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# Latent Membrane Protein 1 of Epstein–Barr Virus Plays an Important Role in the Serum Starvation Resistance of Epstein–Barr Virus-Immortalized B Lymphocytes

**Chan-Hee Park,[1] Hak-Ryul Kim,[2] Ju Kim,[1] Sun-Hee Jang,[1] Kyung-Yeol Lee,[1] Gook-Hyun Chung,[1] and Yong-Suk Jang[1]***

[1]Division of Biological Sciences and the Institute for Molecular Biology and Genetics, Chonbuk National University, Chonju 561-756, Korea
[2]Department of Animal Science and Biotechnology, Kyungpook National University, Daegu 702-701, Korea

**Abstract** We have previously shown that SNU-1103, which is a latency type III Epstein–Barr virus (EBV)-transformed lymphoblastoid cell line (LCL) that was developed from a Korean cancer patient, resists serum starvation-induced $G_1$ arrest. In this study, we examined the role of latent membrane protein-1 (LMP-1) in serum starvation resistance, since LMP-1 is known to be essential for EBV-mediated immortalization of human B lymphocytes. The *LMP-1* gene from SNU-1103 was introduced into the EBV-negative BJAB cell line, and shown to be associated with resistance to $G_1$ arrest during serum starvation. Western blot analyses of the LMP-1-transfected cells revealed several protein alterations as compared to vector-transfected control cells. The expression of key cell-cycle regulatory proteins was affected in the $G_1$ phase: the expression of cyclin D3, CDK2, p27, and E2F-4 was up-regulated, and the expression of cyclin D2, CDK6, p21, and p103 was down-regulated during serum starvation. These results imply that of the several EBV viral genes expressed in EBV-negative B lymphoma cells, LMP-1 mediates resistance to serum starvation-induced $G_1$ arrest. However, we cannot rule out the possibility that other EBV genes are also involved in the cell-cycle progression of the EBV-transformed LCL during serum starvation, since the altered protein expression profile of the LMP-1 transfectants was distinct from that of the SNU-1103 cells that expressed all of the EBV viral proteins. J. Cell. Biochem. 91: 777–785, 2004. 

**Key words:** cell cycle; Epstein–Barr virus; latent membrane protein; serum starvation

Epstein–Barr virus (EBV) is a human Herpes virus that has the ability to immortalize human B lymphocytes. EBV is associated with a variety of human malignant diseases, such as Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease, and lymphoproliferative disorders in immunodeficient individuals [Kawa, 2000; Okano and Gross, 2000]. EBV infection of resting B cells in vitro results in the establishment of an EBV-carrying lymphoblastoid cell line (LCL). In these cells, gene expression is restricted to a limited set of latent EBV genes, which includes six nuclear proteins (EBNA-1, -2, -3A, -3B, -3C, and -LP), two small nuclear RNAs (EBER-1 and -2), and three integral membrane proteins, such as latent membrane protein-1 (LMP-1), -2A, and -2B [Farrell, 1995]. Experiments with recombinant viruses have established that the *EBNA-2*, *-3A*, *-3C*, *-LP*, and *LMP-1* genes are essential for primary B-cell transformation [Kaye et al., 1993; Tomkinson et al., 1993; Kempkes et al., 1995]. Of these, LMP-1, which consists of six transmembrane segments, has been known to play a central role in B-cell immortalization. Particular interest has focused on LMP-1 because it is considered to be a classical oncogene, as evidenced by its ability to transform rodent fibroblast cell lines and human epithelial cells, thereby rendering them tumorigenic [Wang et al., 1985; Baichwal and Sugden, 1988; Hu et al., 1993; Nicholson

Abbreviations used: EBV, Epstein–Barr virus; LCL, lymphoblastoid cell line; LMP, latent membrane protein.

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*Correspondence to: Dr. Yong-Suk Jang, Division of Biological Sciences, Chonbuk National University, Chonju 561-756, Korea. E-mail: yongsuk@chonbuk.ac.kr

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et al., 1997]. Thus, experiments involving *LMP-1* gene transfection into EBV-negative B-lymphoma cell lines have shown that LMP-1 expression causes up-regulation of anti-apoptotic genes, such as *bcl-2* and *mcl-1* [Henderson et al., 1991; Rowe et al., 1994; Wang et al., 1996].

Serum starvation usually leads to cell-cycle arrest in the $G_0$–$G_1$ phase and/or apoptosis after prolonged incubation [Grimm et al., 1996; Bissonnette and Hunting, 1998; Yoshida and Beppu, 1998; Hasan et al., 1999]. However, we have shown previously that serum starvation of the EBV-positive LCL did not lead to cell-cycle arrest, as compared with EBV-negative control cells [Kim et al., 2002]. For example, the EBV-positive LCL continued its cell-cycle progression during serum starvation, while the EBV-negative control cells were arrested at the $G_1$ phase, and several important cell-cycle regulatory proteins were abnormally or differentially expressed in the EBV-positive LCL cells as compared to those of the control EBV-negative cells during serum starvation. These results prompted us to identify the key viral factors in these processes. In this study, we investigated the role of the EBV protein LMP-1, which is a key viral protein for B-cell immortalization, in the serum starvation resistance of EBV-immortalized B lymphocytes. Initially, we introduced the full-length *LMP-1* gene, which had been isolated from the EBV-positive LCL, into EBV-negative BJAB cells. We then observed changes in cell-cycle progression and in the expression of important key cell-cycle regulatory proteins in the LMP-1-transfected cells during serum starvation.

## MATERIALS AND METHODS

### Materials

The chemicals and plastics used in this study were purchased from Sigma Chemical Co. (St. Louis, MO) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ), respectively. Antibodies specific to cell-cycle regulatory proteins were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

### Cells

An EBV-positive LCL, SNU-1103, was developed as described previously [Lee et al., 1998] and provided by the Korean Cell Line Bank (Seoul National University, Seoul, Korea). BJAB, an EBV-negative B lymphoma cell line, was kindly provided by Dr. W.-K. Lee (Myongji University, Yongin, Korea). SNU-1103, BJAB, vector-transfected control cell line, and LMP- 1-transfected cell line were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT). For the serum starvation experiments, cells were harvested in the exponential growth phase, washed once with serum free media, and then plated at a density of $6 \times 10^5$ cells/ml in 6-well plates with fresh medium containing 0.1% FBS. After a 24 hr-incubation, cells were fixed for cell-cycle analyses or lysed for Western blot analyses.

### Generation of LMP-1 Transfecrant

BJAB was resuspended in the growth medium and transfected with pcDNA3.1-LMP1 or pcDNA3.1 as a control by electroporation using a Bio-Rad GenePulser (Bio-Rad Laboratories, Hercules, CA) under the condition of 350 mA and 960 μF in 0.4 cm electoporation cuvette. Twenty-four hours after the transfection, G418 (800 μg/ml) was added to isolate stable transfectants. Finally, the transfectants were selected from outgrowing cells after incubation for 2 weeks in the RPMI-1640 medium containing 10% FBS and 800 μg/ml G418, and the expression of LMP-1 was confirmed by RT-PCR and Western blot analysis using LMP-1-specific monoclonal antibody. PCR amplification was performed using a Perkin-Elmer PCR system 9600 (Perkin-Elmer, Norwalk, CT) as follows: an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 61°C for 30 s, 72°C for 1 min, and then a final extension at 72°C for 10 min. To amplify the gene, 5′-GCG GAT CCC TAG GCG CAC CTG GAG GTG-3′ and 5′-CTA AGC TTT TAG TCA TAG TAG CTT AGC TG-3′ were used as forward and reverse primers, respectively.

### Cell-Cycle Analysis

The cells were harvested, washed with ice-cold PBS, fixed in cold 70% ethanol, and stored for at least 30 min at −20°C prior to staining. Fixed cells were then washed with PBS and stained with a staining solution containing 1.12% sodium citrate (pH 7.4), DNase-free RNase I (500 U/ml), and propidium iodide (50 μg/ml) for at least 2 h at room temperature or overnight at 4°C in the dark. After staining, the cells were harvested and washed twice with PBS and cell-cycle analyses were carried out using a flow

cytometer (FACS Calibur® system, Becton-Dickinson, San Jose, CA).

### Western Blot Analysis

Protein extracts were prepared from cultured cells ($3 \times 10^5$) as described previously [Kim et al., 2002] and protein contents were determined using the Bradford reagent (Bio-Rad) with BSA being a standard protein. Thirty micrograms of total protein were separated on 8, 10, or 12% SDS–PAGE, and then transferred to polyvinyliden difluoride membranes. The membranes were blocked at room temperature with 5%(w/v) powdered skim milk solution for at least 1 hr and incubated with primary antibodies at 4°C. Specific immunoreactive bands were identified using corresponding horseradish peroxidase conjugated secondary antibodies and enhanced with a chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK). The antibodies used were rabbit polyclonal anti-p107 (1/500), rabbit polyclonal anti-p130 (1/500), rabbit polyclonal anti-pRb (1/500), rabbit polyclonal anti-p16 (1/200), mouse monoclonal anti-p53 (1/200), rabbit polyclonal anti-CDK2 (1/500), rabbit polyclonal anti-CDK4 (1/200), rabbit polyclonal anti-CDK6 (1/500), mouse monoclonal anti-cyclin D1 (1/200), mouse monoclonal anti-cyclin E (1/500), mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) (1/500), mouse monoclonal anti-α-tubulin (1/500) as a control. Western blot analyses of the corresponding proteins tested in this study were performed at least twice and their results showed a good consistency. Of those results, best one was presented in this study.

## RESULTS

### LMP-1 Expression in *LMP-1* Gene-Transfected Cells

As an initial step to assess the role of LMP-1 in the serum starvation resistance of the EBV-transformed LCL, the *LMP-1* gene was isolated from the LCL, SNU-1103, and transfected into BJAB, which is an EBV-negative B-lymphoma cell line, and the expression of LMP-1 in the transfectants was confirmed by RT-PCR and Western blot analysis (Fig. 1). A 386-bp PCR product, which corresponded to part of the *LMP-1* gene, was detected in the LMP-1-transfected and SNU-1103 cells (Fig. 1A). However, the corresponding PCR band was absent from the vector-transfected control and BJAB cells. In addition, Western blot analysis showed that the LMP-1 protein was expressed successfully in the LMP-1-transfected and SNU-1103 cells, but not in the vector-transfected control and BJAB cells (Fig. 1B). The bands, which were detected using LMP-1-specific antibody, corresponded to the LMP-1 protein and were calculated to be about 63 kDa in size. These results confirmed that the transfected *LMP-1* gene was expressed correctly within the transfectants.

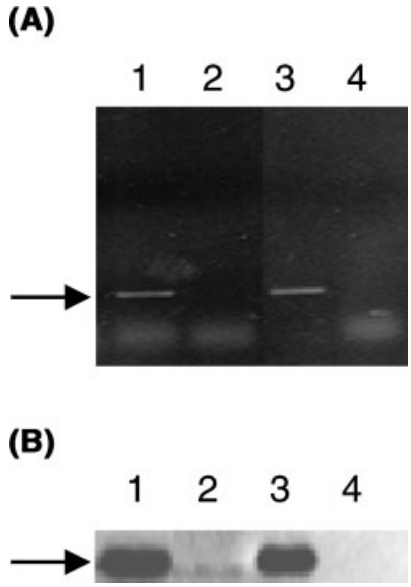


**Fig. 1.** Confirmation of transfected latent membrane protein-1 (LMP-1) gene expression by (**A**) RT-PCR and (**B**) Western blot analyses of *LMP-1* gene-transfected B-lymphoma cells. **Lanes 1–4** represent samples prepared from the *LMP-1* gene-transfected B lymphoma cells, vector-transfected control lymphoma cells, SNU-1103 cells, and control BJAB cells, respectively.

### Effect of LMP-1 on Cell-Cycle Progression During Serum Starvation

The effect of LMP-1 on the cell-cycle progression of asynchronously proliferating LMP-1-transfected cells during serum starvation was analyzed by flow cytometry of ethanol-fixed and propidium iodide-stained cells (Fig. 2). When cultivated in medium that was supplemented with 0.1% FBS, the populations of EBV-negative BJAB cells, and vector-transfected cells were arrested at the $G_1$ phase, as shown in Figure 2. However, under the same culture conditions, the EBV-positive LCL, SNU-1103 cells, and LMP-1-transfected cells continued through the cell cycle without being arrested at

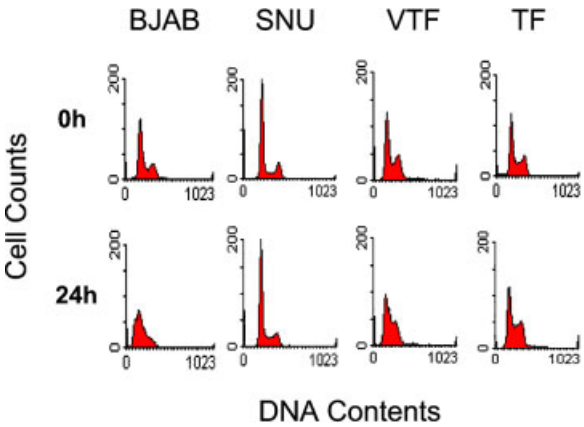


**Fig. 2.** Effect of serum starvation on cell-cycle progression of the control Epstein–Barr virus (EBV)-negative BJAB cells (BJAB), EBV-positive SNU-1103 LCL (SNU) cells, vector control-transfected BJAB cells (VTF), and *LMP-1* gene-transfected BJAB cells (TF). The cells were incubated with 0.1% FBS for the indicated times, stained with propidium iodide, and cell-cycle analysis was performed using flow cytometry, as described in Materials and Methods. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

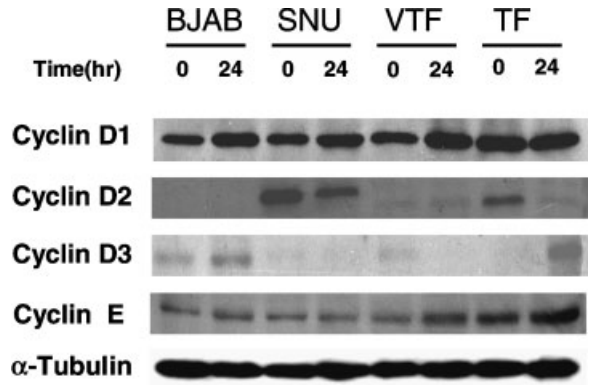


**Fig. 3.** Western blot analyses of $G_1$ cyclin expression in control EBV-negative BJAB cells (BJAB), EBV-positive SNU-1103 LCL (SNU) cells, vector control-transfected BJAB cells (VTF), and *LMP-1* gene-transfected BJAB cells (TF) during serum starvation. The cells were incubated with 0.1% FBS for the indicated times, collected, and lysed. Aliquots (30 μg) of total protein from the cell lysates were loaded and analyzed by 12% SDS–PAGE. The protein bands were transferred to a PVDF membrane, blotted with specific antibodies, and visualized by ECL.

any particular phase, thereby demonstrating resistance to serum starvation. Cell-cycle progression and DNA content cycling of normal and LMP-1 transfected cells were confirmed by synchronization using double thymidine block method followed by cell-cycle progression test (data not shown).

### Effect of LMP-1 on Cyclin Expression During Serum Starvation

Since extraneous LMP-1 expression in the EBV-negative B-lymphoma cells caused serum starvation resistance in terms of cell-cycle progression, we investigated whether the expression of cell-cycle regulatory proteins that are involved in $G_1$ cell-cycle progression was influenced by LMP-1 expression in LMP-1 transfectants during serum starvation. Initially, we investigated the changes in cyclin expression in relation to LMP-1 expression (Fig. 3). Among the three isoforms of the D-type cyclins, cyclin D1 expression was slightly increased during 24 h of serum starvation in all cell lines except the LMP-1 transfectants. In the LMP-1 transfectants, cyclin D1 expression was persistently high during serum starvation. The expression of cyclin D2, which was low in BJAB and vector-transfected control cells, was decreased by continuous serum starvation in LMP-1-transfected and SNU-1103 cells. The expression of cyclin D3 was significantly up-regulated during serum starvation in BJAB cells and LMP-1-transfected cells, but its expression was negligible in SNU-1103 cells. In contrast, the vector-transfected control cells showed down-regulated expression of cyclin D3.

Since cyclin E is known to associate with CDK2 to form the cyclin E-CDK2 complex that drives cells to override the restriction point, it is assumed to be involved in the serum starvation resistance of LMP-1-transfected cells in that it drives cells further into the S phase. As shown in Figure 3, cyclin E expression in LMP-1-transfected cells and vector-transfected control cells was up-regulated during serum starvation. Indeed, the cyclin E expression level in LMP-1-transfectants was already elevated at time zero, while its expression in other cell lines remained constant.

### Effect of LMP-1 on the Expression of CDKs During Serum Starvation

The cyclin-dependent kinases CDK4 and CDK6, which act together with cyclin D in the form of the cyclin D/CDK complex, are required for the phosphorylation of pRb, which in turn activates the cell cycle by passing the $G_1$ phase restriction point. Therefore, in LMP-1 transfectants, as opposed to normal cell lines, some CDKs may be subject to regulation by serum starvation. Therefore, we examined the changes in the expression levels of CDKs and PCNA, the latter of which is known to be associated with the cyclin/CDK complex and

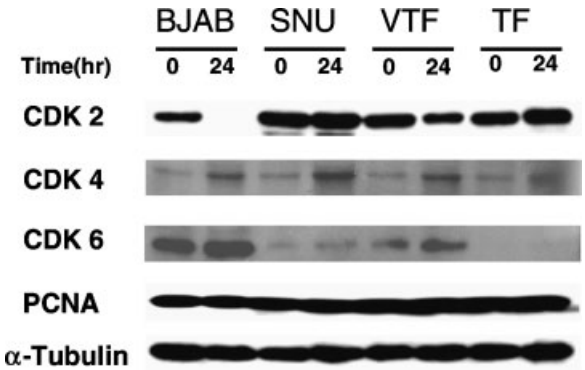


**Fig. 4.** Western blot analyses of the $G_1$ cyclin-dependent kinases and PCNA, which were expressed in control EBV-negative BJAB cells (BJAB), EBV-positive SNU-1103 LCL (SNU) cells, vector control-transfected BJAB cells (VTF), and *LMP-1* gene-transfected BJAB cells (TF) during serum starvation. The cells were incubated with 0.1% FBS for the indicated times, collected, and lysed. Aliquots (30 μg) of total protein from the cell lysates were loaded and analyzed by 12% SDS–PAGE. The protein bands were transferred to a PVDF membrane, blotted with specific antibodies, and visualized by ECL.

functions as a subunit of DNA polymerase δ in both DNA replication and repair [Waga et al., 1994]. As shown in Figure 4, CDK4 expression increased during serum starvation in all the cell lines tested, which indicates that CDK4 expression is independent of LMP-1 expression. However, CDK6 expression was up-regulated in BJAB, SNU-1103, and vector-transfected control cells, although expression levels varied among the different cell lines. Interestingly, CDK6 expression was not detected in the LMP-1 transfectant. CDK2 expression was markedly down-regulated in BJAB cells that were arrested at $G_1$ during serum starvation. Similarly, CDK2 expression in the vector-transfected control cells was down-regulated during serum starvation. In contrast, CDK2 expression in both the LMP-1 transfectants and SNU-1103 cells increased slightly during serum starvation. The expression level of PCNA was unchanged in all cell lines tested during serum starvation, and seemed to be unaffected by LMP-1 expression.

### Effect of LMP-1 on the Expression of CDKIs During Serum Starvation

Since we found several important alterations in the expression of up-regulating cell-cycle proteins in the LMP-1 transfectants during serum starvation, we explored the changes in the expression of CDK inhibitors (CDKIs) in these cells (Fig. 5). For this analysis, we chose

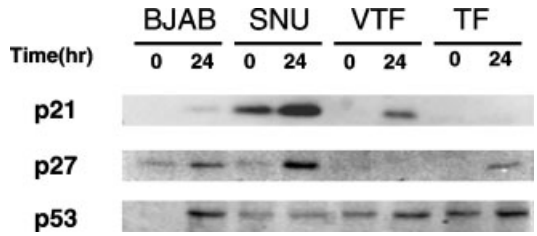


**Fig. 5.** Western blot analyses of $G_1$ cell-cycle inhibitor expression in control EBV-negative BJAB cells (BJAB), EBV-positive SNU-1103 LCL (SNU) cells, vector control–transfected BJAB cells (VTF), and *LMP-1* gene-transfected BJAB cells (TF) during serum starvation. The cells were incubated with 0.1% FBS for the indicated times, collected, and lysed. Aliquots (30 μg) of total protein from the cell lysates were loaded and analyzed by 12% SDS–PAGE. The protein bands were transferred to a PVDF membrane, blotted with specific antibodies, and visualized by ECL.

three CDKIs, $p21^{Cip1}$, $p27^{Kip1}$, and p53, based on our previous results [Kim et al., 2002]. The $p21^{Cip1}$ protein is known to bind and inhibit a wide range of cyclin/CDK complexes, including the cyclin D/CDK4(6) complex, and its expression is regulated by the p53 tumor suppressor protein [El-Deiry et al., 1993]. In SNU-1103, BJAB, and vector-transfected control cells, $p21^{Cip1}$ expression was up-regulated during serum starvation, although the expression levels varied among these cell lines. However, $p21^{Cip1}$ expression was barely detectable in the LMP-1-transfectant. The expression of another CDKI, $p27^{Kip1}$, showed similar variations to those of $p21^{Cip1}$ in the BJAB and SNU-1103 cells. However, $p27^{Kip1}$ expression was not detected in the vector-transfected control cells, in which $p21^{Cip1}$ was increasingly expressed, while the expression of $p27^{Kip1}$ was up-regulated in the LMP-1 transfectant, in which $p21^{Cip1}$ was not detected. The expression of p53 was apparently up-regulated in BJAB and vector-only transfected cells, which did not express LMP-1. However, in SNU-1103 cells and LMP-1-transfectants, both of which carried the *LMP-1* gene, p53 expression remained constant and increased slightly, respectively.

### Effect of LMP-1 on the Expression of pRb Family Proteins and E2F-4 During Serum Starvation

Since we found several remarkable alterations in the expression levels of CDKs, cyclins, and CDKIs during serum starvation after *LMP-1* gene transfection of the EBV-negative B-lymphoma cell line, we deemed it likely that the expression of at least one member of the retinoblastoma protein family might be regu-

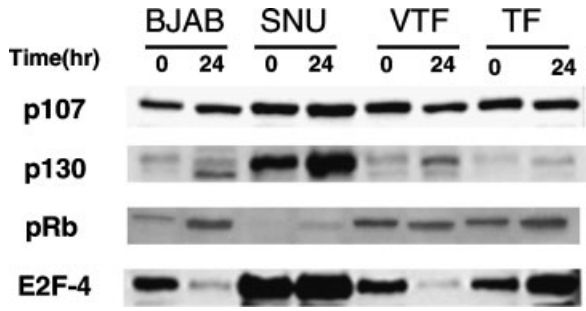


**Fig. 6.** Western blot analyses of the expression of Rb family proteins and E2F-4 in control EBV-negative BJAB cells (BJAB), EBV-positive SNU-1103 LCL (SNU) cells, vector control-transfected BJAB cells (VTF), and *LMP-1* gene-transfected BJAB cells (TF) during serum starvation. The cells were incubated with 0.1% FBS for the indicated times, collected, and lysed. Aliquots (30 μg) of total protein from the cell lysates were loaded and analyzed by 12% SDS–PAGE. The protein bands were transferred to a PVDF membrane, blotted with specific antibodies, and visualized by ECL.

lated, based on the identification of the retinoblastoma protein pRb as a substrate for $G_1$ cyclin–CDK complexes. Thus, we assessed the levels of expression of pRb and other pRb-like proteins (p107 and p130) in LMP-1-transfected and non-transfected B-lymphoma cell lines during serum starvation (Fig. 6). Although there was a certain degree of variation in the expression levels, p107 was expressed continuously during serum starvation in all the cell lines tested. In contrast, p130 expression was relatively high and up-regulated in SNU-1103 cells, while the other cell lines showed low-level p130 expression during serum starvation, and there was no clear correlation between the expression levels of p130 and LMP-1. On the other hand, pRb expression was relatively low in SNU-1103 cells as compared to the other cell lines, although its expression was slightly increased during serum starvation. The pRb expression patterns of the vector-transfected control cells and LMP-1 transfectants were similar in that expression was not significantly altered but the band position was slightly lowered, which indicates that dephosphorylation of pRb occurs during serum starvation. In addition, the BJAB cells showed increased pRb expression and lowered pRb band position during serum starvation. Taken together, these results suggest that pRb expression is not influenced directly by LMP-1 expression. However, it is interesting to note that changes in the expression of E2F-4, which is known to be an important transcription factor that is involved in cell-cycle progression, were LMP-1-dependent. For example, the BJAB and vector-transfected control cells showed remarkably decreased expression of E2F-4, while the SNU-1103 and LMP-1-transfected cells, which carried the *LMP-1* gene, showed up-regulated E2F-4 expression during serum starvation.

## DISCUSSION

One of the characteristics of the EBV-transformed LCL is resistance to serum starvation, whereby the LCL cell cycle proceeds even in low-serum concentrations, while normal animal cells, which include the BJAB cells used in this study, are arrested in the $G_1$ phase under these conditions. In our previous report, we investigated the expression of several cell-cycle regulatory proteins, which included the CDKs, cyclins, and CDKIs, to understand the mechanisms behind the serum starvation resistance of the EBV-transformed LCL. We found several critical alterations in the cell-cycle regulatory protein expression profiles of EBV-transformed LCLs as compared to normal B cells [Kim et al., 2002]. In this study, we addressed the relationship between the expression of the EBV gene *LMP-1*, which is considered to be the critical viral gene in virus-mediated immortalization of B cells, and the serum starvation resistance shown by the EBV-transformed LCL [Cuomo et al., 1992; Vockerodt et al., 2001]. LMP-1-transfected EBV-negative B-lymphoma cells showed cell-cycle progression past the $G_1$ phase, without being arrested at any particular phase, during serum starvation. This suggests that LMP-1 plays a critical role in the resistance to $G_1$ phase arrest that is induced by serum starvation in EBV-immortalized B-lymphoma cells (Fig. 1). In addition, our results indicate that one or more changes in the expression of cell-cycle regulatory proteins occur in LMP-1-transfected cells, as compared to EBV-negative control cells, during serum starvation.

In general, cell-cycle progression past the $G_1$ phase in mammalian cells is controlled by the family of cyclin-dependent kinases, which includes CDK2, CDK4, and CDK6, and their associated cyclins, D1, D2, D3, and E. CDK4 and CDK6, in association with the D-type cyclins, promote the $G_1$-to-S phase transition by phosphorylating pRb, thereby releasing transcription factor E2F in the mid- to late-$G_1$ phase, which is referred to as the restriction

point. If they pass this point, the cells continue to progress to the S phase, or if they do not pass this point they are arrested at $G_1$. Consequently, the D-type cyclins and their associated factors, which include CDK4 and CDK6, and the pRb family members may represent key targets for the altered expression that is associated with LMP-1 transfection of EBV-negative B-lymphoma cells. Some of the changes observed in the LMP-1 transfectants in this study included the ectopic down-regulation of cyclin D2 expression, and the remarkable up-regulation of cyclin D3 expression (Fig. 3). These results are, to some extent, in agreement with our previous report, in that ectopic expression of cyclin D2 and up-regulation of cyclin D3 expression were observed in the EBV-transformed LCL during serum starvation resistance [Kim et al., 2002]. Of the three CDK isomers tested, CDK2 was down-regulated in BJAB and vector-transfected control cells, whereas it was up-regulated in the EBV-transformed LCL and LMP-1 transfectants, which indicates that CDK2 in association with cyclin D3 plays an important role in promoting the $G_1$-to-S phase transition, probably by phosphorylating pRb family proteins (Fig. 4). Therefore, the increased expression of CDK2 in LMP-1 transfectants, in association with the active stimulation of cyclin D3 expression, may induce this cell line to proliferate continuously, even under the conditions of growth inhibition caused by serum starvation. Abnormal over-expression of D-type cyclin and CDK over-expression have been observed in many tumor cell lines [Palmero et al., 1993; Tam et al., 1994; Sinclair et al., 1995]. Furthermore, alterations in the expression and regulation of the CDKs and cyclins have been reported to cause tumorigenicity in various cell lines [Hunter and Pines, 1991; Balasubramanian et al., 1998].

The abnormal expression patterns during serum starvation of cyclins and CDKs in LMP-1 transfectants as compared to vector-transfected control cells may lead to alterations in pRb protein expression, since pRb is known to play an important role during the $G_1$ phase of the cell-cycle. In both sequence and function, pRb is closely related to two other nuclear phosphoproteins, p107 and p130. As with pRb, p130 and p107 may act as negative regulators of cell proliferation through interactions with the E2F transcription factors [Ewen et al., 1991; Li et al., 1993]. In general, pRb appears to be more important than p107 in crossing the restriction point, since pRb is partially phosphorylated by the CDK6(4)/cyclin D complex, and its phosphorylation is completed by the cyclin E/CDK2 complex, while p107 levels increase substantially as the cells progress through the S phase [Kondo et al., 2001; O'Connor et al., 2001]. The results from this study provide additional data on the differential expression and modulation of the three pRb family proteins. For example, of the three pRb family proteins, p130 expression was ectopic and elevated in SNU-1103 cells, but was consistently low in LMP-1 transfectants, while the vector-transfected control cells showed up-regulated expression of p130 during serum starvation (Fig. 6). On the other hand, the expression patterns of the other two pRb proteins, pRb and p107, were not changed by LMP-1 transfection. Interestingly, the expression of the transcription factor E2F-4, which activates the expression of the factors involved in DNA synthesis in the S phase and promotes advancement to the S phase, was increased significantly (Fig. 6). We believe that the over-expressed E2F-4 acts to maintain continuous cell proliferation, and that the expression of E2F-4 is regulated by LMP-1. E2F-4, which is known to be expressed continuously and is associated with the pRb protein until hyperphosphorylation of pRb protein followed by release from the pRb–E2F-4 complex results in its restoration as a transcription factor [Weinberg, 1995], showed opposite expression profiles for the LMP-1 transfectants and vector-transfected control cells. For example, E2F-4 expression was strongly down-regulated in BJAB and vector-transfected control cells that lacked the *LMP-1* gene. However, E2F-4 expression was remarkably up-regulated in LMP-1 transfectants, which is in agreement with its high expression in SNU-1103 cells that carry the *LMP-1* gene. These data suggest that cell-cycle progression past the restriction point in the $G_1$ phase of LMP-1 transfectants is facilitated by abnormal up-regulation of E2F-4 expression, rather than hyperphosphorylation of any particular pRb protein, although the mechanism underlying this abnormality is unclear at present.

The wild-type p53-inducible protein $p21^{Cip1}$ is a universal inhibitor of mammalian CDKs via complex formation with cyclins, CDKs, and PCNA. Another regulator of $G_1$ progression, $p27^{Kip1}$, contains a region with homology to $p21^{Cip1}$, and has the ability to curb the activities

of all the CDKs [Toyoshima and Hunter, 1994]. These inhibitory functions of $p21^{Cip1}$ and $p27^{Kip1}$, when over-expressed in cells, cause cell-cycle arrest at the $G_1$ phase, and progression through $G_1$ is accompanied by reductions in the levels of $p21^{Cip1}$ and $p27^{Kip1}$ expression [Kato et al., 1994; Bissonnette and Hunting, 1998]. However, our results demonstrate divergent expression patterns of $p27^{Kip1}$ and $p21^{Cip1}$ during serum starvation, with the former being up-regulated and the latter being down-regulated in LMP-1 transfectants as compared to their expression levels in vector-transfected control cells (Fig. 5). These results indicate that $p21^{Cip1}$, but not $p27^{Kip1}$, represents a target for the reduction of LMP-1 expression, which affects continuous cell-cycle progression past the $G_1$ phase in LMP-1 transfectants during serum starvation. In addition, these results suggest that the increased expression in SNU-1103 cells of $p21^{Cip1}$, which is one of the active mediators of immortalization and/or serum starvation-induced $G_1$ arrest resistance [Kim et al., 2002], is not caused by LMP-1 alone, but via a complex system involving other factors. Nevertheless, $p27^{Kip1}$ expression was up-regulated in the LMP-1 transfectants to the same extent as in the SNU-1103 cells, which indicates that this CDK inhibitor acts not as a cell-cycle inhibitor, but as an active mediator of the serum starvation-induced $G_1$ arrest resistance in the LMP-1 transfectants. Recently, it has been reported that $p21^{Cip1}$ and $p27^{Kip1}$, depending on their respective levels of production, function as positive regulators by promoting the assembly of active kinase complexes in various tumor cell lines [Fredersdorf et al., 1997; LaBaer et al., 1997; Balasubramanian et al., 1998; Cheng et al., 1999; Kolar et al., 2000]. These observations and the results from this study suggest that LMP-1 expression causes $p27^{Kip1}$, but not $p21^{Cip1}$, to act as an anti-apoptotic factor that maintains unsuppressed cell growth and avoids cell death during serum starvation. This conclusion appears to be supported by a report showing that increased numbers of $p27^{Kip1}$-positive Hodgkin/Reed–Sternberg cells were associated with lower levels of apoptosis in these cells, which suggests a protective activity for $p27^{Kip1}$ [Kolar et al., 2000].

In conclusion, the results from this study demonstrate that LMP-1 expression in EBV-negative B-lymphoma cells induces alterations in the expression patterns of cyclins, CDKs, CDKI, p103, and E2F-4, which lead to resistance to serum starvation-induced $G_1$ arrest. The altered expression of these cell-cycle regulatory proteins depends on the protein species and isomers that are involved in the regulation of cell-cycle progression past the $G_1$ restriction point. Our results imply that the singular expression of LMP-1 in EVB-negative B-lymphoma cells induces resistance to serum starvation-induced $G_1$ arrest. Nevertheless, we cannot rule out the possibility that other EBV genes are involved in the cell-cycle progression of the EBV-transformed LCL during serum starvation, since the altered pattern of protein expression in the LMP-1 transfectant was different from that in SNU-1103 cells that expressed all of the EBV viral proteins.

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