

EBV Latent Membrane Protein 1 Effects on Plakoglobin, Cell Growth, and Migration

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Abstract

Latent membrane protein 1 (LMP1), the major oncoprotein of EBV, is likely responsible for many of the altered cellular growth properties in EBV-associated cancers, including nasopharyngeal carcinoma (NPC). In this study, the effects of LMP1 on cell growth and migration were studied in the context of the EBV-positive C666-1 NPC cell line. In the soft agar transformation and Transwell metastasis assays, LMP1 enhanced cell growth and migration through activation of phosphatidylinositol 3-kinase (PI3K)/Akt and nuclear factor- κ B (NF- κ B) signaling. Inhibitors of PI3K, Akt, and NF- κ B signaling dramatically reduced these enhanced properties. An I κ B α super-repressor also blocked these effects. However, constitutive activation of Akt alone did not alter cell growth, suggesting that both PI3K/Akt and NF- κ B activation are required by LMP1. These enhanced effects required the full-length LMP1 encompassing both the PI3K/Akt-activating COOH-terminal activation region (CTAR) 1 and the nonredundant NF- κ B-activating regions CTAR1 and CTAR2. LMP2A, a latent protein that is also frequently expressed in NPC, similarly activates the PI3K/Akt pathway; however, its overexpression in C666-1 cells did not affect cell growth or migration. LMP1 also decreased expression of the junctional protein plakoglobin, which was shown to be partially responsible for enhanced migration induced by LMP1. This study reveals that in epithelial cells the transforming properties of LMP1 require activation of both PI3K/Akt and NF- κ B and shows that the loss of plakoglobin expression by LMP1 is a significant factor in the enhanced migration. [Cancer Res 2008;68(17):6997–7005]

Introduction

EBV is a ubiquitous γ -herpesvirus that infects >90% of the adult human population and is associated with malignancies of epithelial and lymphocyte origins (1). Cancers linked to EBV include the epithelial cell cancer nasopharyngeal carcinoma (NPC) and B-cell malignancies, including Burkitt's lymphoma, Hodgkin's disease, posttransplant lymphoma, and acquired immunodeficiency syndrome (AIDS)-associated lymphoma (1). These malignancies are associated with the expression of EBV latent genes, which are classified into types I, II, and III (2). Type I latency, typical of Burkitt's lymphoma, has the most restricted expression profile such that only the EBV nuclear antigen 1, *BamHI*-A transcripts, and the untranslated nonpolyadenylated EBERS are expressed. Type II

latency is associated with NPC and Hodgkin's lymphoma and expresses latent membrane proteins 1 and 2 (LMP1, LMP2A, and LMP2B) in addition to the transcripts expressed in type I latency. In type III latency, all of the latency genes are expressed, including *EBNA-2*, *EBNA-3A*, *EBNA-3B*, *EBNA-3C*, and *EBNA-LP*, and are only found in cancers linked to immunosuppression, such as posttransplant lymphoma and AIDS-associated lymphoma. Of these latent proteins, LMP1, EBNA-1, EBNA-2, EBNA-3A, and EBNA-3C are required for EBV-induced B-cell transformation (2).

As LMP1 and LMP2A are frequently detected in EBV-associated NPC, the effects of these genes on growth regulation are thought to contribute to the development of cancer (1). LMP1 can transform Rat-1 and human embryonic lung fibroblasts to form foci, grow in soft agar, and form tumors in nude mice. This property requires the activation of phosphatidylinositol 3-kinase (PI3K)/Akt signaling induced by the COOH-terminal activation region (CTAR) 1 domain (3, 4). In addition, transgenic mice expressing LMP1 in B lymphocytes develop B-cell lymphomas or develop epidermal hyperplasia when expressed in mouse epidermis (5, 6). The effects of LMP1 on cell signaling and expression include activation of transcription factors [nuclear factor- κ B (NF- κ B), extracellular signal-regulated kinase (ERK), p38, and AP1; refs. 7, 8], cytokines [interleukin (IL)-6, IL-8, and IL-10], antiapoptotic proteins (A20 and bcl-2; refs. 9, 10), and, in epithelial cells, proteins that modulate adhesion and invasion [E-cadherin, matrix metalloproteinase 9 (MMP9), and MUC1; refs. 11, 12]. These properties have been largely attributed to two COOH-terminal domains: CTAR1 and CTAR2. Both CTAR1 and CTAR2 can activate I κ B α -dependent canonical NF- κ B signaling, although CTAR1 can also activate the NIK-dependent noncanonical NF- κ B pathway (8, 9, 13). LMP1 CTAR1 also uniquely induces p50/p50 NF- κ B homodimers in association with the transactivating I κ B member bcl-3 (14, 15). This property has been linked to the induction of the epidermal growth factor receptor (EGFR) in the etiology of NPC (14–16). The transforming properties of LMP1 in both fibroblasts and epithelial cells have been shown to require activation of PI3K/Akt and ERK-mitogen-activated protein kinase (MAPK; refs. 3, 7, 17). LMP2A also affects cellular growth properties and can transform several epithelial cell lines and inhibit differentiation. LMP2A has been shown to activate the proto-oncogenic Wnt signaling pathway in a PI3K-dependent manner (18–20). These properties of both LMP1 and LMP2A likely contribute to the development of NPC (10).

To further investigate the transforming properties of LMP1 and LMP2A, their effects on the growth and migration properties of epithelial cells were studied in the context of an EBV-positive NPC cell line, C666-1. C666-1 cells are unique in that they are the only NPC cell line that has retained the EBV episome. These cells express very low levels of LMP1 and LMP2A (21). However, it has been shown that the low level of LMP1 expression and its induction of Akt signaling are still critically required for the survival of C666-1 cells (22). The data presented in this study reveal that enhanced

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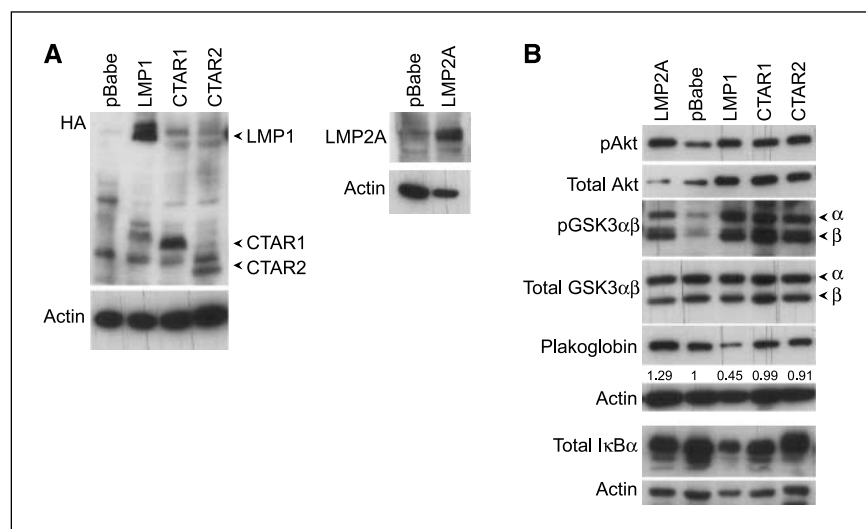


Figure 1. LMP1 down-regulates plakoglobin levels. **A**, stable expression of HA-tagged LMP1, CTAR1, CTAR2, or LMP2A from the pBabe vector in C666-1 cells was analyzed by immunoblot analysis. *Arrows*, LMP1-specific band. **B**, the effects of LMP1, CTAR1, CTAR2, and LMP2A expression on pAkt, pGSK3 $\alpha\beta$, I κ B α , and plakoglobin levels were analyzed by immunoblot analysis. Actin was used as a loading control. Densitometry with ImageJ software was performed to normalize plakoglobin levels to the corresponding actin levels. Fold change of the normalized plakoglobin levels relative to the pBabe control is indicated below the immunoblots.

expression of LMP1 induces growth and migration and these effects require activation of PI3K/Akt and I κ B α -dependent canonical NF- κ B signaling. Interestingly, this study shows that LMP1-mediated down-regulation of plakoglobin is a major factor in promoting enhanced migration.

Materials and Methods

Cell culture and constructs. C666-1 cells were cultured on fibronectin-coated plates in RPMI 1640 supplemented with heat-inactivated 10% fetal bovine serum (FBS) and antibiotic/antimycotic (Life Technologies). Plates were precoated with 10 μ g/mL fibronectin (Sigma) overnight at 4°C. Stable cell lines expressing LMP1, CTAR1, CTAR2, LMP2A, and myristylated Akt (myr-Akt) from the retroviral vector pBabe were established by transduction and selection with 5 μ g/mL puromycin (Sigma). LMP1, CTAR1, CTAR2, and LMP2A constructs in the pBabe vector have been described previously (3, 18). The myr-Akt was subcloned from pCMV6-myrAkt (a kind gift from Channing J. Der, University of North Carolina, Chapel Hill, NC) into pBabe. The I κ B α super-repressor (I κ B α ^{SS32/36AA}) has been previously described (14) and was subcloned from pcDNA3 into pHSCG. Cells expressing the I κ B α super-repressor or the pHSCG retroviral vector control were established by transduction and selected for green fluorescent protein fluorescence by flow cytometry. The myc-tagged full-length plakoglobin construct (p330) expressed from the LK444 mammalian expression vector was a kind gift from Kathleen Green (Northwestern University, Chicago, IL). Stable cell lines expressing plakoglobin were established by transfection with Lipofectamine 2000 (Invitrogen) and selection with 0.5 mg/mL G418 (Life Technologies). The neomycin-resistant plasmid DsRed (Clontech) was used as a control for G418 selection.

Retrovirus transduction. Retrovirus was produced in the 293T packaging cell line by cotransfecting plasmids expressing the protein of interest from a retroviral vector (pBabe or pHSCG), the VSVG envelope protein, and Gag/Pol using FuGENE6 transfection reagent (Roche). Retrovirus was harvested from clarified supernatant 48 h after transfection and C666-1 cells were transduced overnight in the presence of 4 μ g/mL polybrene.

Immunoblot analysis. Preparation of whole-cell lysates has been described previously (3). Protein concentrations were determined with the Bio-Rad detergent-compatible protein assay system. Lysates were separated by denaturing SDS-PAGE and transferred to 0.45 μ m Optitran nitrocellulose membrane (Schleicher & Schuell). Membranes were immunoblotted with the appropriate primary antibody followed by horseradish peroxidase-tagged secondary antibodies (Amersham Biosciences and Dako) and detected with the SuperSignal West Pico System (Pierce).

Antibodies. Rabbit anti-phosphorylated Akt (pAkt; Ser⁴⁷³), anti-phosphorylated glycogen synthase kinase 3 (pGSK3) α/β (Ser^{21/9}), and

anti-Akt were purchased from Cell Signaling. Rabbit anti-I κ B α (clone C-21), rabbit anti-myc (clone A14), goat anti- β -actin (clone I-19), and rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; clone FL-335) were purchased from Santa Cruz Biotechnology. Mouse IgG1 κ anti-GSK3 was purchased from Upstate Biotechnology. Goat anti-plakoglobin was purchased from Abcam. Mouse anti-HA was purchased from Covance. Mouse anti-LMP1 (clones CS1-4) was purchased from DakoCytomation. Rat anti-LMP2 (clone 14B7) was purchased from Ascension.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt assay. 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) cytotoxicity/proliferation assays were performed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's instructions. Cells were seeded in triplicate in a 96-well plate at 2.5×10^4 /mL, 100 μ L per well. MTS reagent was added on days 1 to 4 for 4 h, and absorbance was read at 490 nm and values plotted were subtracted from blanks.

Soft agar colony assay. Soft agar assays were carried out as previously described (19). Briefly, 2×10^5 cells suspended in a semisolid Bacto agar medium (0.5% Bacto agar in culture medium) were seeded on top of a Bacto agar medium underlay to prevent attachment of cells to the culture plate. Suspended cells were overlaid with liquid medium and, where appropriate, grown in the presence of LY294002 (25 μ mol/L), Akt inhibitor I (20 μ mol/L), BAY11-7085 (10 μ mol/L; Calbiochem), or DMSO. Cells were imaged by phase-contrast microscopy at days 4 to 6.

Transwell migration assay. BD Biocoat 8- μ m pore size control cell culture inserts (BD Biosciences) were precoated with 20 μ g/mL fibronectin, and 2×10^5 cells were seeded in the upper chamber in starvation medium (1% FBS). The bottom chamber consisted of starvation medium and 50 μ g/mL fibronectin as chemoattractant. Where indicated, chemical inhibitors were added to both the top and bottom chambers. Following an overnight incubation, cells that had migrated to the lower side of the membrane were fixed in methanol and stained with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes). DAPI-stained cells were visualized with fluorescence microscopy and the average number of migrated cells was calculated from eight representative fields in duplicate inserts using ImageJ software.

Results

LMP1 activates Akt and down-regulates I κ B α and plakoglobin levels. To investigate the effects of LMP1 and LMP2A on the growth of C666-1 cells, full-length LMP1, LMP1-CTAR1 that is deleted for CTAR2 and LMP1-CTAR2 that is deleted for CTAR1, and full-length LMP2A were stably expressed by retroviral transduction. Expression of the HA-tagged LMP1, CTAR1, and CTAR2 constructs

was relatively equivalent (Fig. 1A). Using an LMP2A-specific antibody, expression of LMP2A was confirmed and was increased above the basal levels detected in the pBabe vector control (Fig. 1A). LMP1 activates PI3K/Akt signaling and subsequently inactivates GSK3 β (3, 23). LMP2A also activates PI3K/Akt signaling, leading to inactivation of GSK3 β and activation of β -catenin signaling (18, 24). Activation of PI3K/Akt is required for the transformation by LMP1 and LMP2A (3, 19, 25). To assess the effects of LMP1, CTAR1, CTAR2, or LMP2A on activation of PI3K/Akt in C666-1 cells, activated pAkt and inactivated pGSK3 $\alpha\beta$ isoforms were identified by immunoblotting. The control cell line (pBabe) had detectable levels of pAkt, which was increased by the expression of LMP1, CTAR1, CTAR2, and LMP2A (Fig. 1B). This differs from previous studies where LMP1-CTAR2 was not sufficient to induce Akt activation (3). It is possible that in the context of basal levels of LMP1 expression, overexpression of CTAR2 can complement full-length LMP1 signaling and enhance Akt activation. Phosphorylation and inactivation of GSK3 $\alpha\beta$ was also enhanced in LMP1-, CTAR1-, CTAR2-, and LMP2A-expressing cells, correlating with the enhanced activation of Akt (Fig. 1B).

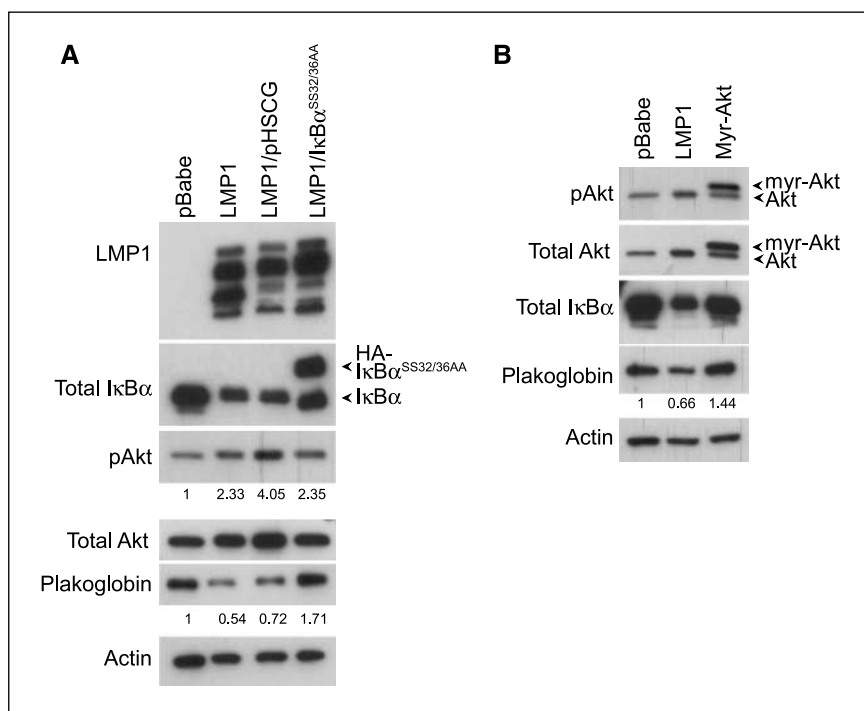
Activation of NF- κ B signaling by LMP1 is required for B-cell transformation but not rodent fibroblast transformation (3, 26, 27). Both CTAR1 and CTAR2 domains of LMP1 have been shown to induce NF- κ B signaling (13, 28); however, the CTAR2 domain is considered the major activating domain that can activate the canonical I κ B α -dependent activation of NF- κ B (29, 30). To assess the activation of the canonical pathway, total levels of I κ B α were detected by immunoblot analysis. Decreased I κ B α was only observed in cells overexpressing full-length LMP1 (Fig. 1B). In the background of basal LMP1 expression, overexpression of CTAR1 or CTAR2 was not sufficient to further decrease I κ B α levels compared with the pBabe control. These findings indicate that overexpression of LMP1, CTAR1, CTAR2, and LMP2A was all able to further activate Akt signaling to comparable levels; however, only full-length LMP1 dramatically affected the canonical NF- κ B

pathway. These differences in signaling properties may induce differences in cellular growth potential.

NPC is a highly metastatic and invasive malignant tumor (10). It has been reported that LMP1 induces epithelial cell migration through down-regulation of proteins involved in cell adhesion, including E-cadherin and up-regulation of proteins involved in opposing cell adhesion, or the degradation of the extracellular matrix, including activation of the ERK-MAPK pathway and the up-regulation of MUC1 and MMP9 (11, 12, 17). To identify whether other proteins involved in cell-cell adhesion contributes to LMP1-induced migration, the levels of plakoglobin were analyzed by immunoblot analysis. Plakoglobin is found at both adherens junctions and desmosomes, and loss of plakoglobin has been associated with breast and ovarian cancers and accounts for increased keratinocyte motility (31, 32). C666-1 cells overexpressing full-length LMP1 had decreased plakoglobin levels. A representative experiment is shown and densitometry revealed that plakoglobin expression was decreased 55% compared with the pBabe control (Fig. 1B). Overexpression of CTAR1, CTAR2, or LMP2A did not decrease plakoglobin levels below the pBabe control (Fig. 1B), suggesting that both CTAR1 and CTAR2 domains of LMP1 are required in the down-regulation of plakoglobin. It seems that in NPC cells, the full-length LMP1 is required to affect multiple pathways linked to oncogenesis, including down-regulation of plakoglobin and the activation of I κ B α -dependent NF- κ B signaling. To investigate any contributions of Akt and NF- κ B signaling to the regulation of plakoglobin levels, additional constructs affecting these pathways were utilized.

To discern the involvement of the canonical NF- κ B pathway on plakoglobin expression, the I κ B α super-repressor (I κ B α ^{SS32/36AA}) was expressed in the LMP1-overexpressing cells. The I κ B α super-repressor contains serine-to-alanine mutations at amino acids 32 and 36 and is unable to be phosphorylated and degraded (14). Expression of the HA-tagged I κ B α super-repressor and total I κ B α was verified by immunoblot analysis to total I κ B α levels (Fig. 2A).

Figure 2. Down-regulation of plakoglobin by LMP1 is dependent on the I κ B α -regulated canonical NF- κ B pathway. **A**, expression and the effects of the HA-tagged I κ B α super-repressor (I κ B α ^{SS32/36AA}) from the pHSCG vector on the down-regulation of plakoglobin of LMP1 were analyzed by immunoblot analysis. Activation of Akt was detected by pAkt. **B**, expression and the effects of the constitutively active myr-Akt and LMP1 on plakoglobin levels were compared by immunoblot analysis. Actin was used as a loading control. Densitometry with ImageJ software was performed to normalize pAkt and plakoglobin levels to the corresponding actin levels. Fold change of the normalized pAkt and plakoglobin levels relative to the pBabe control is indicated below the immunoblots.



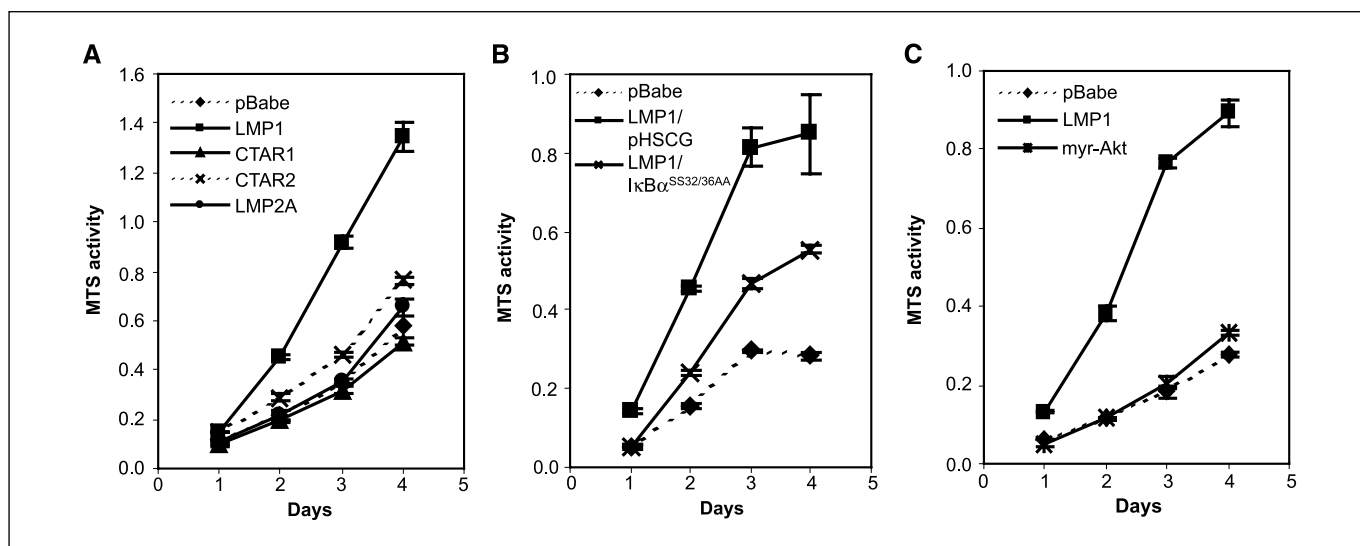


Figure 3. LMP1-induced growth requires both CTAR1 and CTAR2 domains and is dependent on NF- κ B activation. A to C, MTS assays were performed to measure the metabolic activity of C666-1 cells stably expressing LMP1, CTAR1, CTAR2, LMP2A, the constitutively activated myr-Akt, and the pBabe vector control and the effects of the I κ B α super-repressor (I κ B α ^{SS32/36AA}) on the growth of LMP1-expressing cells.

The I κ B α super-repressor stabilized endogenous I κ B α levels, possibly by competitive binding, suggesting that the I κ B α super-repressor was effectively inhibiting degradation of I κ B α (Fig. 2A). LMP1 expression was detected with anti-LMP1-specific antibodies, a combination of four monoclonals that detect LMP1 and multiple degradation fragments (Fig. 2A). Expression of LMP1 in I κ B α ^{SS32/36AA}-expressing cells was comparable with the pHSCG vector control and coexpression of I κ B α ^{SS32/36AA} from the pHSCG retroviral vector did not significantly affect LMP1 expression from the pBabe retroviral vector (Fig. 2A). Expression of the I κ B α super-repressor did not reduce LMP1-mediated phosphorylation and activation of Akt when compared with pBabe levels; however, it did inhibit the effects of LMP1 on reduction of plakoglobin (Fig. 2A).

To determine the effects of Akt activation on plakoglobin expression, a constitutively activated myr-Akt was stably expressed in C666-1 cells. Antibodies for total Akt detected the myr-Akt, which migrated slightly slower than endogenous Akt (Fig. 2B). The myr-Akt was activated as detected by the pAkt levels (Fig. 2B). Expression of the myr-Akt did not affect total I κ B α or plakoglobin levels when compared with the pBabe control (Fig. 2B). These data indicate that the effects of LMP1 expression on plakoglobin require activation of NF- κ B and that activation of Akt alone does not affect plakoglobin expression.

Full-length LMP1 enhances cell growth through activation of PI3K and I κ B α -dependent canonical NF- κ B signaling. To analyze the LMP1-mediated effects on C666-1 growth, MTS and soft agar colony assays were performed. The MTS assay measures the metabolic activity of mitochondrial dehydrogenase as an indicator of cell cycle induction and growth. Full-length LMP1 dramatically induced cell growth and high levels of MTS activity (Fig. 3A). The rate of growth was not altered by CTAR1, CTAR2, or LMP2A expression compared with the pBabe control.

To investigate the contributions of PI3K/Akt and NF- κ B signaling to LMP1-induced growth, inhibitors and constructs activating or suppressing these pathways were used. Expression of the I κ B α super-repressor reduced the enhanced growth of LMP1 cells in the MTS assay, whereas expression of myr-Akt did not enhance cell

growth (Fig. 3B and C). These findings suggest that the canonical NF- κ B pathway is critically involved in LMP1-induced growth but that activation of Akt alone does not alter cell growth.

The soft agar colony formation assay measures anchorage-independent cell growth as an indicator of transformation. Although pBabe control cells were able to form colonies and were visible by 2 weeks after seeding (data not shown), cells over-expressing LMP1 were able to form large colonies by only 4 days after seeding (Fig. 4A). In agreement with the effects on activation of cell signaling pathways, expression of CTAR1, CTAR2, or LMP2A did not enhance growth in soft agar.

To assess the contribution of activated NF- κ B and Akt on LMP1-induced growth effects, inhibitors of PI3K (LY294002), Akt (Akt inhibitor I), and NF- κ B (BAY11-7085) signaling were tested in the soft agar assay. The inhibitors LY294002 and BAY11-7085 have been previously shown to have an inhibitory effect on the growth of LMP1-induced lymphomas (27). The NF- κ B inhibitor BAY11-7085 inhibits the phosphorylation of I κ B α and thus specifically targets the canonical NF- κ B pathway. All of these inhibitors blocked the enhanced soft agar colony growth induced by LMP1 (Fig. 4B). In agreement with previous assays, expression of the I κ B α super-repressor inhibited the LMP1-induced growth in soft agar, whereas myr-Akt did not induce growth (Fig. 4C and D). These data indicate that activation of NF- κ B and PI3K/Akt is required for LMP1-enhanced growth; however, activation of Akt alone is not sufficient to affect the growth of C666-1 cells. The requirement of both of these pathways is also supported by the observation that only full-length LMP1 is able to enhance cell growth and induce both PI3K/Akt signaling and the degradation of I κ B α (Fig. 1B).

Full-length LMP1 enhances migration through activation of PI3K/Akt and I κ B α -dependent canonical NF- κ B signaling. To investigate the effects of LMP1 on cell motility, LMP1-, CTAR1-, CTAR2-, and LMP2A-expressing cells were analyzed using a Transwell migration assay. This assay measures the number of cells that migrate through a porous membrane in the direction of a chemoattractant. Because C666-1 cells are grown on fibronectin-coated culture dishes, fibronectin was used as the chemoattractant.

Full-length LMP1 induced a 5-fold enhancement in migration that was not induced by overexpression of CTAR1 or CTAR2 alone (Fig. 5A). Although LMP2A has been shown to enhance migration in some epithelial cells (33–35), LMP2A did not induce migration in C666-1 cells.

Similar to the growth studies, inhibitors of PI3K (LY294002), Akt (Akt inhibitor I), and NF- κ B (BAY11-7085) signaling inhibited LMP1-induced migration (Fig. 5B). The effect of the inhibitors at blocking Akt activation and I κ B α degradation was determined by immunoblot analysis. LY294002 efficiently blocked activation of Akt as detected by the decreased pAkt levels (Fig. 5C). Akt has been shown to phosphorylate I κ B α and induce its degradation; however, LY294002 treatment only further enhanced the degradation of I κ B α induced by LMP1 (Fig. 5C). This suggests that the decreased migration observed with LY294002 is due to blocking PI3K/Akt signaling without blocking LMP1-induced degradation of I κ B α . Although Akt inhibitor I was not as potent as LY294002 at inhibiting Akt phosphorylation, Akt inhibitor I was able to block phosphorylation and the activation of Akt at 20 μ mol/L, a dose where the strongest effect was observed at inhibiting migration (Fig. 5C). To confirm the inhibition of Akt at 20 μ mol/L, further immunoblot analysis was performed to determine the block in phosphorylation of the Akt target, GSK3 β . A dose-dependent inhibition of GSK3 β phosphorylation was apparent from 10 μ mol/L and was very evident at 20 μ mol/L (Fig. 5C), indicating effective inhibition of Akt activation. Similarly to LY294002 treatment, Akt inhibitor I did not block and even further enhanced the down-regulation of I κ B α by LMP1 (Fig. 5C). This suggests that the inhibition of LMP1-induced migration by Akt inhibitor I was due to inhibition of Akt activation without blocking LMP1-induced degradation of I κ B α .

The BAY11-7085 NF- κ B inhibitor effectively inhibited LMP1-induced enhanced migration and blocked activation of NF- κ B as evidenced by stabilized and increased I κ B α levels. It seems that BAY11-7085 does not affect the activation of Akt by LMP1, such that increasing doses of BAY11-7085 did not affect pAkt levels (Fig. 5C). The requirement of NF- κ B signaling in LMP1-mediated migration was further evaluated in cells expressing the I κ B α super-repressor, where the super-repressor also did not affect LMP1-induced Akt activation (Fig. 2A). The requirement for NF- κ B signaling was confirmed by the inhibition of LMP1-induced migration by the I κ B α super-repressor (Fig. 5D). This supported the BAY11-7085 effects and the requirement for NF- κ B signaling in LMP1-induced migration in the absence of any perturbation of Akt activation.

Although studies with LY294002 showed a requirement for PI3K/Akt signaling, expression of the myr-Akt only enhanced migration slightly above pBabe control levels (Fig. 5D). This suggests that similar to the effects of LMP1 on growth, activation of PI3K/Akt and I κ B α -dependent NF- κ B signaling are required for LMP1-enhanced motility and that activation of Akt alone aids in migration but is insufficient at inducing similar levels of migration as that observed for LMP1.

Restoration of plakoglobin blocks LMP1-induced migration. The loss of E-cadherin has been linked to the increased invasiveness of LMP1-expressing cells (36). Recently, a mutational analysis of LMP1 in Rat-1 cells indicated that LMP1 also down-regulated another cell adhesion protein, plakoglobin, through the TRAF-binding domain in CTAR1 and a region between CTAR1 and CTAR2 (amino acids 220–378; ref. 7). In this study, the loss of plakoglobin only occurred in C666-1 cells expressing full-length LMP1 (Fig. 1A) and this loss correlated with enhanced migration

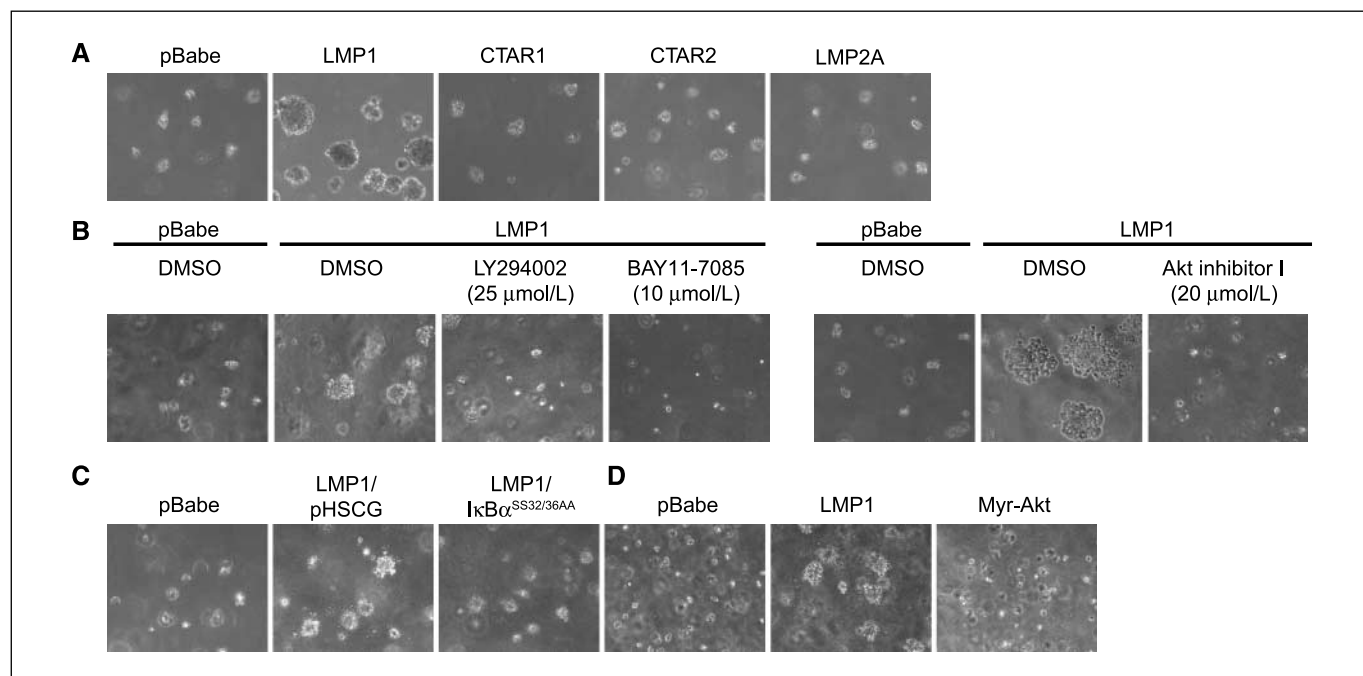


Figure 4. LMP1-enhanced soft agar colony formation requires both CTAR1 and CTAR2 domains and is dependent on PI3K and NF- κ B signaling. **A**, C666-1 cells stably expressing LMP1, CTAR1, CTAR2, LMP2A, or the pBabe vector control were assessed for anchorage-independent growth using the soft agar colony assay. **B**, the signals required by LMP1 for enhanced soft agar colony formation were assessed by using inhibitors of PI3K (LY294002), Akt (Akt inhibitor I), and NF- κ B (BAY11-7085) signaling compared with the DMSO control. **C**, the requirement of canonical NF- κ B signaling for LMP1-induced soft agar colony formation was assessed using the I κ B α super-repressor (I κ B α ^{SS32/36AA}) compared with the pHSCG vector control. **D**, expression of the constitutively active myr-Akt was used to assess whether activation of Akt signaling is sufficient to enhance soft agar colony growth.

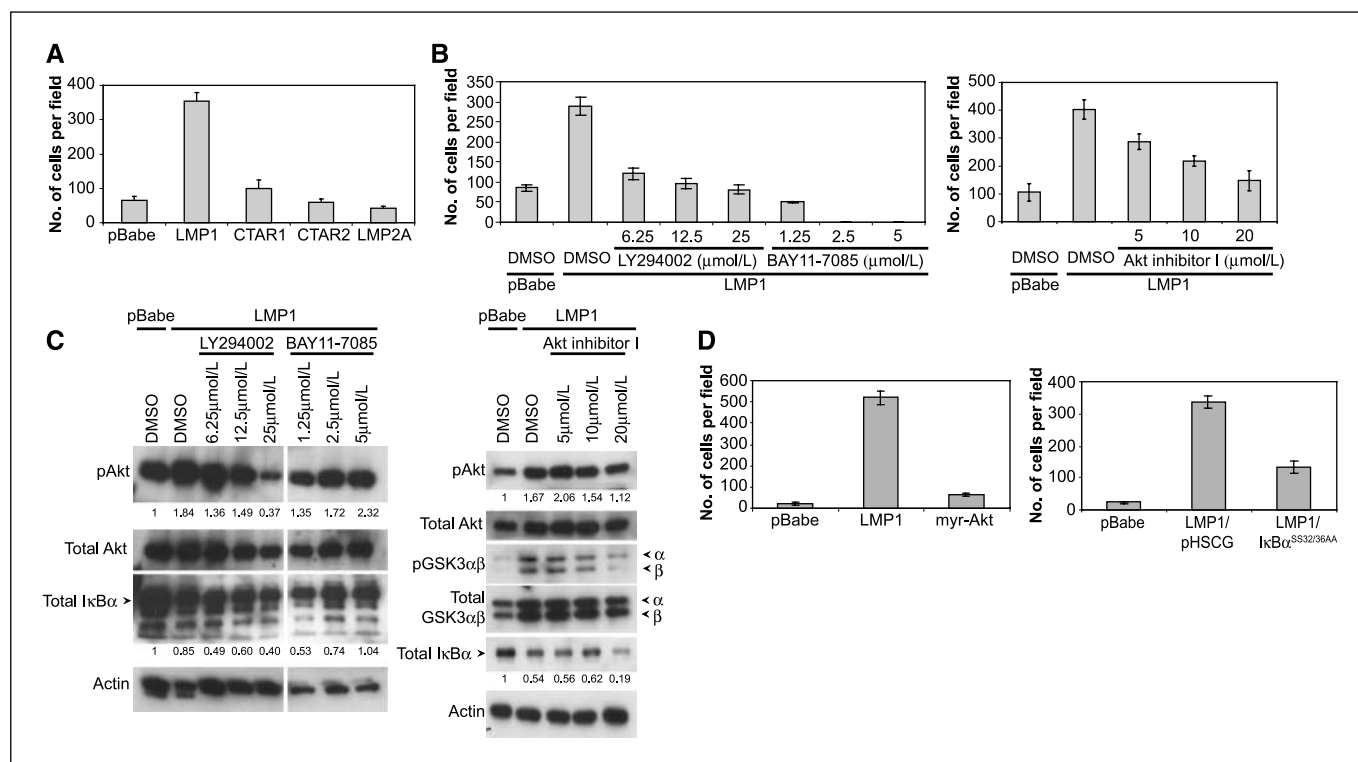


Figure 5. LMP1-induced migration requires both CTAR1 and CTAR2 domains and is dependent on PI3K and NF- κ B signaling. **A**, C666-1 cells stably expressing LMP1, CTAR1, CTAR2, LMP2A, or the pBabe vector control were assessed for metastasis in the Transwell migration assay. **B**, the signals required by LMP1 for enhanced migration was assessed by using inhibitors of PI3K (LY294002), Akt (Akt inhibitor I), and NF- κ B (BAY11-7085) at the indicated concentrations compared with the DMSO control. **C**, the ability of the PI3K inhibitor (LY294002), NF- κ B inhibitor (BAY11-7085), and Akt inhibitor (Akt inhibitor I) to block PI3K/Akt and I κ B α -dependent NF- κ B signaling was assessed by immunoblot analysis after an overnight treatment and blotted for activated pAkt, inactivated pGSK3 α β , and total I κ B α levels. Actin was used as a loading control. Densitometry with ImageJ software was performed to normalize pAkt and I κ B α levels to the corresponding actin levels. Fold change of the normalized pAkt and I κ B α levels relative to the pBabe control is indicated below the immunoblots. *White line*, intervening lanes have been spliced out. **D**, LMP1-induced migration is dependent on I κ B α -dependent NF- κ B signaling and activation of Akt is not sufficient to enhance migration. Expression of the constitutively active myr-Akt was used to assess whether activation of Akt signaling is sufficient to enhance migration. The requirement of canonical NF- κ B signaling for LMP1-induced migration was assessed using the I κ B α super-repressor (I κ B α ^{SS32/36AA}) compared with the pHSCG vector control.

(Fig. 5). In addition, plakoglobin levels were restored in I κ B α super-repressor expressing cells that had reduced migration (Figs. 2A and 5D). Therefore, the role of plakoglobin in LMP1-induced migration was evaluated by restoring plakoglobin levels in LMP1-expressing cells using a wild-type plakoglobin construct. Expression of the myc-tagged wild-type plakoglobin was detected using antibodies against c-myc (Fig. 6A). Expression of the myc-tagged plakoglobin was much higher in LMP1-expressing cells than in the pBabe/DsRed control cells. This may be due to mechanisms that control plakoglobin stability induced by endogenous levels of plakoglobin. Densitometry revealed that plakoglobin levels had been restored, albeit above pBabe/DsRed control levels, in LMP1-expressing cells (6.78-fold above pBabe/DsRed control cells compared with the reduction to 0.6-fold in the parental LMP1-expressing cells; Fig. 6A). Restoration of plakoglobin levels in LMP1-expressing cells did not affect the expression levels of LMP1; however, in the Transwell migration assay, restoration of plakoglobin in LMP1-expressing cells decreased migration ~50% compared with LMP1 alone (Fig. 6B). A slight decrease in migration was also observed when plakoglobin was overexpressed in pBabe control cells (Fig. 6B). These data indicate that plakoglobin levels affect LMP1-mediated migration.

In addition to cell migration, plakoglobin has also been linked with growth-altering effects (31, 32). To determine if the restoration of plakoglobin in LMP1-expressing cells affects other growth

properties, soft agar transformation and MTS assays were performed. Changes in growth were not detected when plakoglobin was restored in LMP1-expressing cells (Fig. 6C and D) in that the number and size of colonies in soft agar and the rate of metabolism were comparable with LMP1-expressing cells that have lost plakoglobin expression. These data indicate that in C666-1 cells, LMP1-induced loss of plakoglobin contributes to migration but is not required for the growth-promoting properties associated with LMP1 function.

Discussion

This study reveals that LMP1 contributes to the transformation of NPC through activation of PI3K/Akt and I κ B α -dependent canonical NF- κ B signaling. Both signaling pathways were required for transformation, and in agreement, both CTAR1 and CTAR2 domains were required for enhanced growth and migration through CTAR1-mediated activation of PI3K/Akt and the activation of canonical NF- κ B signaling primarily from CTAR2. The activation of these pathways has also been previously shown to be required for LMP1-mediated transformation in rodent and human fibroblasts and for transformation of B lymphocytes by EBV (3, 27, 37). The data presented here also indicate that the loss of plakoglobin is an additional target of LMP1 that contributes to LMP1-induced migration.

PI3K activation by LMP1 was required for the induction of growth in soft agar and enhanced migration of C666-1 cells. PI3K is involved in the activation of multiple pathways, including Akt and the MAPK (ERK). It has been recently shown that ERK activation is required for LMP1-mediated transformation of rodent fibroblasts and was specifically activated by LMP1-CTAR1 (7, 17). The activation of PI3K by LMP1 also requires the TRAF-binding site in CTAR1 (3, 7), suggesting that the requirement for PI3K activation in transformation may be due to Akt activation and/or ERK activation. However, a specific inhibitor of Akt blocked LMP1-induced growth of C666-1 cells in soft agar and migration, whereas a MAPK/ERK kinase 1/2 inhibitor (U0126) did not affect LMP1-induced growth and migration (data not shown). In addition, increased levels of activated phosphorylated ERK above pBabe basal levels were not detected in cells expressing full-length LMP1 (data not shown). These findings suggest that the requirement of PI3K signaling in LMP1-mediated transformation in C666-1 cells is due to Akt activation and not ERK induction. Although ERK has been previously shown to be important in LMP1-mediated transformation, it is possible that in the C666-1 carcinoma cells, the stable expression of EBV latent genes, including low levels of LMP1, induces sufficient amounts of ERK activation such that only additional activation of Akt is required for the enhanced growth properties mediated by LMP1.

LMP2A also activates PI3K/Akt signaling and this activation is required for its ability to transform certain epithelial cell lines and inhibit keratinocyte differentiation (19, 24, 25). In C666-1 cells, LMP2A induced activation of Akt but did not induce any changes in growth or migration (Figs. 3A and 4A). Constitutively active myr-Akt was also insufficient to enhance growth or migration (Figs. 3C, 4D, and 5D). LMP2A did not affect $I\kappa B\alpha$ levels, indicating that in C666-1 cells, LMP2A overexpression does not activate canonical NF- κB signaling at the level of $I\kappa B\alpha$ degradation. These findings further support the requirement for activation of both NF- κB and Akt for enhanced growth and migration in the already transformed C666-1 cells. LMP2A-induced migration has been attributed to the activation of ERK and Syk tyrosine kinase signaling, and in primary epithelial cells via the induction of integrin- $\alpha 6$ (33–35, 38). The lack of an effect in migration by overexpressing LMP2A in C666-1 cells may be due to differences in cell lines or indicates that overexpression of LMP2A in C666-1 cells does not further activate signals involved in migration above those induced by basal levels of LMP2A expression.

A critical property to malignant transformation, in addition to growth induction, is the enhancement of migration leading to metastasis, a characteristic often associated with NPC (10). In epithelial cells, LMP1 disrupts cellular adhesion through down-regulation of the intercellular adhesion protein E-cadherin and up-regulation of proteins involved in the disruption of the extracellular matrix, including MMP9 and MUC1 (11, 12). Recently, mapping studies have identified the TRAF-binding domain of LMP1 in the down-regulation of plakoglobin, but the functional consequences of this down-regulation were not determined (7). The data presented here indicate that loss of plakoglobin contributes to LMP1-induced migration but is not required for LMP1-enhanced growth. It is noteworthy that restoration of plakoglobin in LMP1-overexpressing cells was not able to completely inhibit the enhanced migration to pBabe control levels. This finding suggests that other pathways affected by LMP1 contribute to enhanced migration. The inhibition of migration in response to LY294002, Akt inhibitor I, and BAY11-

7085 would suggest that these additional factors are controlled by PI3K/Akt and NF- κB signaling.

Plakoglobin belongs to the armadillo family of proteins and is highly homologous to β -catenin (39–41). Both plakoglobin and β -catenin serve dual roles as adhesive proteins that mediate intercellular junctions and as transcriptional regulators of T-cell factor (Tcf)/lymphoid enhancer factor (Lef)-responsive genes, such as *c-myc* and *cyclin D1* (31). Despite their similarities, they are functionally distinct such that *plakoglobin* and *β -catenin* knockout mice are both embryonic lethal and thus cannot compensate for the loss of the other (39). It is believed that both the adhesive and signaling properties of plakoglobin are important in the development of cancer (31, 41), although whether

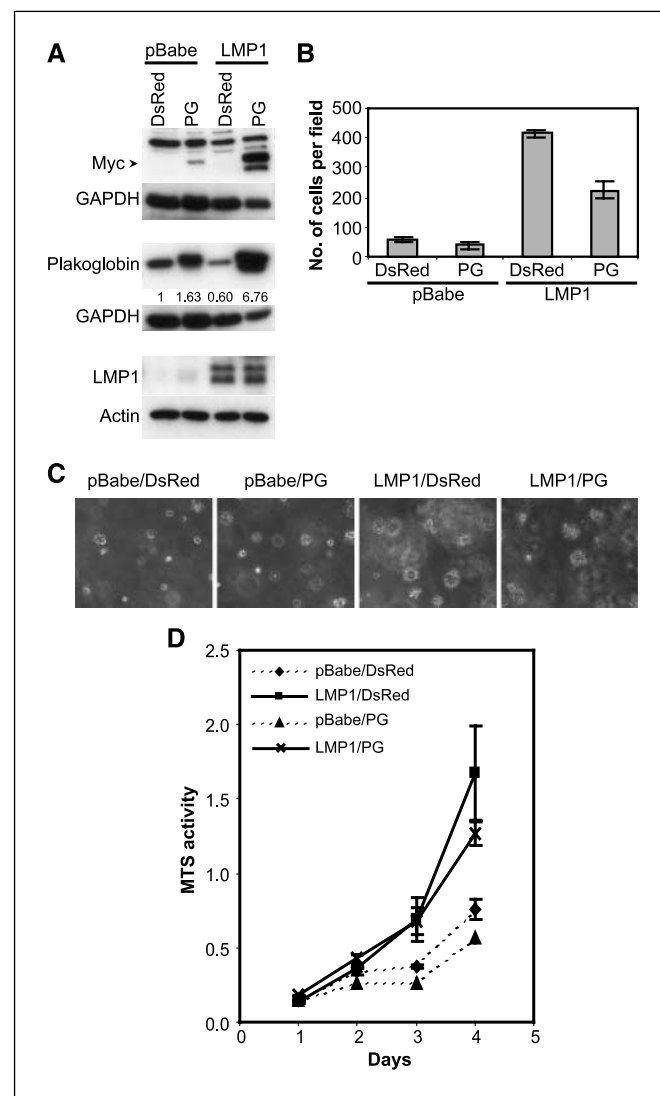


Figure 6. LMP1-induced migration is blocked by restoring plakoglobin levels. **A**, stable expression of LMP1 in the pBabe vector and a myc-tagged plakoglobin (PG) or the DsRed vector control from C666-1 cells was analyzed by immunoblot analysis. Actin and GAPDH were used as loading controls. Densitometry with ImageJ software was performed to normalize plakoglobin levels to the corresponding GAPDH levels. Fold change of the normalized plakoglobin levels relative to the pBabe/DsRed control is indicated below the immunoblots. **B**, the effects of restoring plakoglobin levels on the migration of LMP1-expressing C666-1 cells were assessed by the Transwell migration assay. Soft agar transformation (**C**) and MTS assays (**D**) were performed to assess the effect of expressing a plakoglobin construct on the growth of pBabe and LMP1-expressing C666-1 cells compared with the DsRed vector control.

plakoglobin plays a tumor-suppressive or tumor-promoting role seems to be cell type dependent. Inhibition of growth in lung cancer cells results from plakoglobin blocking β -catenin-mediated Tcf/Lef activity, possibly by sequestering Tcf away from the formation of a functional β -catenin/Tcf complex (42). It is currently unknown whether LMP1-induced loss of plakoglobin enhances migration through disruption of cellular junctions or through regulation of Tcf/Lef-responsive genes. Interestingly, LMP1 also stabilizes β -catenin (43), and in C666-1 cells, β -catenin is increased (data not shown), whereas plakoglobin is decreased. It is possible that the combined effects of plakoglobin and β -catenin may result in enhanced Tcf/Lef-regulated transcription. Although loss of plakoglobin has not been documented in EBV-related carcinomas, an accumulation of nuclear β -catenin is found in NPC and this is thought to be partly mediated through the inactivation of GSK3 β by LMP2A (44).

Plakoglobin is posttranslationally modified by a variety of mechanisms, including phosphorylation by EGFR, Src, Fer, Fyn, and GSK3 β , and is subject to stabilization by *O*-glycosylation (41). Tyrosine phosphorylation of plakoglobin by EGFR, Fer, Fyn, and GSK3 β is destabilizing, whereas phosphorylation by Src stabilizes complexes with desmosomal proteins. In C666-1 cells overexpressing LMP1, AG1478, an inhibitor of EGFR, did not affect Transwell migration (data not shown), suggesting that the down-regulation of plakoglobin by LMP1 is not mediated through EGFR-induced degradation. Because LMP1 induces phosphorylation and inactivation of GSK3 β , it is also unlikely

that GSK3 β is responsible for the effects of LMP1 on plakoglobin. It is presently unknown whether the effects of LMP1 on plakoglobin are due to transcriptional or posttranslational mechanisms.

In summary, this study shows that PI3K/Akt and I κ B α -dependent NF- κ B signaling are both required for LMP1-induced growth and migration in an EBV-positive NPC cell line. Interestingly, the data also indicate that the effect of LMP1 on plakoglobin levels contributes to LMP1-induced migration. These findings indicate that promotion of oncogenesis in cells that express LMP1 not only requires activation of signaling pathways but also results from effects on the expression levels of specific cellular genes. The effects of LMP1 on these adhesion proteins may provide novel targets to inhibit metastasis of NPC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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