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Epstein-Barr Virus (EBV) Latent Membrane Protein 2A Regulates B-Cell Receptor-Induced Apoptosis and EBV Reactivation through Tyrosine Phosphorylation

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Epstein-Barr virus (EBV) is a human herpesvirus that establishes a lifelong latent infection of B cells. Within the immune system, apoptosis is a central mechanism in normal lymphocyte homeostasis both during early lymphocyte development and in response to antigenic stimuli. In this study, we found that latent membrane protein 2A (LMP2A) inhibited B-cell receptor (BCR)-induced apoptosis in Burkitt's lymphoma cell lines. Genistein, a specific inhibitor of tyrosine-specific protein kinases, blocked BCR-induced apoptosis and EBV reactivation in the cells. These findings indicate that LMP2A blocks BCR-induced cell apoptosis and EBV reactivation through the inhibition of activation of tyrosine kinases by BCR cross-linking.

The B-lymphotropic herpesvirus Epstein-Barr virus (EBV) is a ubiquitous human virus (21, 37) that establishes a lifelong persistent infection in memory B lymphocytes (2). It is an important pathogen because of its association with the development of various lymphoid and epithelioid malignancies (21, 29).

Whereas primary infection by EBV is usually asymptomatic during childhood, delayed primary infection can manifest itself in a disease called infectious mononucleosis (21). Whether or not initial infection was symptomatic, the virus subsequently persists in the healthy host for the rest of his or her life as a latent infection of resting memory B cells (38). Based on these observations, a model has been proposed in which the virus uses normal B-cell differentiation processes to establish a persistent infection (3, 38). An important aspect of this model is the germinal center (GC) reaction, in which B cells that encountered a specific antigen with costimulatory signals (CD40 or interleukin-4 receptor engagement) proliferate vigorously and modify their rearranged V genes by somatic hypermutations (25); however, cross-linking of the B-cell receptor (BCR) in the absence of those costimulatory signals induces anergy or apoptosis (35, 40). Only B cells carrying antigen receptors with improved affinity survive and are selected into the pool of memory B cells (31). In the model of persistent infection, EBV primarily infects naïve B cells and induces their proliferation (38). When such cells subsequently undergo a GC reaction and differentiate into memory cells, the virus gains access to the memory B-cell pool, the site of viral persistence. In addition, B-cell fate is predominantly controlled by signals through the BCR. Potentially autoreactive B-cell clones can be purged from the repertoire by the process of clonal deletion (apoptosis) that occurs at various stages during B-cell development in the bone marrow and GC. Latent membrane protein 2A (LMP2A) blocks the accumulation of tyrosine-phosphorylated proteins, calcium mobilization, and EBV reactivation from B cells by BCR cross-linking (18, 32, 33). However, little is known about the effect of LMP2A on the BCR-induced apoptosis in B cells and specifically the effect of LMP2A on EBV reactivation in forms of EBV latency observed in the human host. To further clarify the function of LMP2A in cell survival and EBV latency in GC B cells with limited viral gene expression, we investigated LMP2A-expressing Ramos cell lines. Ramos is an EBV-negative B-cell line that resembles GC B cells (20). In addition, LMP2A-expressing Akata cell lines were also investigated. Akata cells have been used in various studies as a model for studying the mechanisms involved in EBV reactivation and apoptosis by ligation of surface immunoglobulin G (IgG) and express only EBNA1 in readily detected levels (6, 7, 19, 22, 39).

LMP2A and genistein inhibit BCR-induced tyrosine phosphorylation and apoptosis in Ramos cells. To assess the effect of LMP2A on BCR-induced apoptosis, previously constructed LMP2A-expressing Ramos cells and vector control cells were used (12). Ramos cells have been used in various studies as a model for studying the mechanisms involved in apoptosis by ligation of surface IgM, a component of BCR (1, 9, 23). To verify that LMP2A expression did not result in any change in surface levels of IgM, IgM expression was analyzed by flow cytometry (FACS Caliber; Becton Dickinson, San Jose, CA) using CellQuest software (Becton Dickinson) in parental, two vector control, and three LMP2A-expressing Ramos cell clones. For all cell clones tested, the levels of surface IgM were similar (data not shown).

We then examined the effect of LMP2A on BCR-induced cell death in parental, vector control, and LMP2A-expressing Ramos cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) assay. Inhibition of cell viability of parental, vector control, and LMP2A-expressing Ramos cells by BCR ligation was explored either 24 h or 48 h following anti-IgM antibody treatment. In parental and vector control Ramos cells, BCR ligation resulted in cell viability of approximately 60% or 40% of untreated parental or vector control cells (set at 100%) 24 h or 48 h posttreatment, respectively

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(data not shown). Expression of LMP2A resulted in an approximately 20% increase in cell viability for both times following anti-IgM antibody treatment (data not shown).

Caspase activation is an important element in the apoptotic signaling pathway, activation of caspase 3 is increased in BCR-induced apoptosis in Ramos cells, and the cleavage of one of its substrates, poly(ADP-ribose) polymerase (PARP), has been used as an indicator of this activity (1, 8, 28). We examined whether LMP2A inhibits BCR-induced apoptosis by DNA fragmentation (14) and cleavage of PARP (28) using flow cytometry and Western blotting (12) in parental, vector control, and LMP2A-expressing Ramos cells (Fig. 1A and 1B). BCR stimulation induced DNA fragmentation and cleavage of PARP in parental and vector control Ramos (sub-G_1 populations of 15.9% and 16.5%, respectively) at 24 h and (sub-G_1 populations of 19.6% and 19.0%, respectively) at 48 h in a time-dependent manner (Fig. 1A). LMP2A partially inhibited the BCR-induced DNA fragmentation and cleavage of PARP at both 24 h (a greater-than-twofold reduction in apoptotic cells, 16.5% to 6.7%) and 48 h (a greater-than-twofold reduction in apoptotic cells, 19.0% to 7.9%). To verify the DNA fragmentation and cleavage of PARP by BCR ligation were dependent on caspase activity in Ramos cells, cells were pretreated with zVAD-fmk (Calbiochem, La Jolla, CA), a broad caspase inhibitor and, as expected, DNA fragmentation and PARP cleavage were blocked by the addition of zVAD-fmk (Fig. 1C and 1D).

We next examined the mechanism of inhibition of BCR-induced apoptosis by LMP2A. LMP2A blocks BCR-induced tyrosine phosphorylation and EBV reactivation by binding the protein kinases Syk and Lyn as well as altering the activity of other cellular proteins (18, 32, 33). BCR ligation triggers a cascade of signal transduction events; cytoplasmic protein tyrosine kinase (PTK) activation is one of the earliest events (34, 36). In addition, PTKs are known to play a key role in oncogenesis and the control of cell growth and apoptosis (17, 27). To investigate the relationship between BCR-induced tyrosine phosphorylation and apoptosis, we compared the effect of genistein (Calbiochem), a tyrosine kinase inhibitor, on the BCR-induced tyrosine phosphorylation and apoptosis with the effect of LMP2A on BCR-induced tyrosine phosphorylation and apoptosis in Ramos cells (Fig. 2A). Tyrosine phosphorylation and apoptosis in Ramos cells after BCR ligation was blocked by genistein and the LMP2A-mediated inhibitory effects were similar to those in parental Ramos cells treated with 30 or 50 μg/ml of genistein (Fig. 2B). The results from the treatment of LMP2A-expressing Ramos cells with genistein were no different from results with untreated LMP2A-expressing Ramos cells (data not shown), and as would be expected, genistein inhibited cell growth of Ramos cells (data not shown). BCR ligation resulted in approximately 60% viability of Ramos cells at 24 h posttreatment. Genistein treatment resulted in 10 to 20% increase in cell viability following BCR ligation (data not shown). In addition, 30 or 50 μg/ml genistein partially inhibited BCR-induced apoptosis in Ramos cells (16.7% to 10.3% or 8.6%, respectively) and blocked almost entirely BCR-induced cleavage of PARP (Fig. 2C and 2D). These inhibitory effects did not go beyond these values if higher concentrations of genistein or longer treatments were used (data not shown).

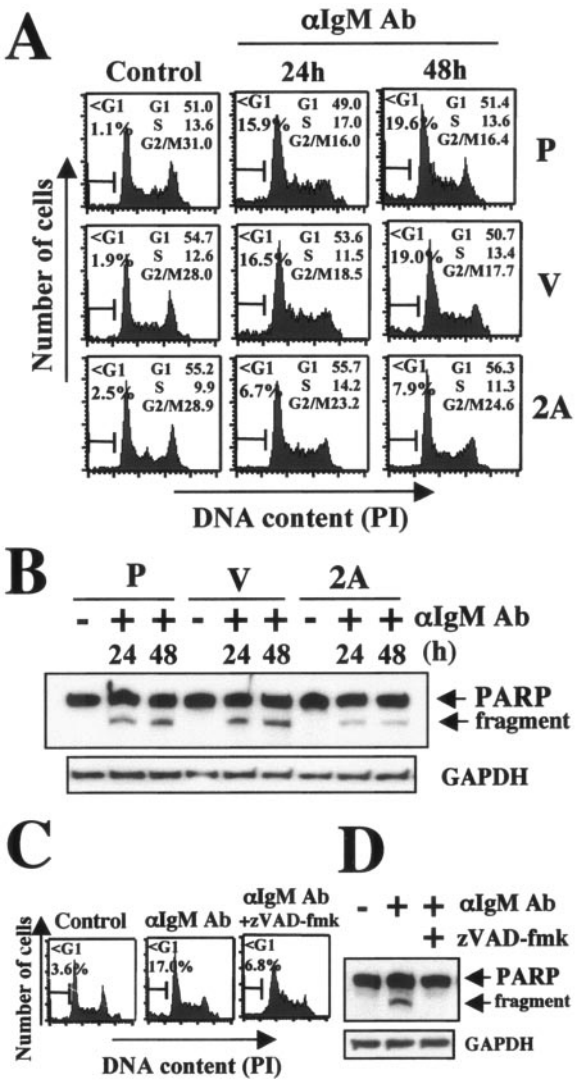

FIG. 1. LMP2A inhibits BCR-induced DNA fragmentation and cleavage of PARP in Ramos cells. (A) Cells were seeded at 3×10^5 cells/ml, and cells were treated without (control) or with 35 μg/ml anti-IgM antibody (αIgAb) for 24 h or 48 h. Cells were analyzed for DNA content by propidium iodide (PI) staining and flow cytometry. Gates employed to ascertain cell cycle distribution and the percentage of cells with a sub-G_1 ($<G_1$) and G_2/M DNA content are shown. These data are representative of three experiments. (B) PARP cleavage was analyzed by immunoblotting with a specific anti-PARP antibody. The full-length 113-kDa and 89-kDa cleaved PARP proteins are indicated. The amount of protein loaded in each lane was assessed by rehybridization of the filter with a specific antibody for human GAPDH. P, parental; V, vector control; 2A, LMP2A expressing. (C) DNA fragmentation and (D) evaluation of cleavage of PARP. Cells (3×10^5/ml) were preincubated for 1 h with or without zVAD-fmk (50 μM), and cells were then treated with 35 μg/ml anti-IgM antibody. After 24 h of incubation, cell cycles and PARP cleavage were analyzed as described in the legend to panel B. The amount of protein loaded in each lane was assessed by rehybridization of the filter with a specific antibody for human GAPDH.

LMP2A and genistein inhibit BCR-induced tyrosine phosphorylation, DNA fragmentation, cleavage of PARP, and EBV reactivation in Akata cells. Since EBV reactivation by BCR ligation correlated with apoptosis, we assessed the effect of

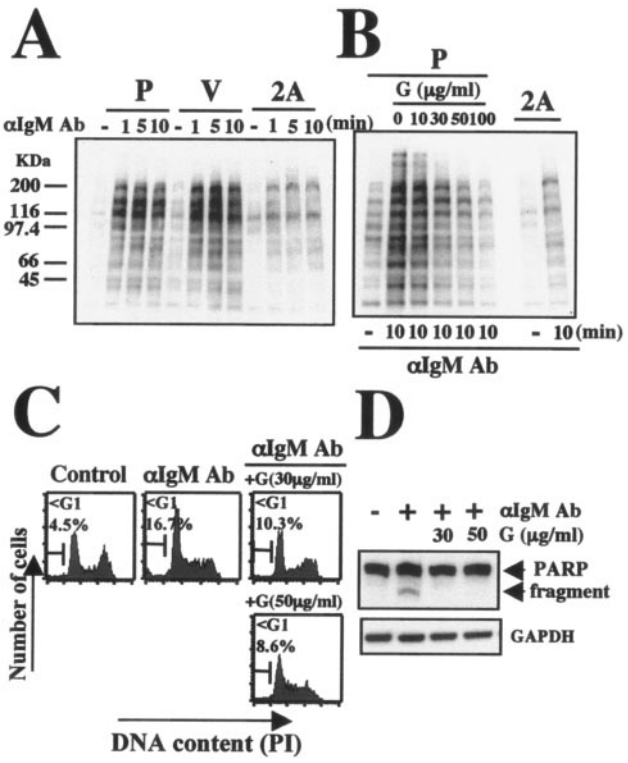

FIG. 2. LMP2A or genistein inhibits BCR-induced tyrosine phosphorylation, DNA fragmentation, and cleavage PARP in Ramos cells. (A) Parental (P), vector control (V), and LMP2A-expressing (2A) Ramos cells (1×10^6/ml) were treated without (control [−]) or in the presence of 35 μg/ml anti-IgM antibody (αIgM Ab) for the indicated times (1, 5, or 10 min). (B) Cells (1×10^6/ml) were preincubated for 30 min with various concentrations of genistein, and then cells were treated with 35 μg/ml anti-IgM antibody for 10 min. Equal amounts of protein from the respective cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The levels of expression of phosphorylated tyrosine were determined by immunoblotting. (C) DNA fragmentation and (D) evaluation of cleavage of PARP. Cells (3×10^5/ml) were preincubated for 30 min with or without genistein, and cells were then treated with 35 μg/ml anti-IgM antibody. After 24 h of incubation, cell cycles and PARP cleavage were analyzed as described in the legend to Fig. 1. The amount of protein loaded in each lane was assessed by rehybridization of the filter with a specific antibody for human GAPDH.

LMP2A on BCR-induced apoptosis and EBV reactivation in Akata cells. LMP2A expression was not detected in the parental Akata cells prior to transduction with a LMP2A retroviral expression vector (30) or the vector control (42; data not shown). As previously described (12), following transduction, hygromycin-resistant stable transfected clones were selected and verified for LMP2A expression by Western blot analysis (data not shown). In addition, surface expression of IgG was detected at similar levels by flow cytometry in all parental, two vector control, and three LMP2A-expressing Akata cell clones (data not shown).

To investigate the relationship between BCR-induced tyrosine phosphorylation, apoptosis, and EBV reactivation, we compared the effects of genistein and LMP2A on BCR-induced tyrosine phosphorylation, apoptosis, and EBV reactivation in Akata cells. In these studies, apoptosis was monitored by flow cytometry and the induction of PARP cleavage, expression of the EBV immediate-early BZLF1 protein product ZEBRA (5, 43), and expression of early lytic antigens (EA) (32) were monitored by Western blotting (Fig. 3). BCR ligation induced apoptosis and cleavage of PARP in parental and vector control Akata cells (sub-G_1 populations of 18.0% and 19.0%, respectively) at 24 h (Fig. 3A and 3B). LMP2A partially inhibited the BCR-induced apoptosis at 24 h (a greater-than-twofold reduction in apoptotic cells, 19.0% to 9.7%) and almost completely blocked PARP cleavage (Fig. 3A and 3B). In addition, BCR ligation induced ZEBRA and EA expression in parental and vector control Akata cells and LMP2A partially inhibited BCR-induced ZEBRA and EA expression in Akata cells (Fig. 3C). Similar to Ramos cells, the amount of genistein to get a similar reduction of tyrosine phosphorylation following BCR ligation to the LMP2A-expressing Akata cells was 50 μg/ml (data not shown). Treatment of Akata cells with 30 or 50 μg/ml genistein partially inhibited BCR-induced apoptosis (18.0% to 11.8% or 18.0% to 10.0%, respectively) and BCR-induced cleavage of PARP (Fig. 3A and 3B). Genistein is a potent inhibitor of cell proliferation and oncogenesis in animal and human cells (4, 10). In contrast to the Ramos cells, there was more PARP cleavage with genistein in Akata cells (Fig. 3B). Genistein-mediated inhibitory effects did not go beyond these values if higher concentrations of genistein or longer treatments were used (data not shown), and the treatment of LMP2A-expressing Akata cells with genistein was no different from results with untreated LMP2A-expressing Akata cells (data not shown). Since zVAD-fmk does not block EBV reactivation but does block apoptosis by BCR ligation in EBV-positive LMP2A-negative BL cell line, Mutu-I (19), we examined the effect of zVAD-fmk on the BCR-induced apoptosis and ZEBRA expression in Akata cells. As might be expected, zVAD-fmk did not block BCR-induced ZEBRA expression (Fig. 3C) but did block BCR-induced apoptosis and cleavage of PARP in Akata cells (Fig. 3D and 3E). In addition, to verify that the zVAD-fmk-mediated antiapoptotic effects were not through the inhibition of BCR-induced tyrosine phosphorylation, we examined the effect of zVAD-fmk on the BCR-induced tyrosine phosphorylation in Akata cells. zVAD-fmk did not block BCR-induced tyrosine phosphorylation in Akata cells (Fig. 3F).

Pharmacological activation of protein kinase C induces cell apoptosis and EBV reactivation in LMP2A-expressing Ramos and Akata cells. To confirm the effect of LMP2A on the BCR signaling pathway, we examined whether LMP2A inhibits BCR-induced p42/p44 mitogen-activated protein kinase (MAPK; ERK1/2) phosphorylation (13, 15, 26), which is downstream of the BCR in Ramos and Akata cells, by Western blot analysis using anti-phospho MAPK (ERK1 and ERK2) antibody and anti-MAPK (ERK1 and ERK2) antibody (Cell Signaling Technology, Beverly, MA). LMP2A partially inhibited the induction of ERK1/2 phosphorylation after BCR ligation (Fig. 4A). A23187 (Calbiochem), a calcium ionophore, and phorbol myristate acetate (PMA; Sigma), direct activators of protein kinase C, induce EBV reactivation in wild-type and LMP2 mutated recombinant EBV-infected lymphoblastoid cell lines (33). To verify if LMP2A-expressing Ramos and Akata cells can be permissive for apoptosis and lytic EBV infection, cell cultures were treated with A23187 and PMA

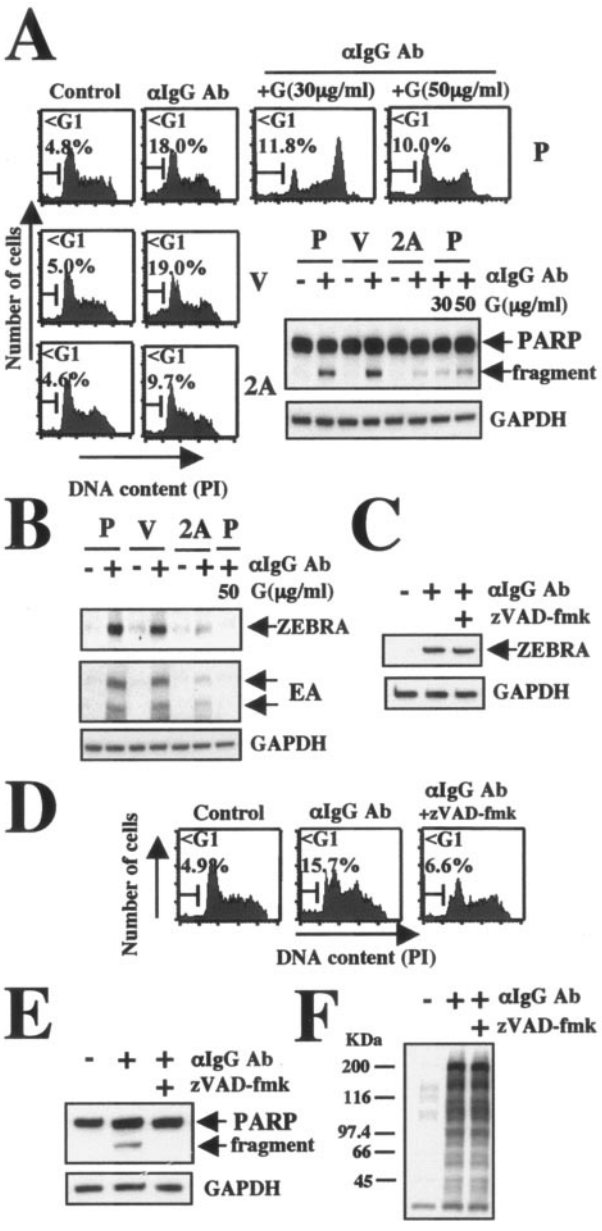

FIG. 3. LMP2A or genistein blocks BCR-induced DNA fragmentation, cleavage of PARP, and EBV reactivation. (A) DNA fragmentation and evaluation of cleavage of PARP. Parental (P), vector control (V), and LMP2A-expressing (2A) Akata cells (5×10^5/ml) were preincubated for 30 min with or without genistein, and cells were then treated with 40 μg/ml anti-IgG antibody (αIgG Ab). After 24 h of incubation, cell cycle analyses and PARP cleavage were analyzed as described in the legend to Fig. 1. (B) EBV reactivation. Cells (5×10^5/ml) were preincubated for 30 min with or without genistein (G; 50 μg/ml), and cells were then treated with 40 μg/ml anti-IgG antibody. After 24 h of incubation, the expression of ZEBRA (38 kDa) and EA (52 kDa and 55 kDa) was analyzed by immunoblotting with a specific anti-ZEBRA antibody or with human EBV serum reactive with EBV early antigens. The amount of protein loaded in each lane was assessed by rehybridization of the filter with a specific antibody for human GAPDH. (C) EBV reactivation, (D) DNA fragmentation, and (E) cleavage of PARP with zVAD-fmk treatment. Cells (5×10^5/ml) were preincubated for 1 h with or without zVAD-fmk (50 μM), and cells were then treated with 40 μg/ml anti-IgG antibody. After 24 h of incubation, cell cycles and the expression of ZEBRA were analyzed as described in the legends to Fig. 1 and panel B. The amount of protein loaded in each lane was assessed by rehybridization of the filter with a specific antibody for human GAPDH. (F) Tyrosine phosphorylation with zVAD-fmk treatment. Cells (1×10^6/ml) were preincubated for 1 h with or without zVAD-fmk (50 μM) followed by treatment with 35 μg/ml anti-IgM antibody for 10 min. Equal amounts of protein from the respective cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The levels of expression of phosphorylated tyrosine was determined by immunoblotting.

(Fig. 4B and 4C). A23187 and PMA strongly induced apoptosis and PARP cleavage similarly in all parental, vector control, and LMP2A-expressing Ramos and Akata cells (sub-G_1 populations of 30 to 40%, respectively) at 24 h (Fig. 4B). In addition, the levels of ZEBRA and EA expression were similar in A23187- and PMA-treated parental, vector control, and LMP2A-expressing Akata cells (Fig. 4C). These results suggest that LMP2A blocks BCR-induced apoptosis and EBV reactivation through the inhibition of BCR-induced tyrosine phosphorylation, an early event of the BCR signaling pathway.

Conclusions. In the analysis described in the current paper, we chose to focus on generating cell lines that had limited viral gene expression, thereby excluding the possible effects of other EBV-encoded, protein-mediated, antiapoptotic effects, such as LMP1 (11, 16, 41), on the analysis performed.

GC reactions may be important for the establishment of EBV latency and persistence and the development of EBV-associated malignancies. It has been proposed that GC passage may be an important step in the strategy of EBV to establish latency in the memory B-cell compartment (3). According to this model, during initial EBV infection, recruitment of EBV-infected naïve B cells into a GC reaction allows the EBV-infected cells to enter the memory compartment (3). The prevention of the induction of apoptosis and the activation of lytic replication, as observed in the current study, are likely key roles for LMP2A in allowing EBV-infected naïve B cells to transit into the pool of long-lived memory B cells. In addition, disruption of the physiologic balance between cell proliferation and death is a universal feature of all cancers. It is interesting to note that the three main types of EBV-associated B-cell lymphomas, Burkitt's lymphoma, Hodgkin lymphoma, and posttransplant lymphomas are predominantly derived from GC B cells or atypical survivors of the GC reaction in most, if not all, cases, indicating that EBV-infected GC B cells are at particular risk for malignant transformation (24).

Previously, we have shown LMP2A promotes cell survival of B cells through the activation of phosphatidylinositol 3-kinase/Akt (12). In this study, we have further shown that LMP2A also promotes cell survival through the inhibition of BCR signal transduction. The protection is not complete and is likely a result of lower expression of LMP2A in the cell lines we tested or incomplete inhibition of signal transduction through the BCR. These studies further highlight how LMP2A regulates EBV latency and persistence through the manipulation of normal signaling pathways in EBV-infected cells. Finally, the prevention of BCR-induced apoptosis by LMP2A is likely key in the development of EBV-associated cancers and may play a role in the development of other diseases in the human host that result from altered immune function.

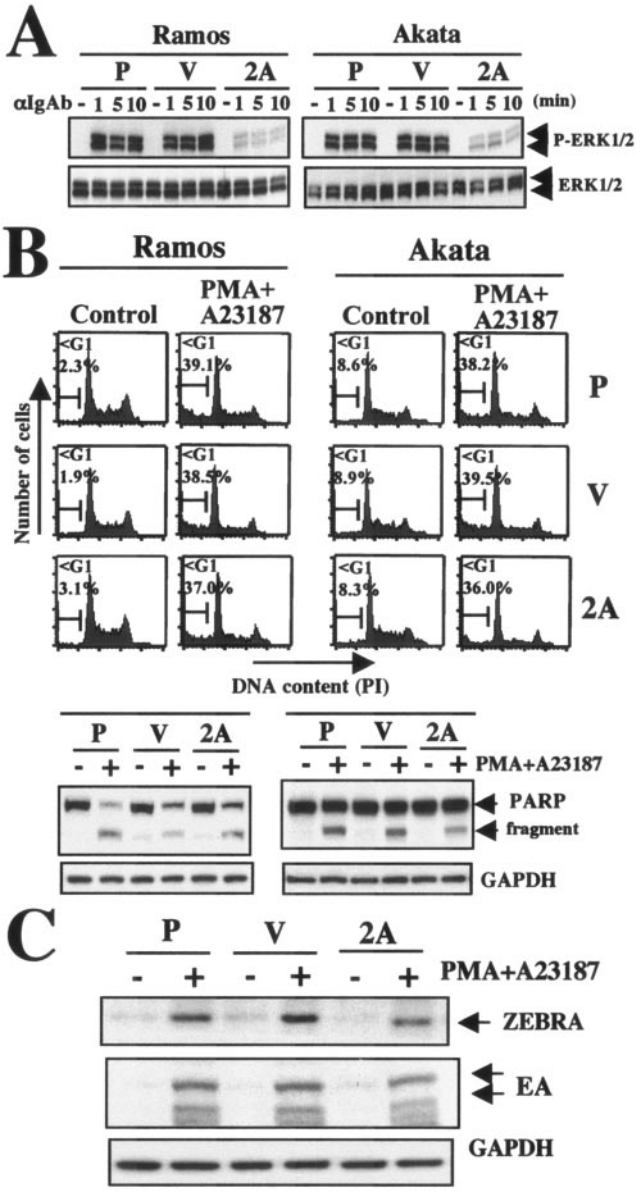

FIG. 4. PMA and calcium ionophore A23187 induce apoptosis and EBV reactivation in LMP2A-expressing Ramos and Akata cells. (A) Parental (P), vector control (V), and LMP2A-expressing (2A) Ramos and Akata cells (1×10^6/ml) were treated without (control [−]) or with of 35 μg/ml anti-IgM antibody or 40 μg/ml anti-IgG antibody for the indicated times (1, 5, or 10 min). Equal amounts of protein from the respective cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. ERK1/2 phosphorylation was detected with anti-phospho MAPK antibody. Upper and lower arrows indicate ERK1 and ERK2, respectively. The lower panels show equal loading of proteins and the expression of total ERK (ERK1/2). (B) DNA fragmentation, evaluation of cleavage of PARP, and (C) EBV reactivation. Parental (P), vector control (V), and LMP2A (2A)-expressing Ramos (3×10^5/ml) and Akata (5×10^5/ml) cells were treated with 20 ng/ml PMA and 7.5 μM A23187. After 24 h of incubation, cell cycles, PARP cleavage, and the expression of ZEBRA and EA were analyzed as described in the legends to Fig. 1 and Fig. 3.

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