The Latent Membrane Protein 1 (LMP1) Oncogene of Epstein-Barr Virus Can Simultaneously Induce and Inhibit Apoptosis in B Cells

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The latent membrane protein 1 (LMP1) of Epstein-Barr virus (EBV) regulates its own expression and the expression of human genes via its two functional moieties; the transmembrane domains of LMP1 are required to regulate its expression via the unfolded protein response (UPR) and autophagy in B cells, and the carboxy-terminal domain of LMP1 activates cellular signaling pathways that affect cellular proliferation and survival. An apparent anomaly in the complex regulation of the UPR and autophagy by LMP1 is that the induction of either pathway can lead to cellular death, yet neither EBV-infected B cells nor B cells expressing only LMP1 die. Thus, we sought to understand how B cells that express LMP1 survive. The transmembrane domains of LMP1 activated apoptosis in B cells, the apoptosis required the UPR, and the carboxy-terminal domain of LMP1 blocked this apoptosis. The expression of the mRNA of *Bcl2a1*, encoding an antiapoptotic homolog of BCL2, correlated directly with the expression of LMP1 in EBV-positive B-cell strains, and its expression inhibited the apoptosis induced by the transmembrane domains of LMP1. These findings illustrate how the carboxy-terminal domain of LMP1 supports survival of B cells in the presence of the deleterious effects of the complex regulation of this viral oncogene.

Proto-oncogenes and viral oncogenes tend to be highly regulated in their expression. The latent membrane protein 1 (LMP1) of Epstein-Barr virus (EBV) is a viral oncogene that regulates itself through a fascinating use of cellular pathways. The amino-terminal six-transmembrane domains (6TM) of LMP1 regulates its own synthesis and degradation cyclically in B cells through its activation of both the unfolded protein response (UPR) and autophagy (38–40). In cells expressing low levels of LMP1, the UPR induces the phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2 α) via protein kinase-like endoplasmic reticulum kinase (PERK) and enhances the translation of activating transcription factor 4 (ATF4) (40). ATF4, in turn, drives the transcription of LMP1 by binding to an ATF/cyclic AMP response element (CRE) element in the promoter of LMP1 (40, 62). Not only does the activation of the UPR via LMP1 block protein synthesis, but it also activates autophagy dose dependently, which facilitates the degradation of LMP1 (37, 39, 40). Autophagosomes are required for the degradation of LMP1, and lysosome inhibitors prevent this degradation (38, 39).

While the 6TM of LMP1 activates the UPR and autophagy, its carboxy-terminal domain drives the proliferation and survival of EBV-infected B cells *in vitro* and *in vivo* (16, 29, 55, 80). LMP1 activates the signaling pathways of nuclear factor- κ B (NF- κ B), activating protein 1 (AP-1), and signal transducer and activator of transcription (STAT), a trait shared with the human cluster of differentiation 40 (CD40) molecule (29). In fact, LMP1 can substitute for the signaling of CD40 in B cells (29, 55, 68).

The UPR is activated after the endoplasmic reticulum (ER) is stressed, such as when the ER is overloaded with unfolded proteins (58). This response is characterized by the upregulation of the chaperone protein, heat shock 70-kDa protein 5 (BiP), and activation of the signaling pathways of inositol-requiring enzyme 1 alpha (IRE1 α), PERK, and ATF6 (58). Proteases and chaperones are activated to degrade misfolded proteins, or fold them properly, respectively (58). However, the UPR induces apoptosis if homeostasis in the ER cannot be achieved (36, 64). For example, eIF2 α is dephosphorylated during the late stages of the UPR and

can translate proapoptotic proteins whose transcription has been induced by the UPR, such as the proapoptotic, B-cell leukemia lymphoma 2 (BCL2) homology 3 (BH3)-only proteins, BCL2 interacting mediator of cell death (BIM) and BH3 interacting death domain agonist (BID) (50, 64). The proapoptotic C/EBP homologous protein (CHOP) is translated during the UPR, promotes apoptosis late in the UPR, and represses the transcription of the antiapoptotic protein, BCL2 (36, 43, 46, 53). The changes in steady-state levels of anti- and proapoptotic proteins affect the integrity of the membrane of both the ER and mitochondria (31, 64–66). For example, localization of BCL2-antagonist/killer (BAK) and BCL2-associated X protein (BAX) to mitochondria is required for ER stress-initiated apoptosis (14, 59, 78, 79). Both at the ER and at the mitochondria, antiapoptotic BCL2 family members sequester BH3-only proteins and inhibit the activity of BAK and BAX (31, 64, 65). It therefore is the balance of proapoptotic (i.e., BCL2) and antiapoptotic (i.e., BAK, BH3-only proteins, and caspases) factors at both the ER and mitochondria that determine the fate of cells during ER stress.

Autophagy is mechanistically linked to the UPR and can counterbalance the expansion of the ER (5, 76). It is unclear whether autophagy is cytoprotective or cytotoxic (33, 69). During the UPR, it appears autophagy is cytoprotective since disrupting autophagy makes some cells more susceptible to apoptosis induced by the

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UPR (51). However, autophagy induces cell death independently of caspases in BAK^{-/-} and BAX^{-/-} mouse embryonic fibroblasts after the UPR is activated (60). Both Beclin1 and autophagy-related 5 homolog (ATG5), components of the basic autophagic machinery, affect apoptosis through autophagy-independent mechanisms (17, 77).

An apparent anomaly in the complex regulation of the expression of LMP1 is that both the UPR and autophagy can lead to apoptosis, and yet neither EBV-infected B cells nor B cells expressing only LMP1 at physiologic levels undergo apoptotic death. We examined how LMP1, in inducing the UPR and autophagy in B cells, blocks apoptosis. We have found that the 6TM of LMP1 does induce apoptosis via its activation of the UPR and that its carboxy-terminal signaling blocks this apoptosis. mRNAs that were differentially expressed in EBV-positive B cells with differing levels of LMP1 and encoding proteins that affect apoptosis were identified. One such transcript encodes an antiapoptotic homolog of BCL2, BCL2-related protein A1 (BCL2A1), whose expression contributes to the survival of lymphocytes and lymphomas (48, 52, 70). The transcription of *Bcl2a1* is activated by the signaling of both CD40 and LMP1 in EBV-negative cells (7, 19, 20). We determined that the expression of BCL2A1 inhibited apoptosis induced by the 6TM of LMP1.

MATERIALS AND METHODS

Cells and culturing conditions. 293T, HeLa, and H1299 cells were cultured in Dulbecco modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with L-glutamine, 10% (vol/vol) fetal bovine serum (FBS; HyClone, Logan, UT), and antibiotics (200 U of penicillin/ml and 200 mg of streptomycin/ml). HeLa are derived from a cervical carcinoma and H1299 from a non-small-cell lung carcinoma (25, 57). BJAB cells are a B-cell line derived from an EBV-negative Burkitt's lymphoma (47). BJAB-LMP1 and BJAB-6TM cells are B-cell clones derived from BJAB cells engineered to conditionally express hemagglutinin (HA)-tagged intact LMP1 (HA-LMP1) or the 6TM derivative of LMP1 (HA-6TM) and were generated as described previously (37). BJAB-6TM/Control (Ctl), BJAB-6TM/nerve growth factor receptor (NGFR)-LMP1, BJAB-6TM/BiP, and BJAB-6TM/BCL2A1 are all populations derived from BJAB-6TM cells. BJAB/Ctl and BJAB/NGFR-LMP1 cells are populations derived from BJAB cells. The EBV-positive B-cell strains 28-2 and 22-5 have been previously described (39). The ability of LMP1 to induce autophagy in these cells has also been previously described (39). The maxi-EBV used to generate both the 28-2 and 22-5 B-cell strains is derived from the 2089 derivative of the B95-8 strain of EBV. The maxi-EBV expresses enhanced green fluorescent protein (eGFP) constitutively and monomeric red fluorescent protein (mRFP) fused to the carboxy terminus of LMP1 (LMP1-mRFP) from the native promoter of LMP1. All B-cell lines were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Invitrogen) supplemented with L-glutamine, 10% FBS, and antibiotics (200 U of penicillin/ml and 200 µg of streptomycin/ml) (R10F). All cell lines were incubated in air containing 5% carbon dioxide (CO₂) at 37°C unless otherwise stated. BJAB-LMP1, BJAB-6TM, BJAB-6TM/Ctl, BJAB-6TM/BCL2A1, BJAB-6TM/BiP, and BJAB-6TM/NGFR-LMP1 were also supplemented with 1 µg of puromycin/ml for the maintenance of the tetracycline repressor-Krüppel-associated box fusion protein (tet-KRAB) and 1,000 µg of G418/ml to maintain the vectors encoding HA-LMP1 or HA-6TM. BJAB, BJAB-LMP1, and BJAB-6TM cells and its derived populations were grown in the presence of doxycycline (Dox) to induce the expression of either HA-LMP1 or HA-6TM. In the experiments reflected in Fig. 1, 2, and 6, a maximum of 1 ng of Dox/ml was used to induce either HA-LMP1 or HA-6TM. During the course of our studies, the stock solution of Dox had degraded. A fresh stock was prepared and 0.3 ng of Dox/ml induced the expression of the 6TM to

similar levels as was observed in Fig. 1, 2, and 6. This change is reflected in the experiments described in Fig. 3 and 7, where a maximum of 0.3 ng of Dox/ml was used. Tunicamycin (TUN) diluted in dimethyl sulfoxide (DMSO) to 0.3, 1, and 2.5 µg/ml was used to induce the UPR chemically in BJAB, BJAB/Ctl, and BJAB/NGFR-LMP1 cells.

Transfection of HeLa and H1299. HeLa and H1299 were plated in 10-cm dishes and grown to ca. 70% confluence. For each dish, 1 µg of a vector encoding eGFP, up to 3 µg of vector, pSG5, encoding the cDNA of LMP1, and pcDNA3.1 up to 4 µg of total DNA were diluted in 500 µl of Opti-MEM (Invitrogen). A 12-µl portion of Lipofectamine (Invitrogen) diluted in 500 µl of Opti-MEM was incubated for 5 min at room temperature and combined with the DNA, and the DNA-Lipofectamine complexes were incubated for 25 min at room temperature. Then, 4 ml of DMEM and 1 ml of transfection mixture were added to cells washed with 1× phosphate-buffered saline (PBS), followed by incubation for 4 h in air containing 5% CO₂ at 37°C. After the incubation, the medium was replaced with 10 ml of fresh DMEM supplemented with 10% (vol/vol) FBS. The efficiency of transfection was determined 24 h later by counting eGFP-positive cells.

Plasmid construction. A retrovirus that encoded the NGFR-LMP1 was constructed. The parent retrovirus (control [Ctl]), pCMMP-MCS-IRES-mRFP, p3313, is a modified Moloney murine leukemia virus plasmid. It was digested with MluI and XhoI and then ligated to an insert containing the sequence encoding NGFR-LMP1 (26). The insert containing NGFR-LMP1 was amplified via PCR with the forward primer (5'-AAG TAC GCG TTT CCA GAA GTA GTG AGG AGG C-3') and the reverse primer (5'-AGT CCT GAC TCG AGA AGC CTA TGA CAT GGT AAT GCC-3') from a vector kindly provided by Wolfgang Hammer-schmidt.

A retrovirus that encoded BiP was constructed. p3313 was digested with MfeI and XhoI and then ligated to an insert encoding BiP. The insert was amplified via PCR from vector, pOTB7, encoding BiP's cDNA (Open Biosystems, Huntsville, AL; clone 5020098, accession number [BC020235](#)), with the forward primer (5'-AAT GCA ATT GTG GCA GGA TGA AGC TCT CCC-3') and the reverse primer (5'-TGC TCT CGA GCC TAA CAA AAG TTC CTG AGT CC-3'). The amplicon was subsequently digested. During the amplification of BiP, a Kozak sequence (CACC) was inserted upstream of BiP's open reading frame.

To construct a retrovirus expressing BCL2A1 tagged with two copies of the myc epitope (2×-myc-BCL2A1), the parent retrovirus, p3313, was digested with MfeI. This product was ligated to cDNA that encoded the 2×-myc-BCL2A1, which was isolated by digestion with EcoRI from the vector, pcDNA3.1 plus 2×-myc-BCL2A1 (kindly provided by Celine Gélinas) (61).

Generation of BJAB-6TM and BJAB populations. Retroviral particles were generated by cotransfecting 293T cells (at 80% confluence) in a 10-cm plate with 3 µg of a vector encoding the Gag and Pol elements of the Moloney murine leukemia virus, 1 µg of a vector encoding NF-κB, 0.5 µg of a vector encoding the vesicular stomatitis virus G protein, and 10 µg of the retroviral backbone vector using 40 µg of polyethyleneimine (25,000 g/mol) in 5 ml of DMEM. Next, 5 ml of DMEM supplemented with 20% FBS was added after 4 h of incubation. After 24 h, 293T cells were irradiated with 30 Gy using a cesium-131 source. BJAB-6TM cells were transduced by plating cells at 10⁶ cells/ml in DMEM supplemented with 10% FBS on top of the irradiated 293T cells for 24 h. Afterward, BJAB-6TM cells were plated in R10F for 2 days and then screened for their expression of mRFP by fluorescence-activated cell sorting (FACS) on a FACS-Vantage SE with FACS-Diva option (BD Biosciences, San Jose, CA). The brightest 10% of mRFP-positive cells were used to generate populations of BJAB-6TM cells expressing the Ctl vector (p3313), 2×-myc-BCL2A1, BiP, or NGFR-LMP1. Also, BJAB/Ctl and BJAB/NGFR-LMP1 cells were generated in the same manner.

Activation of NGFR-LMP1 signaling. BJAB and BJAB-6TM cells engineered to express Ctl vector or NGFR-LMP1 were resuspended to 10⁷ cells/ml in R10F. The cells were then treated with 0.5 µg of unconjugated

FIG 1 6TM of LMP1 induced apoptosis in BJAB cells. (A) BJAB-6TM cells were treated for 2 days with increasing concentrations of doxycycline (Dox), and the expression of HA-6TM (open arrowhead) in BJAB-6TM cells was compared to that of HA-LMP1 (closed arrowhead) in BJAB-LMP1 cells via Western blotting. (B) BJAB, BJAB-LMP1, and BJAB-6TM cells were induced (On) or not induced (Off) with Dox to express the two forms of LMP1 at equal levels for 2 days and then assayed for cleaved PARP by Western blotting. Shown is a representative Western blot of three independent experiments. The expression of cleaved PARP was normalized to the levels of α -tubulin and is represented as the amount of PARP ($\pm$ the SD) relative to that in BJAB-6TM cells induced to express HA-6TM. (C) Caspase activity in induced or uninduced BJAB, BJAB-LMP1, and BJAB-6TM cells was measured as described in Materials and Methods. The activity of caspase-3 and -7 is represented as the RLU for each sample relative to that of BJAB cells cultured in the absence of Dox or uninduced BJAB-LMP1 or BJAB-6TM cells on day 1. Statistics were determined to identify significant changes in BJAB, BJAB-LMP1, and BJAB-6TM clones induced or not induced with Dox (*, $P < 0.05$ [Wilcoxon rank sum, two sided]).

mouse anti-NGFR antibody (Sigma-Aldrich, St. Louis, MO) per 10^6 cells. The cells were incubated at room temperature for 15 min, diluted to 5×10^5 cells/ml in R10F, and then treated with 2 μ g of goat anti-mouse IgG (KPL, Gaithersburg, MD) per 5×10^5 cells. Afterward, the cells were incubated in air containing 5% CO_2 at 37°C. Additional anti-NGFR antibody and goat anti-mouse IgG were supplemented as needed daily to accommodate proliferating cells.

Cell sorting of EBV-positive B cells for LMP1-mRFP. The EBV-positive B-cell strains 28-2 and 22-5 were sorted for their levels of LMP1-mRFP by first selecting for all eGFP-positive cells and then isolating single cells expressing the lowest 5% or highest 5% levels of mRFP or left unsorted. Single cells were sorted on a FACS-Vantage SE with FACS-Diva option.

RNA isolation. RNA was isolated using TRIzol reagent (Invitrogen) as previously described (13). Briefly, up to 10^7 cells were lysed in 1 ml of TRIzol reagent. Chloroform was added, and the lysed cells were shaken to denature the proteins. The solution was centrifuged to clarify the aqueous and organic layers. Total RNA was collected from the aqueous layer and then precipitated in isopropanol with the aid of linear acrylamide (Ambion, Austin, TX) as previously described (23). The precipitated, total RNA was washed twice with ice-cold 70% (vol/vol) ethanol. Afterward, the RNA was air-dried and resuspended in nuclease-free water.

Reverse transcription of mRNAs and microRNAs (miRNAs). Superscript II reverse transcriptase (Invitrogen) was used to reverse transcribe 1 μ g of total RNA into cDNA with an oligo(dT) primer at a final concentration of 5 μ M. Briefly, RNA, oligo(dT), and water to a volume of 12 μ l were incubated at 70°C for 10 min. The samples were cooled on ice. Then, 1 $\times$ first strand buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM

MgCl₂), 1 mM deoxynucleoside triphosphates (dNTP), 10 mM dithiothreitol, and 200 U of Superscript II reverse transcriptase were added to the samples in a final volume of 20 μ l. Samples were reverse transcribed at 42°C for 60 min. The reverse transcription of 2 $\times$ -myc-BCL2A1 was performed with gene-specific primers at a final concentration of 0.5 μ M for experiments that required its detection (see Table S1 in the supplemental material).

Human miRNAs were specifically reverse transcribed using the TaqMan MicroRNA reverse transcription kit (Applied Biosystems, Foster City, CA). Stem-loop primers were obtained from Applied Biosystems. For each miRNA assayed, 200 ng of total RNA was reverse transcribed in 1 $\times$ Multiscribe reverse transcription buffer supplemented with 1 mM dNTPs, 3.8 U of RNase inhibitor, and 50 U of Multiscribe reverse transcriptase (Applied Biosystems).

mRNA microarrays. Total RNA was hybridized to whole genome human microarrays (whole human genome kit, 4 $\times$ 44K features, 60-mer microarrays; Agilent, Foster City, CA) according to the manufacturer's instructions. A total of 150 ng of RNA was reverse transcribed with an oligo(dT)-T7 promoter primer into cDNA using an Agilent Quick-Amp Two-Color kit according to the manufacturer's protocol. The cDNA was then transcribed into cRNA containing CTP labeled with either Cy3 or Cy5 using an Agilent T7 RNA polymerase transcription kit. Equal masses of Cy3- and Cy5-labeled samples were cohybridized to microarrays for the detection of mRNAs and scanned with an Agilent G2505B DNA microarray scanner. Individual spots that were hybridized to mRNAs were evaluated, and the background for each was subtracted using local background subtraction. Arrays were normalized by locally weighted scatterplot smoothing (LOWESS) intraslide normalization (4). Microar-

FIG 2 The apoptosis induced upon the expression of the 6TM is not a gain-of-function. (A) BJAB-6TM/Ctl and BJAB-6TM/NGFR-LMP1 cells were induced (+) to express HA-6TM with 1 ng of Dox/ml or not (-). In addition, the carboxy-terminal signaling of LMP1 was activated (+) with anti-NGFR antibody or not (-). The cells were harvested on day 2 and assayed for their expression of HA-6TM and cleaved PARP, which was normalized to that of α -tubulin. Shown is a representative Western blot of three independent experiments. The expression of the HA-6TM is represented as the fold change in HA-6TM relative to that of uninduced BJAB-6TM/Ctl cells. The amount of cleaved PARP is represented as the fold change in cleaved PARP relative to its expression in BJAB-6TM/Ctl cells induced to express HA-6TM. (B) The activity of caspase-3 and -7 in BJAB-6TM/Ctl and BJAB-6TM/NGFR-LMP1 cells was measured and is represented as the RLU for each sample relative to uninduced BJAB-6TM/Ctl on day two. Statistics were determined to identify significant changes within populations induced or not to express HA-6TM (*, $P < 0.05$ [Wilcoxon rank sum, two sided]). Also, statistics were determined to identify significant changes between the BJAB-6TM/Ctl and BJAB-6TM/NGFR-LMP1 cells that were induced to express similar levels of the 6TM (#, $P < 0.05$ [Wilcoxon rank sum, two sided]).

rays were analyzed with EDGE3 software (71). Statistics were determined using a Student t test and revised-false-discovery-rate (rFDR) correction (3, 24). The expression of mRNAs in 28-2 cells that expressed the lowest 5% or highest 5% of LMP1-mRFP was compared to the expression of the mRNAs in a pooled sample of unsorted 28-2 cells from three independent experiments. The mRNA microarray data discussed here have been deposited in NCBI's Gene Expression Omnibus (1, 21) and are accessible through GEO series accession number [GSE33673](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33673) (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33673>).

miRNA microarrays. miRNAs were isolated along with total RNA and hybridized to microarrays for the detection of miRNAs. Briefly, miRNAs were mixed with a set of Spike-In miRNAs (Exiqon, Woburn, MA), treated with calf intestinal phosphatase, and then labeled with the fluorophores Hy3 and Hy5 (Exiqon). miRNAs labeled with either Hy3 and Hy5 were hybridized to the miRCURY locked nucleic acid microRNA arrays (v.11.0; Exiqon) and scanned on an Agilent G2505B DNA microarray scanner. Individual spots that were hybridized to miRNAs were evaluated, and the background for each was subtracted using local background subtraction (4). The arrays were normalized by LOWESS intraslide normalization. The expression of miRNAs in 28-2 cells that expressed the lowest 5% or highest 5% of LMP1-mRFP was compared to the expression of the miRNAs in a pooled sample of unsorted 28-2 cells from three independent experiments. The miRNA microarray data discussed in this publication has been deposited in NCBI's Gene Expression Omnibus (1, 21) and are accessible through GEO series accession number [GSE33972](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33972) (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33972>).

Real-time PCR. Reverse-transcribed cDNA was amplified and detected by real-time PCR under the following conditions: 1 $\times$ AmpliTaq Gold PCR master mix (Applied Biosystems), 0.5 μ M concentrations of each primer, 0.2 μ M probe, 1 $\times$ ROX reference dye (Invitrogen), and water to 20 μ l. The PCR cycling conditions were 50°C for 2 min and 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 60 s. The probes were labeled with fluorescein amidite (FAM) and either carboxytetramethylrhodamine (TAM) or Iowa Black FQ quencher (IABkFQ) at their 5' and 3' ends, respectively, and were purchased from IDT DNA (Coralville,

IA). Sequences for all primers and probes used are listed in Table S1 in the supplemental material. Measurements were made with the ABI Prism 7900 thermocycler and analyzed using SDS2.2.2 software (Applied Biosystems). Standard curves to determine the amplification factor of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), and *Bcl2a1* were generated by amplifying serial dilutions of their respective cDNA products (data not shown). cDNA of *Gapdh* and *Bcl2a1* was amplified 2.12- and 1.99-fold each cycle, respectively. All other cDNAs were assumed to amplify 1.8-fold each cycle. The expression of the mRNAs was determined in three steps. First, the cycle at which the amplification curve passed a threshold of detection (C_T) was calculated. Second, the C_T value of the amplified cDNA for each sample was compared to the C_T value of a control sample by the $\Delta\Delta C_T$ method (42). Finally, the expression of all reverse-transcribed and amplified mRNAs was normalized to the expression of the mRNA of *Gapdh*. The results are reported as the mean expression of the mRNA relative to a control sample. Error bars represent the standard deviation (SD) of the mean from at least three independent experiments.

The protocol to analyze the cDNA of miRNAs was similar to that described above with the following modifications: each 20- μ l PCR contained 1 μ l of 20 $\times$ primer-probe mix for each miRNA assayed (Applied Biosystems) and 1 $\times$ TaqMan Universal Master Mix, no UNG AmpErase (Applied Biosystems). Individual reactions were performed for each miRNA as previously reported (10). Probes were labeled with FAM and TAM on the 5' and 3' ends, respectively. The levels of the miRNAs were normalized to the human snoRNA, U38B.

Western blotting. The cells were harvested and resuspended in a 1:1 volume of 1 $\times$ NET (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA) and 2 $\times$ sample buffer (100 mM Tris [pH 6.8], 10% [wt/vol] sodium dodecyl sulfate [SDS], 10% [vol/vol] glycerol, 50 μ l of β -mercaptoethanol/ml, 0.04% [wt/vol] bromophenol blue). Lysates were sonicated to reduce the viscosity of the solution. Lysates were run on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel, electrically transferred to nitrocellulose, and blocked overnight at 4°C in BLOTTO (5% [wt/vol] nonfat milk, 0.075% [vol/vol] Tween 20, 1 $\times$ PBS) with the following

FIG 3 BiP partially rescued BJAB cells expressing the 6TM from apoptosis. (A) BJAB cells were treated with either DMSO or 2.5 μ g of tunicamycin (TUN)/ml for 24 h, and then phospho-eIF2 α , total eIF2 α , and α -tubulin were each measured by Western blotting. Shown is a representative immunoblot of three independent experiments. The ratio of phospho-eIF2 α -to-total eIF2 α was calculated and normalized to the levels of α -tubulin and is represented as the fold change in expression relative to that in DMSO-treated BJAB cells. (B) The activity of caspase-3 and -7 in DMSO- or TUN-treated BJAB cells was measured for 2 days and is represented as the number of RLU relative to that in DMSO-treated BJAB cells. Statistics were determined in panels A and B to identify significant changes between DMSO- and TUN-treated BJAB cells (*, $P < 0.05$ [Wilcoxon rank sum, two sided]). (C) BJAB-6TM/Ctl and BJAB-6TM/BiP cells were induced to express HA-6TM with 0.3 ng of Dox/ml (+) or not (-). The expression of HA-6TM, BiP, ATF4, and cleaved PARP was assayed and normalized to the levels of α -tubulin. The amount of HA-6TM, BiP, and ATF4 is represented as the fold change in expression relative to that in uninduced BJAB-6TM/Ctl cells. The amount of cleaved PARP is represented as the fold change in expression relative to BJAB-6TM/Ctl cells in which the expression of 6TM was induced. (D) Also, the activity of caspase-3 and -7 was measured in BJAB-6TM/Ctl and BJAB-6TM/BiP cells. Caspase activity is represented as the RLU normalized to the RLU in uninduced BJAB-6TM/Ctl assayed on day 2. Statistics were determined for the data in panels C and D to identify significant differences within a population (*) or between populations of cells treated with similar amounts of Dox (#, $P < 0.05$ [Wilcoxon rank sum, two sided]).

exceptions: (i) eIF2 α and phospho-eIF2 α were resolved by using SDS-12% PAGE, and (ii) Western blots used to detect 2 $\times$ -myc-BCL2A1 were incubated with the myc-antibody overnight rather than using BLOTTO. The blots were probed with primary antibody, followed by secondary antibody conjugated to alkaline phosphatase. Secondary antibodies included donkey anti-mouse at 1:1,000 (Jackson ImmunoResearch Laboratories, West Grove, PA) and goat anti-rabbit at 1:1000 (Jackson ImmunoResearch Laboratories). The bands were visualized using a solution of 0.13 mg of BCIP (5-bromo-4-chloro-3-indolylphosphate) *p*-toluidine salt/ml and 0.33 mg of nitroblue tetrazolium chloride/ml. Western blots were scanned and quantified using ImageQuant 5.2 software (GE Healthcare, Piscataway, NJ). The expression of each protein was normalized to the amount of α -tubulin. Experiments that were performed at least in triplicate were reported as the mean of the normalized expression $\pm$ the SD.

The primary antibodies included mouse monoclonal anti-LMP1 antibody at 1:500 (Dako, Carpinteria, CA; clone CS1.4), mouse monoclonal anti-HA antibody at 1:2,000 (Sigma-Aldrich), rabbit polyclonal anti-poly-ADP-ribose polymerase (PARP; p85) antibody at 1:1,000 (Promega, Madison, WI), rabbit polyclonal anti-CREB-2 (ATF4) antibody at 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA; clone C-20), mouse monoclonal anti-BiP antibody at 1:500 (BD Biosciences), mouse monoclonal anti-total eIF2 α (Invitrogen) at 1:2,000, rabbit polyclonal anti-phosphorylated eIF2 α (Invitrogen) at 1:500, unmodified mouse monoclonal anti-myc antibody (clone 9B11; Cell Signaling Technology, Beverly, MA) at 1:1,000, and mouse monoclonal anti- α -tubulin antibody (Sigma-Aldrich) at 1:10,000.

CaspaseGLO 3/7 assay. Apoptosis was measured with the CaspaseGLO 3/7 assay (Promega). The CaspaseGLO 3/7 assay measures activity of

caspase-3 and -7 using light as a surrogate marker. Briefly, total cells were lysed and incubated for 1 h in the presence of luciferase, magnesium ion, and aminoluciferin covalently bound to the Asp-Glu-Val-Asp (DEVD) amino acid sequence. A total of 500 BJAB, BJAB-LMP1, and BJAB-6TM cells and their derivatives were assayed. Active caspase-3 and -7 cleave after the second aspartyl residue, releasing the aminoluciferin, a substrate of luciferase. Relative light units (RLU) were measured using a Monolight 3010 luminometer (BD Biosciences). The results are reported as the mean normalized RLU from at least three independent experiments. Error bars represent the SDs of means.

Statistical analysis. Statistical tests (Wilcoxon rank sum) were performed using Mstat 5.01 provided by Norman Drinkwater (18).

RESULTS

The 6TM of LMP1, but not intact LMP1, induced apoptosis in the EBV-negative B-cell line BJAB. The 6TM of LMP1 dose dependently induces the UPR and autophagy in B cells (37, 39, 40). LMP1 uses these pathways to regulate its synthesis and degradation in EBV-positive B cells. However, B cells do not die when they express intact LMP1, although their growth can be slowed (34, 37). These observations are striking given that the UPR and autophagy can activate pathways of programmed cell death. We sought to determine how LMP1 could regulate its expression in this complex manner without causing B cells to die by apoptosis. BJAB cells were engineered to express conditionally either the 6TM of LMP1 (BJAB-6TM) or intact LMP1 (BJAB-LMP1) tagged

FIG 4 The carboxy-terminal signaling of LMP1 inhibited apoptosis induced with tunicamycin (TUN). BJAB cells were transduced with a control vector (BJAB/Ctl) or a vector that expressed NGFR-LMP1 constitutively (BJAB/NGFR-LMP1). These cells were tested for their apoptotic response to increasing concentrations of TUN. BJAB/NGFR-LMP1 cells were additionally treated with anti-NGFR antibody to activate the carboxy-terminal signaling of LMP1. (A) The activity of caspase-3 and -7 was measured via luminescence, and this activity is reported as the RLU relative to BJAB/Ctl cells cultured in the absence of TUN. (B) Cleaved PARP was measured in the two cell populations via Western blotting, and its levels were normalized to the expression of α -tubulin. The ratio of cleaved PARP to α -tubulin is represented as the fold change relative to BJAB/Ctl cells treated with 1 μ g of TUN/ml. Statistics ($P < 0.05$ [Wilcoxon rank sum, two-sided]) were determined to identify significant changes in caspase activity and cleaved PARP within the populations treated with differing concentrations of TUN (*) or between populations treated with the same concentration of TUN (#).

with HA (37). HA-LMP1 and HA-6TM were expressed to similar levels in these cell clones induced with Dox (Fig. 1A).

These cell clones were used to study the mechanisms by which B cells survive the UPR and autophagy induced by LMP1. BJAB-LMP1 and BJAB-6TM were grown with or without Dox to induce or not induce, respectively, the expression of the variants of LMP1 (Fig. 1A). Apoptosis, as reflected via caspase activity, was quantified in two ways. First, cleaved PARP, a product of proteolysis by caspases, was measured via Western blotting (Fig. 1B). Second, the cleavage of a luminescent substrate of activated caspase-3 and -7 was measured (Fig. 1C). Apoptosis was detected as early as 2 days after inducing the expression of 6TM. The amount of cleaved PARP in induced BJAB-6TM cells was 6-fold greater than in uninduced cells (Fig. 1B). Similarly, induced BJAB-6TM cells had 8- and 33-fold more caspase activity after 2 and 3 days, respectively, than uninduced cells (Fig. 1C). In contrast, intact LMP1 did not cause apoptosis of BJAB cells. These findings indicated one or more of the functions of the 6TM (aggregation for signaling, localization to lipid rafts of Golgi, or the induction of the UPR and autophagy), but not intact LMP1, induced apoptosis (39–41).

The apoptosis induced by the 6TM is not a gain-of-function but is masked by the signaling of the carboxy-terminal domain of LMP1. Unlike the 6TM, the expression of intact LMP1 did not activate apoptosis in BJAB cells (Fig. 1B and C). We hypothesized that signaling from the carboxy-terminal tail of LMP1 prevented apoptosis that was activated by the 6TM. Alternatively, it was possible that the 6TM alone had gained an ability not present in intact LMP1. To determine whether the apoptosis caused by the 6TM was a gain-of-function mutation, BJAB-6TM cells were engineered to express a derivative of LMP1, NGFR-LMP1, constitutively (BJAB-6TM/NGFR-LMP1). NGFR-LMP1 contains the amino terminus of NGFR fused to the carboxy terminus of LMP1, and the carboxy-terminal signaling of LMP1 is conditionally activated when anti-NGFR antibody cross-links the NGFR moieties (26, 27). This construct can replace the signaling of intact LMP1, drives proliferation, and inhibits apoptosis in EBV-positive B-cell strains (16, 26). In addition, we engineered BJAB-6TM cells to express the parental vector, pCMMP-MCS-IRES-mRFP, as a negative control (BJAB-6TM/Ctl). The expression of the NGFR-

LMP1 in BJAB cells was confirmed to be similar to that of intact LMP1 by Western blotting (data not shown).

BJAB-6TM/Ctl and BJAB-6TM/NGFR-LMP1 cells were induced with Dox to express similar levels of HA-6TM (Fig. 2A). Activation of the carboxy-terminal signaling of LMP1 rescued BJAB cells induced to express HA-6TM from apoptosis (Fig. 2). Similarly to BJAB-6TM cells, apoptosis was observed within 2 days in BJAB-6TM/Ctl cells induced to express HA-6TM (Fig. 2). The apoptosis increased and on day 4 was 60-fold greater in induced BJAB-6TM/Ctl cells than in uninduced cells (Fig. 2B). Signaling from the carboxy terminus of LMP1 inhibited caspase activity by 84% each day and reduced the amount of detectable cleaved PARP by 72% (Fig. 2). Collectively, these data indicated that apoptosis activated by the 6TM of LMP1 was not a gain-of-function and that the carboxy-terminal signaling of LMP1 inhibited the apoptotic phenotype induced by the 6TM.

The apoptosis activated by the 6TM was induced, at least in part, by the UPR. It seemed likely that the 6TM of LMP1 caused apoptosis via its activation of the UPR and of autophagy. In order to test this likelihood, it was necessary to ascertain whether the UPR could cause apoptosis in BJAB cells or not. We sought to induce the UPR chemically with tunicamycin, which causes ER stress (22). BJAB cells were treated with tunicamycin, or its carrier, DMSO, for 2 days. The increased ratio of phospho-eIF2 α to total eIF2 α in BJAB cells treated with tunicamycin confirmed that the UPR was induced (Fig. 3A). After 1 and 2 days, caspase activity was 12- and 28-fold greater, respectively, in BJAB cells treated with tunicamycin than with DMSO (Fig. 3B). This finding indicated that the UPR was a candidate for inducing apoptosis.

We next sought to determine whether inhibiting the UPR would block the apoptosis induced by the 6TM of LMP1. To do so, the chaperone protein, BiP, was expressed exogenously in BJAB-6TM (BJAB-6TM/BiP) cells. The dissociation of BiP from PERK, IRE1 α , and ATF6 activates the UPR (58). The level of the introduced BiP was ~2-fold more than was endogenously expressed in BJAB-6TM/Ctl cells (Fig. 3C). The ability of BiP to repress the UPR was measured via the translation of ATF4, which was decreased by 30% in the presence of exogenous BiP (Fig. 3C). After 2 days of expressing the 6TM, the level of cleaved PARP was de-

FIG 5 The expression of LMP1-mRFP correlated with the differential expression of mRNAs encoding proteins that regulate apoptosis. (A) The EBV-positive B-cell strain 28-2 was sorted by flow cytometry for the 5% of cells expressing the lowest (Low 5%) or highest (High 5%) levels of LMP1-mRFP. Monomers of

creased by 60% in the presence of exogenous BiP (Fig. 3C). Similarly, BiP inhibited the activity of caspase-3 and -7 by 25% after 2, 3, and 4 days of inducing the expression of the 6TM (Fig. 3D). These data show that repressing the UPR blocked the apoptosis induced by the 6TM of LMP1.

The carboxy-terminal signaling of LMP1 inhibited apoptosis induced by the UPR in BJAB cells. That the carboxy-terminal domain of LMP1 can inhibit apoptosis and its 6TM can activate apoptosis via the UPR led us to predict that the carboxy terminus could inhibit apoptosis induced by the UPR when activated independently of the 6TM. To test this prediction, BJAB cells were engineered to express NGFR-LMP1 (BJAB/NGFR-LMP1) or not (BJAB/Ctl), and apoptosis was measured during their treatment with increasing concentrations of tunicamycin. Tunicamycin induced the UPR to cause apoptosis in BJAB cells, and yet the caspase activity was greatly inhibited by the carboxy-terminal signaling of LMP1 (Fig. 4A). Even when BJAB cells were treated with the highest amount of tunicamycin (1 μ g/ml), the carboxy-terminal signaling of LMP1 inhibited the UPR-induced apoptosis by 90%. Similarly, the cleavage of PARP was inhibited by 90% when the carboxy-terminal signaling of LMP1 was activated in tunicamycin-treated BJAB cells (Fig. 4B). These findings substantiated the prediction that the carboxy terminus of LMP1 can inhibit apoptosis induced by the UPR.

The signaling of LMP1 correlates with the expression of human miRNAs and mRNAs. The carboxy-terminal signaling of LMP1 strongly inhibited apoptosis induced by both 6TM and tunicamycin, supporting its role in overcoming UPR-activated apoptosis in EBV-positive B cells. We sought to identify miRNAs and mRNAs that correlated with the expression of LMP1 in the EBV-positive B-cell strain, 28-2, and might contribute to the inhibition of apoptosis, or regulate the UPR and autophagy.

28-2 cells contain EBV carrying a derivative of full-length LMP1 in which mRFP is fused to its carboxy terminus. The mRFP allows for sorting of single cells based on their levels of LMP1. Cells were sorted for those expressing the highest 5% or lowest 5% of LMP1-mRFP, which was confirmed by Western blotting for LMP1 (Fig. 5A). Thus, cells selected for the highest 5% levels of LMP1-mRFP expressed 6-fold more of it than those sorted for the lowest 5% levels of this protein. The expression of LMP1-mRFP correlated dose dependently with markers of the UPR (Fig. 5B); the levels of BiP and ATF4 were 2- and 3.5-fold greater, respectively, in cells containing the highest levels of LMP1-mRFP than in those containing the lowest levels. These results are consistent with the dose-dependent induction of the UPR via LMP1 (40).

RNA was harvested from 28-2 cells that were unsorted or sorted for the lowest 5% or highest 5% levels of LMP1-mRFP and then assayed for the differential expression of human miRNAs and mRNAs via microarrays. In total, 10 known miRNAs were found to be differentially expressed 1.5-fold or more ($\log_2$ scale) between cells expressing the lowest and highest levels of LMP1-mRFP (Ta-

TABLE 1 miRNAs whose expression correlated with LMP1-mRFP in 28-2 cells

miRNA	Fold change (high vs low LMP1-mRFP)	<i>P</i> ^a
hsa-miR-1290	2.24	1.11E-02
hsa-miR-146b-5p	2.16	2.60E-02
hsa-miR-146a	2.28	2.99E-02
hsa-miR-378	1.58	3.56E-02
hsa-miR-205	1.59	3.64E-02
hsa-miR-886-3p	1.58	3.89E-02
hsa-miR-1246	1.81	4.15E-02
hsa-miR-29b	1.68	4.61E-02
hsa-miR-148a	0.43	2.29E-02
hsa-miR-424	0.61	2.49E-02

^aAs determined by Student *t* test.

ble 1). Stem-loop reverse transcription and real-time PCR were performed on miR-146a and miR-424. In addition, we assayed for the expression of miR-150, a miRNA that correlates inversely with the expression of LMP1 in EBV-negative B cells (8). miR-146a and miR-150 were confirmed to correlate directly and inversely with the expression of LMP1-mRFP, respectively (Fig. 5C). However, miR-424 did not correlate with the expression of LMP1-mRFP in 28-2 cells (data not shown). The signaling of LMP1 has been previously reported to positively regulate the expression of miR-146a, but miR-146a has no documented role in the UPR, autophagy, or apoptosis (8, 49). Likewise, no role in the UPR or autophagy has been ascribed to the eight other miRNAs that correlated with levels of LMP1 by microarray. *c-Myb*, a proto-oncogene and direct target of miR-150 was not found to be differentially expressed in mRNA microarrays (data not shown) (74). Therefore, we chose not to pursue miR-150 as a gene contributing to the inhibition of apoptosis via LMP1.

A total of 900 mRNAs were differentially expressed 1.5-fold or greater ($\log_2$ scale) between the 28-2 cells expressing low or high levels of LMP1 (Student *t* test, rFDR correction; *P* [two sided] < 0.01). GO ontology was used to identify 60 transcripts that encoded proteins affecting apoptosis, the UPR, and autophagy (Fig. 5D) (9). Reverse transcription and real-time PCR were used to measure the expression of 11 mRNAs whose expression correlated with the expression of LMP1. The expression of 7 of the 11 tested mRNAs was confirmed to correlate with the expression of LMP1-mRFP (Fig. 6B). When LMP1-mRFP was expressed at its highest levels in 28-2 cells, four mRNAs were increased in their expression more than 2-fold (*Tnfrsf11b*, *Bcl2a1*, *Cflar*, and *Pik3cd*), and three decreased to <50% (*Tnfrsf10*, *Ccr5*, and *Ifi27*) of the levels in the 28-2 cells that had the lowest levels of LMP1-mRFP. *Bcl2a1* was the only BCL2 homolog whose expression correlated directly with LMP1-mRFP in 28-2 cells. *Bcl2* and *Bclxl* were not differentially expressed, whereas the expression of *Mcl1* was correlated inversely

LMP1-mRFP are indicated by the arrowhead. Above the arrowhead are dimers of LMP1 that were no longer conjugated to the mRFP. The expression of LMP1-mRFP is represented as the fold change relative to its expression in unsorted 28-2 cells. The Western blots are representatives of six independent experiments. (B) 28-2 cells sorted for their levels of LMP1-mRFP were assayed for their expression of BiP and ATF4 via Western blotting with levels of each protein normalized to α -tubulin and represented as the fold change in expression relative to that in unsorted cells. (C) The expression of miR-146a and miR-150 was measured via stem-loop reverse transcription and real-time PCR in sorted 28-2 cells. The statistics for panels A and C were determined to identify significant changes (*, *P* < 0.05; #, *P* < 0.01 [Wilcoxon rank sum, two sided]). (D) mRNAs were isolated from sorted 28-2 cells and hybridized to microarrays. The expression of nearly 900 transcripts correlated with the expression of LMP1-mRFP (≥ 1.5 -fold change, $\log_2$ scale, Student *t* test, rFDR correction, *P* [two sided] < 0.01). Of these 900 transcripts, a subset of 60 mRNAs that are involved in apoptosis, the UPR, or autophagy were identified and are shown in panel D.

FIG 6 The expression of the mRNA of *Bcl2a1* correlated with the signaling of LMP1 in EBV-positive and -negative B cells. (A) 22-5 cells were sorted by flow cytometry for the 5% of cells expressing the lowest (Low 5%) or highest (High 5%) levels of LMP1-mRFP. The expression of the LMP1-mRFP was normalized to α -tubulin and is represented as the fold change relative to the expression in cells expressing the lowest levels of LMP1-mRFP. The Western blots shown are representatives of three independent experiments. (B) mRNAs encoding *Ifi27*, *Tnfsf10*, *Ccr5*, *Tnfrsf11b*, *Bcl2a1*, *Pik3cd*, and *Cflar* were measured for their correlation with LMP1-mRFP in 28-2 and 22-5 cells. The expression of each mRNA is represented as the fold change in expression relative to that of unsorted 28-2 cells or to that of 22-5 cells expressing the lowest 5% of mRFP. (C) The expression of the mRNA of *Bcl2a1* (i), *Cflar* (ii), and *Pik3cd* (iii) was measured in BJAB, BJAB-LMP1, and BJAB-6TM cells that were treated with Dox (On) to induce the expression of the variants of LMP1 or left untreated (Off). (D) The expression of the mRNA of *Bcl2a1* was measured BJAB-6TM/Ctl and BJAB-6TM/NGFR-LMP1 cells treated with (+) or without (–) Dox and anti-NGFR antibody. The expression of the mRNA of *Bcl2a1* is represented as the fold change relative to the expression in untreated BJAB, BJAB-LMP1, and BJAB-6TM cells in panel C and BJAB-6TM/Ctl cells in panel D. Statistics were determined to identify significant changes in expression (*, $P < 0.05$; #, $P < 0.01$ [Wilcoxon rank sum, two sided]).

with LMP1 (Fig. 5D). In addition, reverse transcription and real-time PCR were used to assay for the differential expression of *Pten*, *Bak1*, *Xbp1*, and *Pik3c3*, but none was observed, and these findings were not consistent with the mRNA microarrays (data not shown). The expression of LMP1 correlates with the splicing of the mRNA of *Xbp1* in EBV-positive and -negative B cells, but the analysis we performed did not differentiate between the spliced and unspliced forms of the mRNA (40). Other mRNAs encoding genes likely involved in UPR-induced apoptosis were identified, including *Casp9*, *Birc2*, *Tp63l*, and *Scotin* (Fig. 5D) (6, 11, 30, 32, 45, 54, 56, 67, 72, 81).

BCL2A1 is a bona fide candidate for mediating the survival of B cells that express LMP1. It was predicted that for candidate genes to contribute to the inhibition of apoptosis induced by the 6TM, their expression would correlate with the expression of intact LMP1 in two additional models: (i) an independent EBV-

positive B-cell strain and (ii) BJAB-LMP1 cells, but not BJAB-6TM cells. These criteria were met by three of the seven mRNAs confirmed by real-time PCR to correlate with the expression of LMP1 in 28-2 cells (Fig. 6B). 22-5 cells, an EBV-positive B-cell clone similar to 28-2 cells, were sorted for levels of LMP1-mRFP via flow cytometry (Fig. 6A). The expression of *Bcl2a1*, *Pik3cd*, and *Cflar* correlated directly with the expression of LMP1-mRFP in 22-5 cells (Fig. 6B). Furthermore, the expression of both *Pik3cd* and *Cflar* was 3-fold greater in BJAB cells induced to express HA-LMP1 than uninduced cells, and the expression of *Bcl2a1* increased 8-fold in the same cells (Fig. 6C). However, when the expression of HA-6TM was induced in BJAB cells, the levels of the mRNAs of *Bcl2a1* and *Cflar* remained unchanged, whereas the amount of mRNAs of *Pik3cd* mRNA was reduced by 65%.

The mRNAs of *Cflar* and *Pik3cd* encode caspase-8 and FADD-like apoptosis regulator, abbreviated as c-FLIP, and

FIG 7 The constitutive, exogenous expression of BCL2A1 inhibited apoptosis in BJAB cells that also expressed the 6TM. (A) BJAB-6TM/Ctl and BJAB-6TM/BCL2A1 cells were treated with 0 (–), 0.1 (+), or 0.3 (++) ng of Dox/ml to induce the expression of HA-6TM. Cells were harvested after 2 days to assay for the expression of HA-6TM, 2×-myc-BCL2A1, and cleaved PARP. The expression of each protein was normalized to the levels of α-tubulin. The expression of the HA-6TM and 2×-myc-BCL2A1 is represented as the fold change in expression relative to that in uninduced BJAB-6TM/Ctl cells. The expression of cleaved PARP (± the SD) is represented as the fold change in expression relative to that in BJAB-6TM/Ctl cells induced to express HA-6TM with 0.3 ng of Dox/ml. (B) The expression of the mRNA encoding 2×-myc-BCL2A1 was measured via real-time PCR. The expression of the mRNA is represented as the fold change in expression relative to that in uninduced BJAB-6TM/Ctl cells. (C) Caspase activity in BJAB-6TM/Ctl and BJAB-6TM/BCL2A1 cells induced to express HA-6TM dose dependently was measured. The caspase activity was normalized to the RLU of uninduced BJAB-6TM/Ctl cells on day 2. Statistics ($P < 0.05$ [Wilcoxon rank sum, two sided]) were determined to identify significant differences within populations treated with different concentrations of Dox (*) or between populations treated with the same concentration of Dox (#).

phosphoinositide-3 kinase delta (PI3K Δ), respectively. If c-FLIP were to contribute to the LMP1-mediated inhibition of apoptosis, its levels should increase in BJAB-LMP1 cells, but not BJAB-6TM cells, upon the induction of the derivatives of LMP1 with Dox. There was no change in the levels of c-FLIP when HA-LMP1 was induced in BJAB cells, as determined via Western blotting (data not shown). Western blotting was also used to quantify the phosphorylation of v-akt murine thymoma viral oncogene homolog 1 (AKT), which is a reflection of the activity of PI3K Δ . The phosphorylation of AKT was unaffected by HA-LMP1 in BJAB cells (data not shown). These findings indicated LMP1 did not inhibit apoptosis via the increased expression of *Pik3cd* or *Cflar*.

In addition, we measured the expression of the mRNA of *Bcl2a1* via reverse-transcription and real-time PCR in BJAB-6TM cells expressing NGFR-LMP1 or not (Ctl). When anti-NGFR antibody was added to cells expressing NGFR-LMP1 in the absence of Dox, there was a 20-fold increase in the expression of the mRNA of *Bcl2a1* compared either to cells left untreated or to BJAB-6TM/Ctl cells regardless of their treatment (Fig. 6D). When both anti-NGFR antibody and Dox were added, the levels of the mRNA of *Bcl2a1* increased 5-fold more in BJAB-6TM/NGFR-LMP1 cells than in the BJAB-6TM/Ctl cells. Altogether, the observations in the B-cell strains 28-2 and 22-5 and in derivatives of BJAB expressing variants of LMP1 indicated that *Bcl2a1* was the

only candidate for contributing to the LMP1-mediated inhibition of apoptosis among the genes tested.

The constitutive, exogenous expression of BCL2A1 inhibited apoptosis in BJAB cells that also expressed the 6TM of LMP1. *Bcl2a1* was an attractive candidate for inhibiting apoptosis induced by the 6TM of LMP1 because it binds and inhibits the activities of both truncated BID (tBID) and BAK, proapoptotic proteins whose steady-state levels increase during the UPR (61). Thus, the ability of BCL2A1 to inhibit the activities of tBID and BAK could block UPR-induced apoptosis. Also, LMP1 has previously been reported to activate the transcription of the mRNA of *Bcl2a1* in B cells via NF- κ B, and *Bcl2a1* was the only mRNA encoding an antiapoptotic member of the BCL2 family to correlate directly with the expression of LMP1 (Fig. 5D) (7, 19).

For these reasons, the ability of BCL2A1 to inhibit apoptosis induced by the 6TM was tested. BJAB-6TM cells were transduced with a vector that expresses 2×-myc-BCL2A1 (Fig. 7A and B). The mRNA encoding the 2×-myc-BCL2A1 protein was expressed at 35-fold-greater levels in this population of cells (BJAB-6TM/BCL2A1) than in uninduced BJAB-6TM/Ctl cells (Fig. 7B). Both BJAB-6TM/Ctl and BJAB-6TM/BCL2A1 cells were treated with increasing concentrations of Dox to induce the expression of HA-6TM, which they did at similar levels (Fig. 7A).

We were unable to repress the expression of *Bcl2a1* with shRNAs; however, the exogenous expression of BCL2A1 inhibited

FIG 8 The expression of LMP1 induced apoptosis in epithelial cells. (A) HeLa and H1299 cells were transfected with 0, 0.3, 1, or 3 μ g of cDNA encoding LMP1. The expression of LMP1 and cleaved PARP was normalized to α -tubulin and is represented as the fold change in expression relative to HeLa or H1299 cells transfected with 3 μ g of cDNA encoding LMP1. (B) Caspase activity was measured 24 h posttransfection and is represented as the mean normalized RLU relative to untransfected HeLa or H1299 cells. Statistics were determined to identify significant changes (*, $P < 0.05$ [Wilcoxon rank sum, two sided]).

apoptosis induced by the 6TM. After 2 days of expressing the 6TM with 0.1 or 0.3 ng of Dox/ml, only 50 and 40% of the PARP were cleaved, respectively, in BJAB cells expressing 2 $\times$ -myc-BCL2A1 compared to BJAB-6TM/Ctl cells (Fig. 7A). The activity of caspase-3 and -7 was similarly repressed by 2 $\times$ -myc-BCL2A1 in the presence of the 6TM of LMP1, where BCL2A1 inhibited apoptosis by 35% each day in both Dox treatments (Fig. 7C). These findings indicate that BCL2A1 inhibits apoptosis induced by the 6TM of LMP1.

The expression of the mRNA of *Bcl2a1* did not correlate with the expression of LMP1 in epithelial cells in which LMP1 induces apoptosis. The signaling of LMP1 in epithelial cells may not parallel its signaling in B cells. For example, LMP1 does not induce the UPR in the EBV-negative, NPC-derived cell line, HONE, as it does in B cells (33). We therefore sought to determine whether LMP1 was capable of inducing apoptosis and activating the transcription of *Bcl2a1* in two EBV-negative epithelial cell lines, H1299 and HeLa.

Both H1299 and HeLa were transfected with increasing amounts of a vector encoding intact LMP1. For both H1299 and HeLa, 60% of the cells were transfected after 24 h as assayed by the expression of eGFP encoded by a cotransfected vector (data not shown). Western blots performed on lysates from cells harvested at 24 h posttransfection showed LMP1 was expressed at increasing levels in both cell lines (Fig. 8A). Intact LMP1 induced apoptosis in a dose-dependent manner in both H1299 and HeLa, as assayed by determining the activity of caspase-3 and -7 and the cleavage of PARP (Fig. 8). The expression of the mRNA of *Bcl2a1* was undetectable when assayed via real-time PCR (data not shown), a finding consistent with the notion that the capacity of LMP1 to induce the mRNA of *Bcl2a1* in B cells contributes to inhibiting its induction of apoptosis in those cells.

DISCUSSION

The LMP1 oncogene is regulated through its effects on two cellular pathways. The 6TM induces both the UPR and autophagy dose dependently in B cells and, in doing so, LMP1 can regulate its own expression (37–40). This regulation needs to be compatible with EBV's successful, long-term infection of B cells and, accordingly, the activation of the UPR and autophagy via LMP1 does not cause B cells to die. We have now found in B cells that signaling from the carboxy-terminal moiety of LMP1 inhibits the apoptosis that its

6TM induces as a consequence of its activation of the UPR and autophagy.

How does LMP1 inhibit its activation of apoptosis? The signaling of CD40 likely provides some clues. CD40 activates the transcription of mRNAs that encode antiapoptotic proteins (2). LMP1 activates the same pathways as does CD40, activates the transcription of many of the same mRNAs as does CD40, and can replace the signaling of CD40 in B cells *in vivo* (15, 28, 29, 35, 55, 68, 80). The analysis of microarrays showed that the expression of LMP1 in EBV-positive B cells correlated with the expression of mRNAs that affect apoptosis (Fig. 5D). The levels of seven of these mRNAs found to correlate with the expression of LMP1 were validated via reverse transcription and real-time PCR (Fig. 6B). Both the mRNAs encoding *Pik3cd* and *Cflar* correlated directly with the expression of LMP1-mRFP (Fig. 6B). HA-LMP1 had no effect on the phosphorylation of AKT in BJAB cells or on the levels of CFLAR protein (Fig. 6B). Therefore, we concluded they could not generally contribute to the inhibition of apoptosis conferred to B cells by the carboxy-terminal domain of LMP1. Among the mRNAs that were affected by LMP1-mRFP, the one encoding BCL2A1, a homolog of BCL2, was the only candidate of the antiapoptotic BCL2 family that correlated directly with levels of LMP1-mRFP (Fig. 5D). These findings are supported by data that LMP1 does not regulate the expression of BCL-2 nor BCL-xL in B cells. The expression of mRNAs encoding *Bcl2* and *Bclxl*, or the proteins, BCL2 and BCL-xL, do not change detectably when primary B cells are infected with EBV (44; M. Sandburg, unpublished data). Similarly, the mRNA of *Bcl2a1*, but not other BCL2 homologs, correlates with the conditional activation of the carboxy-terminal signaling of LMP1 in EBV-positive B-cell strains (16). In addition, the expression of the mRNA of *Bcl2a1*, but not that of *Bclxl*, is greater in lymphoblastoid cell lines (LCLs) than EBV-negative Burkitt's lymphoma cell lines (BLs) (7).

We investigated *Bcl2a1* further because its transcription is activated by both LMP1 and CD40 in EBV-negative B cells (7, 19, 20). Activation of its transcription is dependent on NF- κ B and signaling from the carboxy-terminal transactivating region 2 of LMP1 (20). BCL2A1 is also expressed at greater levels in B-cell chronic lymphocytic leukemia cells (B-CLLs) than in normal B cells, helping to render B-CLLs resistant to apoptosis (48, 52, 70). Likewise, the exogenous expression of BCL2A1 in EBV-positive MutuI cells inhibits apoptosis that is induced by serum starvation

FIG 9 Comparison of how apoptosis is inhibited after the induction of the UPR by the BCR or LMP1. The independent signaling of the 6TM of LMP1 and its carboxy-terminal tail resembles the interaction between stimulated BCR and stimulated CD40 in immature B cells with anti-BCR and anti-CD40 antibodies, respectively. The stimulation of the BCR with anti-BCR antibody can activate the UPR, which then induces apoptosis. Similarly, the 6TM of LMP1 activates the UPR constitutively in the absence of a ligand, which also induces apoptosis. Constitutive signaling from the carboxy-terminal domain of LMP1, like the conditional signaling of CD40, inhibits apoptosis induced by the UPR. *Bcl2a1* is one gene both LMP1 and CD40 activate, and BCL2A1 inhibits the UPR-induced apoptosis activated by LMP1.

(19). Our results show that the exogenous expression of BCL2A1 inhibited apoptosis in BJAB cells induced to express HA-6TM, illustrating the induction of BCL2A1 by LMP1 is one way by which LMP1 inhibits the apoptosis that it induces (Fig. 7 and 8).

The interplay between the 6TM of LMP1 in inducing apoptosis and its carboxy terminus in inhibiting apoptosis is similar to some of the combined functions of the B-cell receptor (BCR) and CD40 in immature B cells (Fig. 9). The stimulation of the BCR in primary B cells isolated from mice and in WEHI-231 B cells induces the UPR and autophagy (63, 73, 75). The activation of the UPR in immature B cells also induces apoptosis and stimulated CD40 can rescue these B cells from the UPR-induced apoptosis (12, 63, 75). The induction of the UPR and autophagy by LMP1 can, under some conditions, induce apoptosis, as can a stimulated BCR. Two findings support this conclusion. First, EBV-negative BJAB cells induced to express the 6TM supported the UPR and died by apoptosis (Fig. 1 and 3C) (38–40). Second, apoptosis induced by the 6TM of LMP1 was partially inhibited when BiP, a chaperone protein and mediator of the UPR, was expressed exogenously (Fig. 3C and D). This partial inhibition likely reflects only a partial inhibition of the UPR by BiP in these experiments. The exogenous expression of BiP in WEHI-231 cells inhibits apoptosis after stimulation of the BCR (75). However, unlike the BCR, which requires binding to immunoglobulin to stimulate its signaling, the 6TM constitutively induces the UPR, autophagy, and apoptosis in a dose-dependent manner (Fig. 3C and 5B) (38, 40). Intact LMP1 induces the UPR and autophagy, but also antiapoptotic genes, including *Bcl2a1*, to inhibit apoptosis and allow EBV-infected B cells to survive to proliferate (Fig. 9).

Unlike its 6TM, the carboxy-terminal domain of LMP1 cannot function independently. Rather, the carboxy-terminal domain must be oligomerized to signal, much like CD40 (28). Therefore, the carboxy-terminal signaling of intact LMP1 is directly linked to the activities of its 6TM and is influenced by phenotypes in B cells generated by the 6TM, including the UPR and autophagy. In contrast, the signaling of CD40 occurs independently of the signaling of BCR. Thus, differences in the phenotypes of B cells signaling via

intact LMP1 or CD40 could be explained by the environments in which they signal.

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