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Epstein-Barr Virus Represses the FoxO1 Transcription Factor through Latent Membrane Protein 1 and Latent Membrane Protein 2A▽

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Epstein-Barr virus (EBV) infection is associated with the development of many B-cell lymphomas, including Burkitt's lymphoma, Hodgkin's lymphoma, and posttransplant lymphoproliferative disease. The virus alters a diverse range of cellular molecules, which leads to B-cell growth and immortalization. This study was initiated to investigate the interplay between EBV and a proapoptotic transcription factor target, FoxO1. In this report, we show that EBV infection of B cells leads to the downregulation of FoxO1 expression by phosphatidylinositol 3-kinase-mediated nuclear export, by inhibition of FoxO1 mRNA expression, and by alteration of posttranslational modifications. This repression directly correlates with the expression of the FoxO1 target gene *Bcl-6* and inversely correlates with the FoxO1-regulated gene *Cyclin D2*. Expression of the EBV genes for latent membrane protein 1 and latent membrane protein 2A decreases FoxO1 expression. Thus, our data elucidate distinct mechanisms for the regulation of the proapoptotic transcription factor FoxO1 by EBV.

Epstein-Barr virus (EBV) is a member of the human γ-herpesvirus family. Greater than 90% of the adult population worldwide is infected with the virus. In the majority of cases, EBV infection is asymptomatic for the lifetime of the host due to cytotoxic T-lymphocyte-mediated targeting of infected cells (42). EBV primarily infects B cells but has also been reported to infect T cells and epithelial cells. Primary infection with EBV during adolescence or adulthood can be accompanied by the development of a self-limiting T-cell lymphocytosis known as infectious mononucleosis. However, EBV is also potentially oncogenic. The virus has been detected in malignancies of lymphoid as well as epithelial origin (41). The EBV genome is detected in most cases of posttransplant lymphoproliferative disease, where patients are immunosuppressed and thus cannot control the proliferation of virus-infected cells (36). Essentially every case of endemic Burkitt's lymphoma (BL) is EBV positive, in contrast with between 10 and 90% of sporadic cases. The virus has also been implicated in cases of Hodgkin's lymphoma, nasopharyngeal carcinoma, and some T-cell lymphomas.

The in vitro infection of primary B cells with EBV leads to the establishment of immortalized lymphoblastoid cell lines (LCLs). The cooperative actions of several EBV genes contribute to this effect by generating survival and proliferation signals (56). Activation of the transcription factor NF-κB and the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) signaling pathway has been shown to play an important role in the regulation of survival and proliferation of B cells (6). While many of the targets of NF-κB have been characterized, the nuclear targets of PI3K are relatively poorly characterized for EBV-immortalized B cells.

Proapoptotic forkhead box class O (FoxO) transcription factors are direct targets of PI3K-mediated signal transduction in a variety of cell systems. Phosphorylation of members of this transcription factor family by PKB, the main downstream effector of PI3K activity, results in the nuclear exclusion and inhibition of transcriptional activity (3, 9, 49). FoxO transcription factors coordinate cell cycle progression and cell survival by the activation of antiproliferative genes, such as those encoding $p27^{Kip1}$ and cyclin G2, as well as proapoptotic genes, such as those encoding Fas ligand (FasL), Bcl-6, and the Bcl-2 family member Bim (9, 13, 15, 20, 33, 50). Initial identification of this transcription factor family in humans occurred when three members were identified at chromosomal translocations in human tumors, namely, FoxO1 (FKHR) in alveolar rhabdomyosarcomas, FoxO3a (FKHR-L1) in acute myeloblastic leukemia, and FoxO4 (AFX) in acute lymphocytic leukemia (5, 11, 21, 22, 38). These discoveries were the first indications that FoxO transcription factors have a role in tumor development. Recent studies have demonstrated that a loss of FoxO activities due to protein degradation contributes to cellular transformation of primary breast cancer tumors (23) and mouse primary lymphomas (24).

This study was initiated to investigate the interplay between FoxO1 and EBV. We focused on one member of the FoxO family, FoxO1, and have shown that it is downregulated in EBV-infected B cells. This repression was found to correlate with the expression of two FoxO1 target genes, *bcl-6* and *cyclin D2*. PI3K regulation of FoxO1 protein levels and subcellular localization were also found to differ between EBV-negative and -positive B-cell lines. In addition, two EBV proteins, latent

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membrane protein 1 (LMP1) and LMP2A, have been identified as sufficient for the downregulation of FoxO1 expression.

MATERIALS AND METHODS

Cell culture. All cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics (200 U/ml penicillin and 200 μg/ml streptomycin) and were maintained at 37°C in a 5% CO_2 humidified incubator.

Nuclear and cytosolic protein extraction. Nuclear and cytosolic extracts were generated using a modification of a previously published protocol (8). Following the application of inhibitors as indicated, 1×10^7 cells were harvested and placed on ice. They were washed in 1 ml of hypotonic buffer (10 mM HEPES [pH 7.9], 1.5 mM magnesium chloride, 10 mM potassium chloride, 10 mM phenylmethylsulfonyl fluoride [PMSF]) and centrifuged at 10,000 × *g* for 1 min. Cells were lysed in 100 μl of hypotonic buffer with 0.1% Nonidet P-40 and placed on ice for 5 min. The mixture was centrifuged for 5 min at 10,000 × *g*. The supernatant was removed and retained as the cytosolic fraction. The remaining pellet was incubated with 60 μl of high-salt buffer (20 mM HEPES [pH 7.9]), 420 mM NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 0.5 mM PMSF) to release the transcription factors from the DNA. This mixture was incubated on ice for 15 min and centrifuged for 5 min at 10,000 × *g*. The supernatant was retained as the nuclear fraction and stored at −20°C until further use.

Transfection, Western blotting, and antibody detection. For transient transfections, 1×10^7 DG75 cells from a suspension culture were transfected by electroporation using a Bio-Rad Genepulser II electroporator at 300 V and 950 μF at room temperature in 500 μl growth medium. Following electroporation, the cells were transferred to 3.5 ml of fresh medium per sample and incubated at 37°C in a 5% CO_2 humidified incubator for 20 h. Total protein lysates were generated using the passive lysis buffer provided with the Promega Dual Luciferase reporter assay system (E-1910). The lysates were clarified by centrifugation at 13,000 × *g* for 5 min, and the soluble fraction was added to an equal volume of 2× gel sample buffer (0.1 M Tris buffer, pH 6.8, 0.2 M dithiothreitol, 4% sodium dodecyl sulfate [SDS], 20% glycerol, 0.1% bromophenol blue) and boiled for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham) for immunoblotting. Specific antibody-protein complexes were detected using alkaline phosphatase-conjugated secondary antibodies and CDP-Star (Tropix) chemiluminescence reagent.

Antibodies to poly(ADP) ribose polymerase (PARP; sc-7150), cyclin D2 (sc-593), Bcl-6 (sc-858), and calregulin (sc-11398) were obtained from Santa Cruz Biotechnology and used at a concentration of 200 ng/ml. An antibody to FoxO1 (9462) was purchased from Cell Signaling Technologies and used at a 1/1,000 dilution of the stock supplied. An antibody to actin (A-2066), obtained from Sigma, was used at a 1/1,000 dilution of the stock supplied. Anti-LMP1 (CS.1-4) (45), anti-EBNA2A (PE2) (55), and anti-LMP2A (14B7) (18) monoclonal antibodies have been described previously.

DNA affinity precipitation. Nuclear extracts were diluted with 20 volumes of salt-free buffer (50 mM Tris-HCl, pH 8, 0.25 mM EDTA, 10 mM NaF, 25% glycerol, 0.5 mM PMSF, 10 μl/ml phosphatase inhibitor cocktail I [P-2850; Sigma] and phosphatase inhibitor cocktail II [P-5726], 1 mM $NaVO_4$). Streptavidin-conjugated agarose beads (30 μl of a 50% slurry) and a biotinylated double-stranded oligonucleotide (1 μg) were added to the lysate, which was rotated for 1 h at 4°C. The mixture was centrifuged at 12,000 × *g*, and the supernatant was removed. The beads were washed in buffer three times, and the proteins were eluted from the beads by the addition of 2× gel sample buffer (0.1 M Tris buffer, pH 6.8, 0.2 M dithiothreitol, 4% sodium dodecyl sulfate, 20% glycerol, 0.1% bromophenol blue) for one-dimensional analysis by SDS-PAGE. Proteins were eluted from the beads by the addition of sample buffer {7 M urea, 2 M thiourea, 0.4% 3-[(3-cholamidopropyl)-dimethylamino]-1-propanesulfonate (CHAPS)} for analysis by two-dimensional (2D) electrophoresis. Separated proteins were transferred to PVDF membranes and analyzed using specific antibodies. The sequence of the oligonucleotide corresponding to the *bim* promoter was CAG AGTTACTCCGGTAAACACGCCAGGGAC (15).

2D electrophoresis. DNA affinity-precipitated proteins were eluted from streptavidin-coated agarose beads using 100 μl sample buffer (7 M urea, 2 M thiourea, 0.4% CHAPS). A 7-cm pH 3-10 NL Immobiline Drystrip gel (IPG; Amersham) was rehydrated for 12 h at 20°C with 80 μl of the eluted sample in a total volume of 125 μl of sample buffer supplemented with 50 mM dithiothreitol, 1% bromophenol blue, and 0.5% IPG pH 3-10 NL buffer (Amersham). Isoelectric focusing (IEF) of the samples was performed on an Ettan IPGphor II IEF system using the following program: 1 h at 500 V; 2 h at 1,000 V (gradient); 1 h at 1,000 V and 2 h at 8,000 V (gradient); and 8 h at 8,000 V. IPG strips were then equilibrated for 15 min in equilibration buffer (1× NuPAGE LDS sample buffer; Invitrogen) containing 10× NuPAGE sample reducing agent (0.5 ml) (Invitrogen). The IPG strips were subsequently equilibrated for 15 min in equilibration buffer containing 125 mM iodoacetamide. Equilibrated IPG strips were transferred to the IPG wells of NuPAGE 4 to 12% bis-Tris Zoom gels (Invitrogen) and separated in the second dimension for 1 h at 200 V. Separated proteins were subsequently transferred to PVDF membranes and analyzed by Western blotting as described previously.

Real-time quantitative reverse transcription-PCR. Total cellular RNA was isolated using an RNeasy kit (QIAGEN) according to the manufacturer's instructions. RNAs were treated with RNase-free DNase I (QIAGEN) for subsequent real-time quantitative PCR. Total RNA was reverse transcribed with Superscript III reverse transcriptase (Invitrogen). The resulting cDNAs were amplified using an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) with the following primer pairs: *FOXO1*-sense (TGG ACA TGC TCA GCA GAC ATC), *FOXO1*-antisense (TTG GGT CAG GCG GTT CA), *L19*-sense (GCG GAA GGG TAC AGC CAA T), and *L19*-antisense (GCA GCC GGC GCA AA). *L19*, a nonregulated ribosomal housekeeping gene, was used as an internal control to normalize input RNA. All measurements were performed in triplicate (28).

Plasmids. The pSG5 empty vector, pSG5-LMP1 (25), pSG5-LMP1^{AAA} (14), and pSG5-LMP2A (29) have been described previously. The Bcl-6ΔBcl-6-luciferase reporter was a kind gift from Tracy Tang and Laurence Lasky (Genentech Inc., South San Francisco, Calif.) and has been described previously (16, 50). The phRL-SV40 luciferase reporter vector was purchased from Promega (E-6261).

Inducible expression of LMP1, LMP2A, and EBNA2A in stable transfectants. Stable DG75 transfectants containing an inducible LMP1, LMP2A, or EBV nuclear antigen 2A (EBNA2A) gene (17) were maintained under drug selection and in 1 μg/ml tetracycline until required. Prior to each experiment, cells were washed five times in RPMI 1640 medium and were recultured without drug selection and in the presence or absence of 1 μg/ml tetracycline for a period of either 24 or 48 h. For the generation of total cell lysates, cells were counted on a hemocytometer and resuspended in 50 μl phosphate-buffered saline per 10^6 cells. An equal volume of 2× gel sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 0.4 M sodium 2-mercaptoethane sulfonate, 4% SDS, 0.02% bromophenol blue) was added, and the cells were sonicated using a W385 sonicator (Heat Systems Ultrasonics). Following sonication, samples were heated at 100°C for 5 min. The solubilized proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Amersham) for immunoblotting as described above.

RESULTS

FoxO1 expression is downregulated by EBV infection. To investigate the relationship between EBV infection and FoxO1 expression, nuclear FoxO1 protein levels were analyzed by Western blotting, using cell lines with different EBV status and a specific antibody. PARP protein levels were also analyzed to verify equal protein loading, as PARP is found in the nucleus and PARP expression levels are not affected by EBV status. BL41, BL41+B95.8, and IARC-171 are from the same individual and thus have the same genetic background. They differ in their patterns of EBV gene expression and the presence of the *myc* translocation in BL41 that is typical of Burkitt's lymphoma cells (44). BL41 is an EBV-negative BL line. BL41+B95.8 is the same line after infection with the B95.8 strain of EBV. IARC-171 is an EBV B95.8-immortalized LCL derived from the same patient as BL41 (Fig. 1a). Our results show high levels of FoxO1 protein expression in the EBV-negative BL line (BL41). In contrast, FoxO1 expression was markedly downregulated in EBV-immortalized B cells (IARC-171). To investigate whether the presence of EBV was the cause of downregulation, FoxO1 expression was also analyzed in BL41+B95.8 cells. Less FoxO1 was detected in BL41+B95.9 cells than in BL41 cells, suggesting that EBV gene products have a role in downregulating FoxO1 protein expression.

EBV infection of B cells can lead to the establishment of distinct latency programs as a result of the different expression

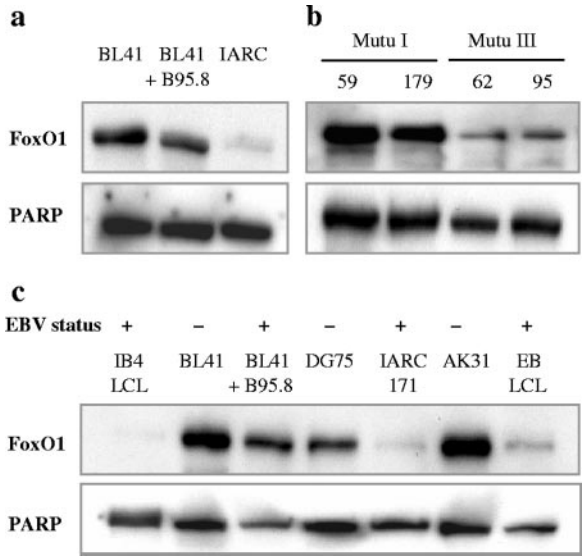

FIG. 1. EBV infection downregulates FoxO1 expression. Nuclear protein extracts were generated from the following cell lines: (a) a Burkitt's lymphoma cell line (BL41), an EBV-infected Burkitt's lymphoma line (BL41+B95.8), and a lymphoblastoid B-cell line (IARC-171); (b) an EBV-positive Burkitt's line expressing latency I genes (Mutu I, clones 59 and 179) and an EBV-positive Burkitt's line expressing latency III genes (Mutu III, clones 62 and 95); and (c) EBV-negative BL lines (BL41, DG75, and AK31), an EBV-positive BL line (BL41+B95.8), and lymphoblastoid cell lines (IB4-LCL, IARC-171, and EB-LCL). Proteins were resolved by SDS-PAGE, and FoxO1 protein levels were determined by immunoblotting with a specific FoxO1 antibody. PARP was used as a loading control.

patterns of viral genes (27). To establish whether the latency program affects FoxO1 expression, protein levels were compared using four EBV-positive sublines of a BL tumor. Mutu I cells express only EBNA1 and two small polyadenylated RNA molecules (EBER1 and EBER2), a restricted pattern of viral gene expression termed latency I. Mutu III cells express the full complement of EBV latency genes (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP, LMP1, LMP2, EBER1, EBER2, and BamHI A rightward transcripts), termed the latency III pattern (43). Our results clearly demonstrate that a latency III pattern of viral gene expression is required for the repression of FoxO1 protein levels (Fig. 1b).

Additional EBV-negative and -positive lines were also tested for FoxO1 protein expression (Fig. 1c). Three EBV-negative Burkitt's lymphoma lines were analyzed. BL41 is described above. DG75 is another EBV-negative BL line (2). AK31 is an EBV-negative subclone of Akata (26), an EBV-positive BL line displaying a latency I pattern of gene expression. Again, FoxO1 protein expression was high in EBV-negative tumor lines. Three LCLs were also tested. IARC-171 is described above. IB4-LCL is an LCL generated from cord blood B cells immortalized with EBV B95.8 (46). EB-LCL is an LCL generated in-house by infection of primary B cells from the blood of a healthy donor with the B95.8 strain of EBV. In accordance with previous results, FoxO1 expression was very low in EBV-immortalized B cells. The level of FoxO1 expression in the BL41+B95.8 cells was again between those observed in BL41 and IARC-171 cells.

PI3K inhibition increases nuclear FoxO1. One mechanism whereby FoxO activity is regulated is by nuclear exclusion and subsequent degradation once it is in the cytoplasm. Stimulation of the PI3K pathway has a central role in this process by activating the kinase activity of PKB. FoxO transcription factors contain three consensus PKB phosphorylation sites that, when phosphorylated, target the proteins for nuclear exclusion, thereby increasing the survival potential of the cell (4). In order to investigate the role of PI3K in regulating the subcellular localization and protein levels of FoxO1, a specific inhibitor of PI3K, LY294002, was employed (52). BL41 and IARC-171 cells were either left untreated or treated with increasing doses of LY294002. Nuclear and cytosolic FoxO1 protein levels were subsequently analyzed by Western blotting.

Although basal levels of FoxO1 were relatively high in the nuclei of BL41 cells, this could be increased further following PI3K inhibition by LY294002 for 1 h (Fig. 2a). In parallel with this increase, a decrease in cytosolic FoxO1 was observed following LY294002 treatment. In contrast, no nuclear increase or cytosolic decrease in FoxO1 level could be detected in the LCL (IARC-171) after 1 h of treatment with the PI3K inhibitor (data not shown). Only after treatment with LY294002 for 24 h could an increase in FoxO1 nuclear protein be detected (Fig. 2b). This increase was not accompanied by a decrease in cytosolic FoxO1. These results show that PI3K inhibition can increase the FoxO1 level in both cell types, but with distinct mechanisms, suggesting a difference in the ways in which PI3K regulates FoxO1 activity in cells with different EBV statuses.

The slow kinetics of upregulation of FoxO1 by treatment with LY294002 in IARC-171 cells suggests a different mechanism of regulation in these cells. For this reason, we investigated whether FoxO1 repression by EBV could occur at the transcriptional level. Total RNAs were purified from BL41, BL41+B95.8, and IARC-171 cells, and FoxO1 mRNA activity was measured relative to that of a housekeeping gene (L19) by real-time PCR. The results showed that steady-state levels of FoxO1 mRNA were reduced by half in EBV-infected cells compared to those in EBV-negative cells (Fig. 2c), indicating that FoxO1 is downregulated at the transcriptional level by EBV.

FoxO1 can bind DNA and is posttranslationally modified in EBV-infected cells. DNA binding is required for transcription factors to regulate target genes and downstream effects. It was therefore important to check whether the FoxO1 protein detected was capable of binding DNA for mediation of its effects on cell proliferation and survival. DNA affinity precipitation experiments were therefore carried out using an oligonucleotide containing a forkhead response element (5′-TAAACAC-3′) from the *bim* promoter (15). FoxO1 molecules from nuclear extracts of BL41 and BL41+B95.8 cells, either left untreated or treated with LY294002 (20 μM) for 1 h, were DNA affinity precipitated using the *bim* promoter oligonucleotide. FoxO1 DNA binding was subsequently analyzed by Western blotting (Fig. 3a). DNA binding of FoxO1 to the *bim* promoter oligonucleotide was detected in untreated BL41 and BL41+B95.8 cells, confirming that the FoxO1 protein detected is capable of binding to target promoter sequences. The effect of PI3K inhibition on DNA binding was also tested. Following LY294002 treatment, the amount of DNA-bound FoxO1 was significantly increased, to similar degrees, in both BL41 and

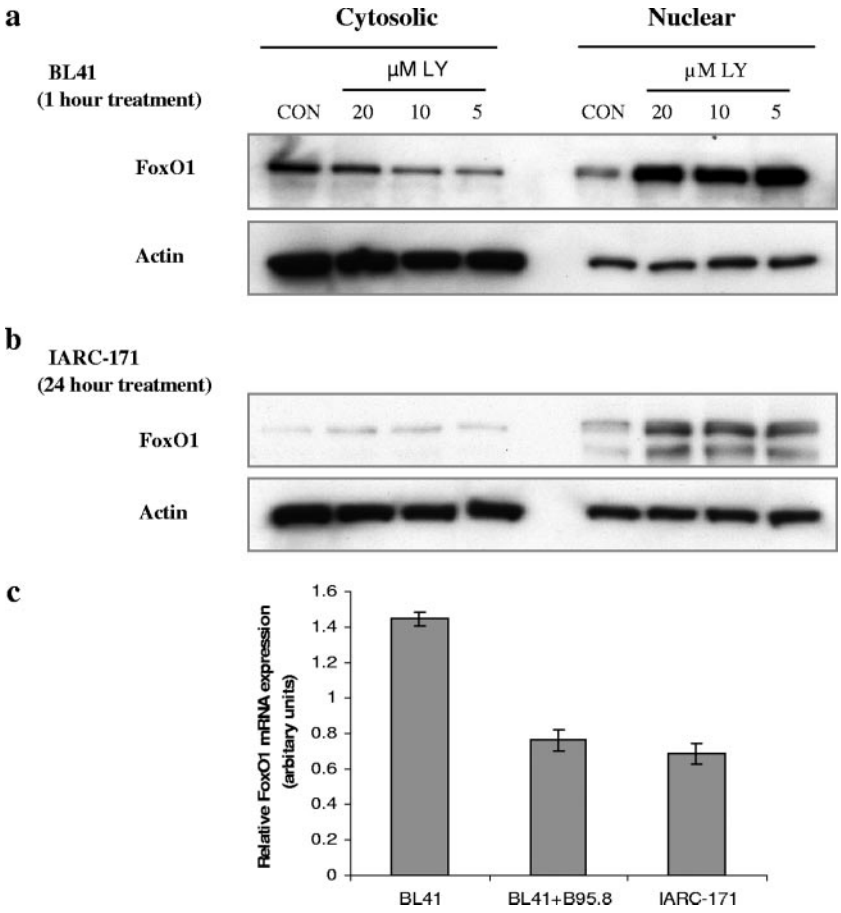

FIG. 2. PI3K inhibition can increase nuclear FoxO1 in all cell types, but with different kinetics. Nuclear and cytosolic protein extracts were generated from (a) BL41 and (b) IARC-171 B-cell lines. Proteins were resolved by SDS-PAGE and immunoblotted with the anti-FoxO1 antibody. Cells were either left untreated or treated with various concentrations of LY294002 (20 μM, 10 μM, and 5 μM) for either 1 h (BL41) or 24 h (IARC-171). Human anti-actin was used as a loading control. (c) The expression of FoxO1 RNA was analyzed by real-time PCR and normalized to the level of L19. The results shown are the averages of triplicate results.

BL41+B95.8 cells. This suggests that a direct correlation exists between the amount of FoxO1 localized within the nucleus and DNA binding.

The role of posttranslational modifications in the regulation of transcription factor activity is well established. Phosphorylation events are well known to have important effects on the activities of many transcription factors. FoxO members contain multiple PKB phosphorylation sites that mediate subcellular localization and DNA binding activity. Both ubiqitinylation and acetylation have also been reported to modulate members of the FoxO family by directing degradation and attenuation of DNA binding, respectively (24, 32, 39). To establish how many isoforms of FoxO1 exist in the nuclei of EBV-negative and -positive BL41 cells, FoxO1 was analyzed by 2D electrophoresis (Fig. 3b). DNA affinity-precipitated FoxO1 eluted from *bim* promoter oligonucleotides was isoelectrically focused, using a pH 3-to-10 nonlinear immobilized pH gradient, and subsequently separated in the second dimension by SDS-PAGE. Western blot detection of FoxO1 with a specific antibody revealed that multiple isoforms of FoxO1 bind to DNA in BL41+B95.8 cells. The isoelectric points (pI) of the different FoxO1 isoforms range between 5.4 and 6.1, and the isoforms are present in various amounts. The most abundant isoform has a pI of approximately 5.7. In contrast, only one isoform of FoxO1 could be detected in BL41 cells, with a lower pI value of approximately 4.7. We can therefore conclude that FoxO1 is posttranslationally modified and that multiple isoforms of FoxO1 are capable of binding DNA in EBV-positive cells (BL41+B95.8) but not in an EBV-negative B-cell line (BL41).

FoxO1 expression correlates with Bcl-6 expression and inversely correlates with cyclin D2 expression. Having established that the FoxO1 protein detected was capable of binding DNA, it was then important to determine whether DNA binding initiated transcriptional activation and to measure the effect of EBV on target gene activity. To address these issues, expression levels of FoxO1 target genes were analyzed by using various cell lines (Fig. 4). Bcl-6 is a sequence-specific transcriptional repressor of proteins mediating lymphocyte apoptosis and differentiation, and *bcl-6* transcription is activated by FoxO1 (50). Nuclear Bcl-6 from various cell lines was analyzed by Western blotting. A direct correlation between FoxO1 and Bcl-6 protein levels was observed, with a high level of Bcl-6 expression in cells with high FoxO1 expression and less Bcl-6 detected in EBV-transformed LCL cells. The Bcl-6 levels did

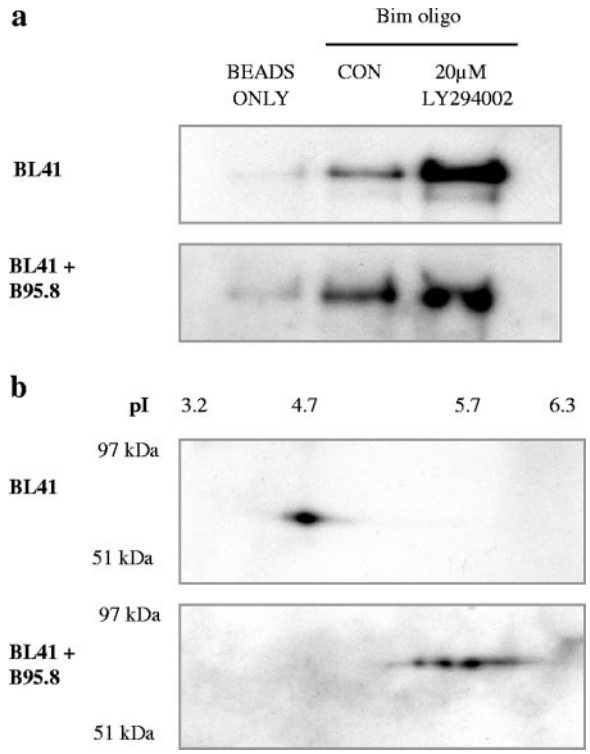

FIG. 3. FoxO1 can bind DNA and is present in multiple forms. Nuclear protein extracts were generated from BL41 and BL41+B95.8 cells. Nuclear proteins were subsequently DNA affinity precipitated using streptavidin-coated agarose beads with or without biotinylated *bim* oligonucleotides. Bound proteins were eluted, resolved by SDS-PAGE, and immunoblotted using the anti-FoxO1 antibody. (a) BL41 and BL41+B95.8 cells left untreated or treated with LY294002 (20 μM, 1 h). (b) DNA affinity-precipitated (using the *bim* oligonucleotide) proteins from BL41 and BL41+B95.8 cells treated with LY294002 (20 μM, 1 h) were analyzed by 2D electrophoresis. Eluted proteins were isoelectrically focused using a pH 3-to-10 nonlinear immobilized pH gradient and separated in the second dimension by SDS-PAGE. FoxO1 isoforms were detected by immunoblotting with the anti-FoxO1 antibody.

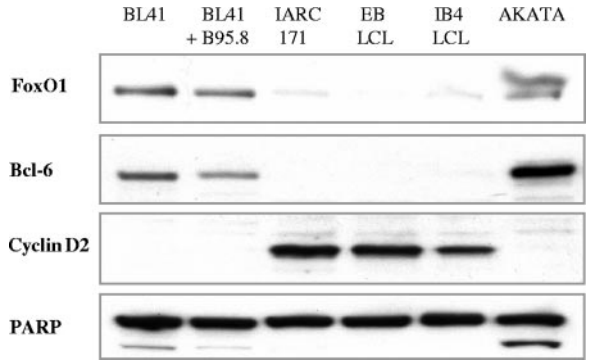

FIG. 4. Expression of FoxO1 correlates with expression of Bcl-6 and inversely correlates with expression of cyclin D2. Nuclear protein extracts were generated from the following B-cell lines with various EBV statuses: BL41, an EBV-negative BL line; BL41+B95.8, an EBV-positive BL line (latency III); three EBV-positive LCLs (IARC-171, EB-LCL, and IB4-LCL); and Akata, an EBV-positive BL line expressing the latency I pattern. Proteins were resolved by SDS-PAGE and subsequently analyzed by immunoblotting with specific antibodies to FoxO1, Bcl-6, and cyclin D2. PARP was used as a loading control.

not correspond with EBV status per se, as Akata cells, which are EBV positive but have a latency I pattern of gene expression, expressed high levels of FoxO1 and Bcl-6. This agrees with the data for Mutu I cells and suggests that one or more of the EBV genes expressed in latency III are likely to regulate FoxO1. Cyclin D2 protein levels were also analyzed. Cyclin D2 is a cell cycle protein required for progression through the G_1 phase of the cell cycle and has been shown to be repressed transcriptionally by FoxO (16, 47). Western blot analysis showed an inverse correlation between cyclin D2 expression and the expression of both FoxO1 and Bcl-6. These data suggest that FoxO1 is transcriptionally active and that the repressive effect that EBV has upon FoxO1 also extends to its target genes.

Both LMP1 and LMP2A can downregulate FoxO1 expression. The establishment of EBV latency requires the expression of a repertoire of EBV-carried latent genes. LMP1 is the major transforming protein of EBV and is required for the transforming effects of EBV on primary B cells. LMP1 mimics constitutively activated CD40, a member of the tumor necrosis factor receptor superfamily, mediating ligand-independent signaling through a range of key signaling pathways essential for survival, including the NF-κB, mitogen-activated protein kinase, Jun N-terminal protein kinase (JNK), p38, and JAK/STAT pathways as well as the PI3K pathway (56). LMP2A, which is also encoded by EBV, is another potent activator of the PI3K pathway, acting as a constitutively activated B-cell receptor (BCR), thereby inhibiting normal signaling through the BCR (34, 48). Since the activities of both LMP1 and LMP2A have been shown to activate PI3K signaling, it was reasonable to suspect that they may have a role in the repression of FoxO1.

To test this hypothesis, DG75 cells, which express high levels of FoxO1, were transiently transfected with increasing amounts of either an LMP1 or LMP2A expression vector. After 20 h, cells were harvested and lysed, and FoxO1 and Bcl-6 protein levels were analyzed by Western blotting. DG75 cells transfected with LMP1 demonstrated reduced levels of FoxO1 and Bcl-6 protein expression in a dose-dependent manner (Fig. 5a). A reporter plasmid carrying a *Bcl-6* promoter containing a FoxO consensus binding sequence (50) was also repressed by LMP1 in a dose-dependent manner (data not shown). Protein analysis of LMP2A-transfected cells showed a similar decrease of FoxO1 expression. A clear downregulation of Bcl-6 was also detected with transfection of 10 μg of LMP2A. However, at the lower doses of 1 and 5 μg, LMP2A did not repress Bcl-6 protein expression (Fig. 5b). Transfection of DG75 cells with an EBNA2A expression vector did not repress either the protein level of FoxO1 or Bcl-6 or the transcriptional activity of the Bcl-6 gene (not shown).

To further analyze the effects of LMP1, LMP2A, and EBNA2A on protein levels of FoxO1 and Bcl-6, protein expression levels were analyzed in stable DG75 transfectants containing an inducible LMP1, LMP2A, or EBNA2A gene. Protein levels were compared with those detected in two lymphoblastoid cell lines, namely, IARC-171 and an early-passage LCL (CMc) generated from the B cells of a healthy donor with the B95.8 strain of EBV (Fig. 6). In the presence of tetracy-

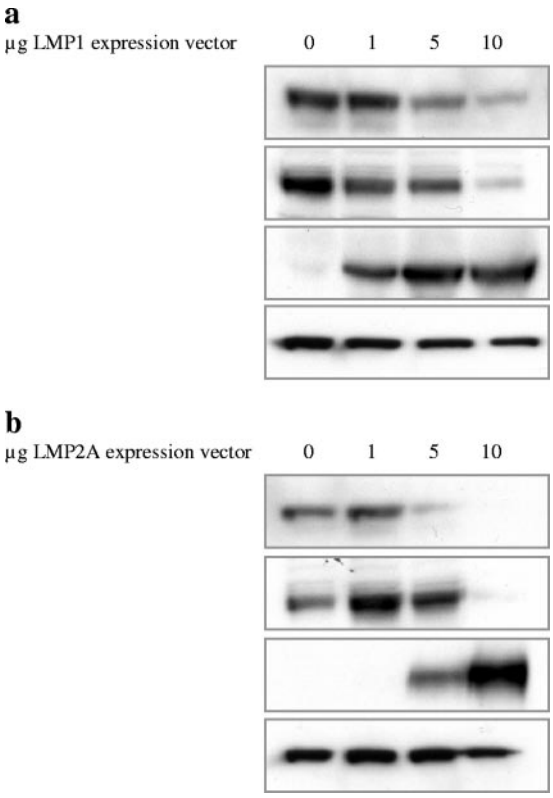

FIG. 5. Roles for LMP1 and LMP2A in FoxO1 downregulation. DG75 cells were transfected with various amounts of (a) LMP1 and (b) LMP2A expression vectors. Cells were harvested, and lysates generated were analyzed by SDS-PAGE and Western blotting. Protein levels of FoxO1 and Bcl-6 were analyzed using specific antibodies. LMP1 and LMP2A protein levels were also checked using specific antibodies. Actin was used as a loading control.

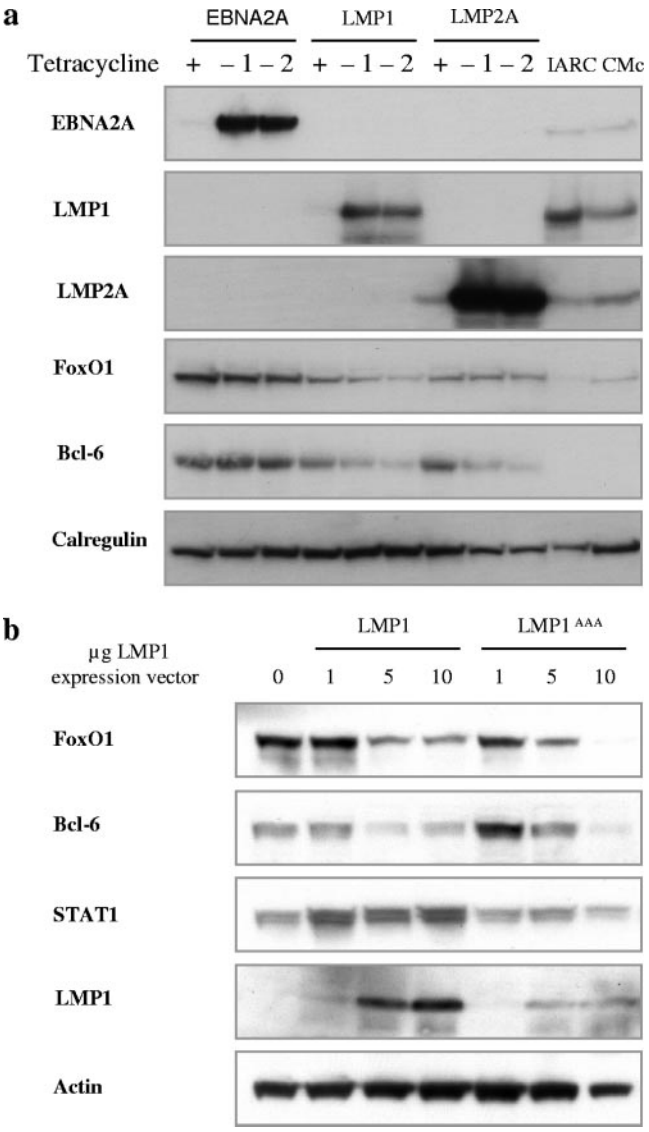

FIG. 6. Inducible LMP1 and LMP2A downregulate FoxO1 and Bcl-6. (a) Stable DG75 transfectants containing an inducible EBNA2A, LMP1, or LMP2A gene were maintained under drug selection and with 1 μg/ml tetracycline until required for experiments. When required, cells were washed five times with RPMI 1640 medium and were recultured without drug selection and in the presence or absence of 1 μg/ml tetracycline for a period of either 24 (−1) or 48 h (−2). Protein levels of FoxO1 and Bcl-6 were subsequently analyzed using specific antibodies. Protein expression levels were also analyzed in the IARC-171 and CMc lymphoblastoid cell lines. EBNA2A, LMP1, and LMP2A protein levels were also checked using specific antibodies. Calregulin was used as a loading control. (b) In a similar experiment to that described in the legend to Fig. 5, three different amounts of an expression vector for wild-type and a mutant LMP1 ($LMP1^{AAA}$) were transiently transfected into DG75 cells. Cells were harvested, and the lysates generated were analyzed by SDS-PAGE and Western blotting. Protein levels of FoxO1, Bcl-6, LMP1, and STAT1 were analyzed using specific antibodies. Actin was used as a loading control.

cline, protein expression of FoxO1 and Bcl-6 was clearly detectable in EBNA2A-, LMP1-, and LMP2A-inducible lines, although FoxO1 expression was higher in the EBNA2A line and the parent DG75 cells (not shown). Upon induction of LMP1 gene expression by reculturing the cells in the absence of tetracycline, clear decreases in both FoxO1 and Bcl-6 levels were detected at 24 h and, to a greater degree, at 48 h. The induction of LMP2A expression did not significantly reduce FoxO1 expression. However, the FoxO1 level was lower in the DG75 cells that contained LMP2A, even when they were cultured in the presence of tetracycline, than in cells with the tetracycline transactivator alone (not shown) or cells expressing EBNA2A. The cells clearly also expressed LMP2A. However, the induction of higher levels of LMP2A caused a decrease in Bcl-6 protein levels, suggesting that LMP2A may contribute to the regulation of this protein in a FoxO1-independent fashion. Similar to the LMP2A level, the FoxO1 level in the LMP1-inducible line was reduced compared to that in the EBNA2A line. Together, these observations demonstrate the sensitivity of FoxO1 protein expression to the residual low levels of both LMP1 and LMP2A signals in the presence of tetracycline. Induction of EBNA2A did not affect the expression of FoxO1 or Bcl-6. The results from the transiently transfected and stable lines demonstrate that both LMP1 and LMP2A can downregulate the expression of FoxO1.

LMP1 gave a more dramatic downregulation of FoxO1 than did LMP2A. There has been significant analysis of LMP1 sig-

naling that has identified at least two different signaling domains, CTAR1 and CTAR2. CTAR1 has been shown to activate PI3K in epithelial cells and fibroblasts (12, 30). To determine whether CTAR1 can regulate FoxO1, we expressed a mutant that had three amino acids changed in this domain, entitled LMP1^{AAA}, where proline 204, glycine 206, and threonine 208 were all mutated to alanine. Three different amounts of an expression vector for wild-type LMP1 or LMP1^{AAA} were transiently transfected into DG75 cells, and the cells were left for 24 hours. After this time, the cells were harvested and lysed by the addition of gel loading buffer. The protein extract was resolved by SDS-PAGE and transferred to a PVDF membrane, and the levels of four different proteins were detected by specific antibodies (Fig. 6b). Both wild-type LMP1 and the LMP1^{AAA} mutant were able to cause a decrease in FoxO1 and Bcl-6 protein levels. However, only wild-type LMP1 was able to induce STAT1, a transcription factor that is increased by LMP1 through an NF-κB pathway (40). These data show that distinct domains of LMP1 are involved in the distinct regulation of transcription factor protein levels and that this may not be due to the direct activation of PI3K by this molecule.

DISCUSSION

This study was performed to investigate the interplay between EBV and FoxO1. Our data clearly demonstrate that EBV has a repressive effect on FoxO1 protein expression in B cells and that this effect can be mediated by both latent membrane proteins 1 and 2A. The repression of FoxO1 may be due to the ability of these membrane proteins to activate PI3K signaling, although our data show that EBV can also repress FoxO1 mRNA levels, suggesting alternative mechanisms for the regulation of this molecule.

FoxO1 has a well-established role in the regulation of cell survival, and thus it is logical to suspect that it is a target of regulation by EBV. It is important, however, that all of the cell lines used in this study proliferate, suggesting that FoxO1 regulation can be circumvented, perhaps by the c-*myc* oncogene, in Burkitt's lymphoma cells. In this study, a correlation was observed between EBV status and the activities of the FoxO target genes *Bcl-6* and *cyclin D2*, which are essential for B-cell proliferation and development. The level of Bcl-6, a direct target gene for activation by FoxO (50), correlated with FoxO1 expression. However, this does not prove that Bcl-6 is a direct target in these cells. The introduction of a FoxO1 transgene into an LCL, probably in some system that allows regulated gene expression, could be used to directly identify FoxO1 target genes in EBV-immortalized cells. Interestingly, Bcl-6 is a transcriptional repressor of both lymphocyte differentiation and apoptosis and is the most frequently targeted proto-oncogene in non-Hodgkin's lymphomas (54). LMP1 is sufficient for this pathway, as inducible expression of LMP1 and transfection of a plasmid encoding LMP1 were enough to repress both FoxO1 and the target gene encoding Bcl-6. An inverse correlation between LMP1 and Bcl-6 has previously been described at the transcriptional and protein levels for the B cells of transgenic mice expressing LMP1 or a chimeric LMP1-CD40 molecule (37). Other studies have also observed the repressive effect of CD40 activation or EBV genome expression on Bcl-6 expression in B cells and dendritic cells (1, 10, 35). The function of Bcl-6 repression by LMP1 is believed to be germinal center suppression, allowing exit of EBV-infected cells from the germinal center. This study identifies FoxO1 as a molecular intermediate by which LMP1 can regulate Bcl-6.

The role of LMP2A in the regulation of FoxO1 and Bcl-6 is more complex. The activation of PI3K through LMP2A has previously been shown to induce phosphorylation of FoxO1 in epithelial cells (34), which subsequently targets FoxO1 for degradation. However, the effects of LMP2A on the total protein levels of both FoxO1 and Bcl-6 had not been defined previously. A clear downregulation of FoxO1 protein expression was observed in this study upon LMP2A transient transfection, but this did not translate to a corresponding dose-responsive decrease in Bcl-6 expression. However, induction of LMP2A in stable transfectants did result in a clear repression of Bcl-6 protein expression. This may be due to differences in the time or level of expression. In the system with stable expression, more LMP2A is expressed for a longer time than the 20 h studied for the transient transfections.

In contrast to Bcl-6, an inverse correlation was observed between cyclin D2 protein expression and that of FoxO1. We have previously shown that PI3K regulates the cyclin D2 protein (7) and the *cyclin D2* promoter (53), so this may be due, in part, to FoxO1. However, the repressive effect of FoxO transcription factors on cyclin D2 transcription may be indirect (47), perhaps through Bcl-6, via an interaction with the STAT5 transcription factor (16). The *cyclin D2* gene has a complex promoter and is subject to regulation by a diverse range of cellular stimuli (31, 53).

Our data have revealed a difference in the kinetics of FoxO1 regulation by the PI3K pathway between EBV-negative BL cells and LCLs. In IARC-171 cells, 24 h of incubation with LY294002 was required to detect an increase in nuclear FoxO1, but 1 h was sufficient for an increase of a similar level in the nuclei of EBV-negative BL41 cells. This suggests the presence of extra mechanisms for the repression of FoxO1 in EBV-immortalized cells, an observation supported by the detection of lower levels of FoxO1 mRNA in these cells. A difference in the contributions of the PI3K pathway towards the survival of these two cell types is evident when analyzing cell survival after treatment with the PI3K inhibitor (7). EBV-negative BL lines rapidly undergo apoptosis; in contrast, LCL lines do not die but are growth arrested. This difference in sensitivity to LY294002 is also seen for other agents and may be due, in part, to the delay in induction of FoxO1 as well as other molecules, such as NF-κB, that are increased in EBV-immortalized cells.

Our 2D-electrophoretic analysis of DNA-bound FoxO1 showed that posttranslational modification patterns are distinct for EBV-positive B cells (BL41+B95.8) compared with those for EBV-negative B cells (BL41), suggesting a further level of control of FoxO1. Interestingly, there was also more than one band present in IARC-171 nuclear extracts, particularly when they were treated with LY294002 (Fig. 2a). Changes in molecular weight are often indicative of posttranslational modification. However, the small amount of FoxO1 in these cells precluded the analysis of FoxO1 by 2D gel electrophoresis. It is possible that the multiple isoforms of FoxO1 detected bound to DNA in EBV-positive cells are differentially phosphorylated forms, but this is unlikely, as phosphorylation tar-

gets FoxO1 molecules for nuclear exclusion and thus would not be easily detected by this assay. Acetylation of nuclear FoxO proteins has also been reported to affect the transcriptional programs controlled by FoxO proteins due to interference with the balance of coactivator and corepressor recruitment (19, 39, 51). Again, a role for the PI3K pathway is evident, as acetylation was reported to increase the sensitivity of FoxO1 to phosphorylation, contributing to degradation (32). A recent study demonstrated the role of ubiquitinylation by the Skp2/Cul-1/F-box-protein–ubiquitin complex in the targeting of FoxO1 molecules for degradation following PKB activation (24). However, the pattern of spots observed did not show a significant increase in the molecular weight of the protein, which would be suggested following ubiquitinylation. Thus, while pathways activated by EBV lead to differential modifications of FoxO1, as detected in BL41+B95.8 cells, that may repress transcriptional activity, their identity is unknown and currently under investigation.

Integrating the data generated in this study provides evidence that EBV regulates FoxO1 expression by three distinct mechanisms, i.e., the prototypic repression of FoxO1 by PI3K, alternative posttranslational modifications observed in BL41 cells infected by EBV (BL41+B95.8 cells), and a repression of FoxO1 expression at the mRNA level. These diverse mechanisms ensure that FoxO1 expression and activity are inhibited. The existence of these distinct mechanisms suggests that repressing FoxO1 activity is an important part of EBV infection of B cells.

In summary, our data identify FoxO1 as an EBV-regulated transcription factor and, as such, add to a body of work demonstrating the suppression of proapoptotic pathways by EBV. Furthermore, we have identified FoxO1 as one of the few proteins regulated by both LMP1 and LMP2A. Putting this in context, LMP1 and LMP2A have been shown to activate a diverse range of signaling pathways contributing to cell survival. These include the activation of tyrosine kinases, mitogen-activated protein kinases, JNK, p38, the transcription factor NF-κB, and the PI3K pathway. Distinctly, this study describes another nuclear target by which EBV latent membrane proteins work to shift the balance of anti- and proapoptotic pathways towards survival. The interplay between EBV and FoxO1 is likely to contribute to the characteristic apoptotic resistance of immortalized B cells in the context of EBV-associated malignancies.

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