectious Epstein-Barr virions present in the viral stocks by a factor of 10 (Fig. 2C). However, comparing Raji and EBV-negative Daudi cells also revealed that the latter are less consistent with respect to EBV infection, as indicated by a generally higher standard deviation of the slope of linear regression and slightly inferior goodness of fit. EBV-negative Daudi cells were reported to contain a variable fraction of apoptotic cells and are more difficult to maintain (27), which might explain our observation. Taken together, an MOI of 0.1 ensures that each B-lymphocyte is infected with at least one functionally intact Epstein-Barr virion on average.

(ii) Infections of B lymphocytes with an MOI of 0.25 were well tolerated and provided cell numbers very close to the optimal virus dose, i.e., an MOI of 0.1 (Fig. 2B), indicating that there is little viral interference if any. We therefore hypothesized that infection with two virus stocks with an MOI of 0.1 each is tolerated and should yield a major fraction of doubly infected primary B cells.

![Figure 3](http://jvi.asm.org/)

**FIG 3** The KSHV genes K1 and K15 partially rescue BCR<sup>−</sup> B cells from apoptosis. (A) Unsorted, sorted BCR<sup>+</sup> (CD<sup>3</sup>−, λ<sup>+</sup>/κ<sup>+</sup>) and BCR<sup>−</sup> (CD<sup>3</sup>−, λ<sup>−</sup>/κ<sup>−</sup>) B cells were infected with the indicated EBV strains. At the given time points, the absolute numbers of activated proliferating cells in the LCL gate was determined by flow cytometry (Fig. 2A). The means and standard deviations of three independent experiments are shown. (B) The numbers of BCR<sup>−</sup> cells (y axis, log<sub>2</sub> scale) in the LCL FACS gate after infection with four different EBV strains was plotted over time (x axis). The doubling time is the slope of the linear fit function.
lymphocytes on average according to the model of Gaussian normal distribution.

Therefore, we infected unsorted primary B cells, sorted BCR\(^{-}\) (CD3\(^{-}\), \(\lambda^{-}/\kappa^{-}\)), or BCR\(^{-}\) (CD3\(^{-}\), \(\lambda^{-}/\kappa^{-}\)) B cells with our four single virus stocks or with both K1 EBV and K15 EBV as indicated in Fig. 4. In three independent experiments with B cells from three different donors, the proliferation rate of BCR\(^{-}\) or BCR\(^{-}\) B cells singly or dually infected with K1 EBV and K15 EBV did not differ. This finding did not prove but did suggest that K1 and K15 have similar, noncomplementing functions. In this model and under our experimental conditions, K1 and K15 did not act additively or synergistically in infected primary human B cells. In contrast to similar infection experiments shown in Fig. 3, we had to compromise on the purity of sorted BCR\(^{-}\) primary B lymphocytes in order to obtain sufficient numbers of B cells to be used in the three experiments shown in Fig. 4. As a consequence, BCR\(^{-}\) (CD3\(^{-}\), \(\lambda^{-}/\kappa^{-}\)) B cells infected with ∆LMP2A EBV appear to be contaminated with a low fraction of BCR\(^{-}\) cells, which obscured the cellular phenotype reported earlier by us (4) and shown in Fig. 3A (bottom panel).

**K1 and K15 do not block BCR signaling.** K1 and K15 were reported to interfere with BCR signaling upon ectopic expression in B cells (20, 21, 41; see reference 16 for a recent review). The influence of LMP2A, K1, and K15 on BCR-induced phosphorylation of Syk and PLC\(_{\gamma2}\), two key signal mediators of BCR, as well as calcium influx, were investigated in established LCLs, which had been generated by infecting adenoid B cells with wt 2089 EBV, ∆LMP2A, K1, or K15 mutant EBV strains. The BCR of the LCLs was cross-linked for 10 min with an antibody directed against surface immunoglobulin or the cells were left untreated. The LCL clone A16, which is BCR negative and infected with wt EBV (28) was included as negative control. Intracellular pSyk and pPLC\(_{\gamma2}\) levels were detected with suitable antibodies as shown in Fig. 5A. In BCR\(^{-}\) LCLs infected with wt EBV, ∆LMP2A EBV, K1 EBV, or K15 EBV stocks, BCR cross-linking induced Syk and PLC\(_{\gamma2}\) phosphorylation, indicating that neither LMP2A nor K1 and K15

![FIG 4](http://jvi.asm.org/)
KSHV Membrane Proteins Mimic BCR Functions

A

wt (clone A16) (hlgM-,hlgG+)

wt (hlgM-,hlgG+)

ΔLMP2A (hlgM+,hlgG-)

K1 (hlgM+,hlgG-)

K15 (hlgM+,hlgG-)

% of Max

pSyk

x6.1

x6.3

x3.0

x3.3

x1.6

Calcium-flux

time (min)

C

B

wt (clone A16)

wt

ΔLMP2A

K1

K15

130KDa 1.0 0.9 0.9 1.0 0.8

PLCγ2 (150KDa)

70KDa 1.0 0.9 1.4 1.6 1.0

Syk (72KDa)

55KDa

Tubulin (50-55KDa)
inhibited phosphorylation of BCR signaling molecules. The BCR−
w.t EBV-infected LCLs (clone A16) did not show a significant in-
crease in Syk and PLCγ2 phosphorylation after antibody-mediated
BCR activation, as expected. In addition, the total levels of Syk and PLCγ2 in the five LCLs investigated in Fig. 5A were ana-
lyzed by Western blotting immunodetection revealing similar lev-
els (Fig. 5B). This finding indicates that the changes in the phos-
phorylation levels of Syk and PLCγ2 do not reflect differences in
the steady-state levels of these proteins.

Next, we analyzed calcium influx as a second function of BCR-
emanating signals in this model (Fig. 5C). BCR was cross-linked
with an α-hlgG/A/M F(ab)2 antibody fragment. The clone A16
LCL did not show an increase in cytoplasmic calcium levels after
antibody-mediated BCR cross-linking and served as a negative
control. LCLs infected with wt EBV but not ΔLMP2A EBV showed
impaired calcium mobilization after BCR cross-linking as re-
ported (22), but BCR cross-linking in LCLs generated with K1
EBV or K15 EBV induced calcium influx, suggesting that K1 and
K15 proteins did not block BCR-activated calcium signaling in
this model.

LMP2A, K1 and K15 control a related set of cellular genes in
newly infected B cells. Phenotypes of B cells infected with wt EBV,
K1 EBV, or K15 EBV were remarkably similar but differed from
ΔLMP2A EBV-infected cells in the experiments shown in Fig. 3
and 4. We hypothesized that LMP2A, together with K1 and K15
could regulate related sets of downstream cellular genes. There-
fore, we infected unsorted B cells with the four virus stocks, wt
EBV, K1 EBV, K15 EBV or ΔLMP2A EBV, adjusted to yield sim-
ilar rates of EBV-activated and growth-transformed B cells. At 8
days p.i. activated B cells in the lymphoblast gate were collected by
FACS sorting, and their RNAs were analyzed with the aid of the
Affymetrix GeneChip Human Gene 1.0 ST Array covering the
whole transcriptome of approximately 29,000 annotated human
genes. B cell preparations from four different donors were infected
and analyzed accordingly.

A standard principal component analysis (PCA) showed that
the measured gene expression profiles were highly biased by spe-
cific effects arising from the four individual donors of the B lym-
phocytes (Fig. 6A). Thus, we applied ICA (43) to the data, a
method to uncover processes that are hidden by superimpos-
ing processes. ICA is a general computational method that has
been further developed to disclose complex biological processes
and mechanisms from microarray data in models, which study
conditions of differentially expressed genes (43, 44). ICA is an
unsupervised, hypothesis-free approach capable of extracting mi-
croarray data to yield statistically independent components, e.g.,
genes that define novel biological processes or regulatory net-
works (45).

ICA can yield modes of gene expression indicative of the
underlying biological processes but it can also be applied to
group samples into functional categories identifying regulatory
networks (44, 46). In comparison to PCA, wherein the data are
transformed to linearly uncorrelated principal components, ICA searches for statistically independent sources. In brief and
as shown in Fig. 6B, in a matrix (X) of gene expression data
where each row corresponds to a sample and each column is the
expression profile of a gene, the goal is to find a matrix (S) of
source profiles that reflects the underlying biological processes.

Under the assumption that these processes are statistically in-
dependent in the first approximation and following a linear
mixing model, ICA can be used to find a mixing matrix (A)
such that X = AS (45, 47). The columns of the mixing matrix
form so-called feature profiles that display the contribution of
the corresponding source to the samples (Fig. 6C). A specific
source profile of interest can now be identified by comparing a
design vector reflecting a desired quality available from the
experimental design with the corresponding feature profile
(44).

Subsequent clustering, another type of unsupervised analysis,
groups genes that appear to contribute to the same independent
source profile assuming that these profiles represent biological
processes. Here, we were interested to identify the source profile
characteristic of ΔLMP2A EBV-infected primary B cells and the
set of genes that mainly reflects a differential response to the
LMP2A mediated signal. We therefore identified the feature pro-
file with the strongest correlation to our experimental setup (Fig.
6B and C).

It has been shown that meaningful biological information can
be deduced from source profiles by selecting its most active genes
(46). From the related source profile, we identified two clusters of
genes with a source expression below or above the mean plus 3.5
times the standard deviation (see Fig. 6C). A stringent threshold of
3.5 was chosen in order to obtain two clusters of about 50 genes
each.

The heat map in Fig. 7A shows cellular genes upregulated in B
cells infected with wt EBV, K1 EBV, or K15 EBV compared to
genes in ΔLMP2A EBV-infected cells. Vice versa, genes in the heat
map in Fig. 7B were upregulated in cells infected with ΔLMP2A
EBV. One-way hierarchical clustering of the 16 samples yielded
two dendrograms in Fig. 7A and B that clearly separated the four
samples infected with ΔLMP2A from the 12 samples with wt
EBV-, K1 EBV-, and K15 EBV-infected cells. Profiles of genes
regulated in K1 EBV- and K15 EBV-infected cells were very simi-
lar; their relations merely reflected individual, donor-specific
characteristics, as revealed by the indexed pairs in Fig. 7 (C1 and
D1 from donor 1, C2 and D2 from donor 2, etc.). Figure 7A and B
clearly indicated that K1- or K15-infected samples were more
closely related to wt EBV-infected cells but only distantly related to
ΔLMP2A-infected cells.

Box plots with the distributions of gene expression of the 16

FIG 5 The KSHV proteins K1 and K15 do not inhibit BCR signaling. LCLs were established by infection of primary B cells with wt EBV, ΔLMP2A EBV, K1 EBV, and K15 EBV. An LCL (clone 16) generated from infection with w.t 2089 EBV and unable to express a BCR was included as negative control. (A) The LCLs were cross-linked with 25 μg of α-hlgG/A/M antibody/ml for 10 min or left untreated. Intracellular phosphorylated Syk (pSyk) and PLCγ2 (pPLCγ2) were stained with phosphospecific antibodies. Histograms of flow cytometry analysis are shown. Numbers next to the graphs display the fold increases in the mean fluorescence intensities of the pSyk and pPLCγ2 signals after BCR stimulation. (B) Western blot immunodetection of the total levels of Syk and PLCγ2 in the LCLs used in panel A. Three independent experiments were carried out and Western blot bands corresponding to Syk and PLCγ2 were quantified and normalized against tubulin levels. The protein levels of clone A16 were set to one. (C) The LCLs were loaded with 3 μM Indo-1, and the violet/blue ratio of Indo-1 was measured over time. After 1 min of baseline measurement, the BCR was cross-linked with 25 μg of α-hlgG/A/M F(ab)2 fragment/ml.

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samples supported this interpretation and revealed a remarkable downregulation (Fig. 7C) or upregulation (Fig. 7D) of the expression of clustered genes in cells infected with ΔLMP2A EBV compared to wt EBV, K1 EBV, and K15 EBV.

We combined the two clusters and performed a one-way cluster analysis of ~100 cellular genes in the 16 samples shown in Fig. 7E. As in the heat maps in Fig. 7A and B, two major clusters could be deduced that encompass genes coinduced in wt EBV-, K1 EBV-, and K15 EBV-infected cells but downregulated in ΔLMP2A EBV-infected cells and vice versa (see the top and bottom halves of the heat map in Fig. 7, respectively). In essence, this finding again underscores that the expression of LMP2A, K1, or K15 activates...
similar pathways and probably executes related functions in this infection model with primary B lymphocytes.

**DISCUSSION**

Wild-type EBV but not ΔLMP2A EBV rescued infected BCR~GC~ B cells from apoptosis (4), indicating that an active LMP2A signal is required to support the survival of these B cells at the initial stage of infection. It is likely that LMP2A provides early antiapoptotic functions in conjunction with the two viral bcl-2 homologues, BHRF1 and BALF1 (48), expressed in the prelatent phase of EBV infection (49). The presumed concerted action of several viral genes dedicated to cellular survival suggests that infected cells have to cope with different proapoptotic signals, e.g., DNA damage response signals (50) and interferon induction (32), which the...
KSHV Membrane Proteins Mimic BCR Functions

virus must counteract for its success. LMP2A might complement the two redundant viral BCL-2 proteins (48) and induce cellular target genes (Fig. 7) that indirectly support B cell survival, as reported in transgenic mice (51) and Burkitt’s lymphoma cell lines expressing LMP2A (52).

The KSHV proteins K1 and K15 have been reported to have multiple functions depending on the host cell (see reference 16 for a recent review). For example, K1 and K15 were found to interfere with BCR signaling (20, 21, 41), but cross-linking of BCR \(^\pm\) LCLs infected with K1 EBV or K15 EBV induced calcium influx and phosphorylation of Syk and PLC\(\gamma\)2, indicating that K1 or K15 do not block BCR-emanating signals in our model (Fig. 5). However, we cannot discard the possibility that the EBV recombients expressed levels of K1 or K15 proteins sufficient to mimic a “trickling” BCR (53) but insufficient to antagonize BCR-dependent signaling, as observed after ectopic expression of K1 or K15 (20, 21, 41).

The functions of LMP2A, K1, and K15 were analyzed in two different approaches: we studied the survival and proliferation of infected BCR \(^\pm\) and BCR \(^\pm\) B cells over time and obtained gene expression profiles of unsorted, mainly BCR \(^\pm\) B cells shortly after infection. With both approaches we found that K1 and K15 activities are indistinguishable (Fig. 4 and 5) and closely related to phenotypes and functions of LMP2A (Fig. 7). Microarrays have become standard tools to provide insights into gene regulation and the underlying biological processes. Their biomathematical analysis and interpretation, however, is not standardized. Deliberately, we used ICA, an unsupervised, hypothesis-free approach, to analyze the microarray data sets from B cells of four individual donors infected with the four virus stocks. We wanted to challenge our initial observation in which K1 and K15 expression evoked comparable, yet noncomplementing phenotypes in BCR \(^\pm\) cells, replacing LMP2A at least partially (Fig. 3). Reassuringly, ICA in conjunction with subsequent hierarchical clustering, another unsupervised technique of data analysis, confirmed our initial observation (Fig. 7). The microarray data nicely recapitulated our findings in BCR \(^\pm\) B cells and identified two different groups: samples infected with wt EBV, K1 EBV, and K15 EBV and the group of four infected samples with \(\Delta\)LMP2A EBV (Fig. 7). Dendrograms reflect the relationship of the different samples and indicated that K1 and K15 samples are almost indistinguishable in this experimental setting but related to LMP2A \(^\pm\) and yet very distinct from LMP2A \(^\pm\) samples.

Interestingly, certain target genes of LMP2A, K1 and K15 shown in Fig. 7E underscore the functional contribution of this group of herpesviral oncogenes to B cell survival and activation. For example, CD80 is highly upregulated in wt EBV, moderately induced in K1 and K15 but downregulated in \(\Delta\)LMP2A EBV-infected B cells. This finding is further supported by CD86 surface expression measured by FACS that parallels expression measured by FACS that parallels expression measured by FACS that parallels

\(\Delta\)LMP2A EBV (54), K1, and K15, suggesting that the three encoded viral proteins uniformly contribute to B cell activation.

Similarly, PIK3R5, the regulatory subunit of phosphatidylinositol 3-kinase (PI3K), which itself is critically involved in LMP2A signaling (55, 56), is upregulated by LMP2A, K1, and K15 but downregulated if LMP2A is absent. This observation suggests that K1 and K15 indeed activate PI3K-dependent signaling pathways (57) as LMP2A does. B cell survival depends on PI3K-dependent signaling (58), which might explain the antiapoptotic effects of LMP2A, K1, and K15 proteins seen in our model.

**CARD16 and CASP1** encoding COP1 and caspase 1, respectively, are critically involved in activating apoptosis signals and are upregulated in \(\Delta\)LMP2A EBV-infected B cells but not in other samples, suggesting that LMP2A might prevent the initial upregulation of proapoptotic cellular regulators upon infection. The three selected examples only skim the data uncovered by our microarray analysis but suggest that our model is informative reflecting an important aspect of EBV’s and KSHV’s biology.

Models of B cell infection have been scarce and limit our knowledge of KSHV-induced B cell lymphomagenesis (see reference 24 for a recent review), although studies with activated primary cells from peripheral blood mononuclear cells or tonsils showed a 5 to 10% rate of KSHV infection (59–61). In this setting, KSHV supported the short-term proliferation of tonsillar B cells only, suggesting that this virus contributes to cellular survival (59). Both K1 and K15 are expressed in PELs and have been linked to the cellular transformation and/or maintenance of latent B cell infection with KSHV (16). It is not clear whether EBV or KSHV infect human BCR \(^\pm\) cells in vivo, but in vitro these cells only survive and proliferate, eventually, if infected with EBV strains expressing LMP2A, K1, or K15, thus supplying the proapoptotic B cells with a BCR-like survival signal.

Our findings suggest that K1 and K15 are instrumental in KSHV-associated B cell lymphoma and might explain the existence of BCR \(^\pm\) PEL cells (14, 15). K1 and K15 might also contribute to other malignancies, including Kaposi’s sarcoma or multicentric Castleman’s disease, because K1 is regularly expressed in biopsy specimens of these entities (62).

**ACKNOWLEDGMENTS**

We thank Dietmar Martin and Kerstin Maier for critical and important support with the microarray hybridizations and data acquisition, Christine Gobel for help and expertise in preparing, purifying, and infecting primary B cells, and Dagmar Pich for experimental advice and assistance during cell sorting experiments.

M.G. is an Alexander-von-Humboldt Fellow. Our work was supported by funds from the Deutsche Forschungsgemeinschaft (SPP1230, SFB1054/TP B05, SFB1064/TP A13, and SFB-TR36/TP A04), the National Institutes of Health (grant CA70723), and Deutsche Krebshilfe (grants 107277 and 109661).

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