LMP1 Strain Variants: Biological and Molecular Properties

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The ubiquitous herpesvirus Epstein-Barr virus (EBV) is linked to the development of several malignancies, including nasopharyngeal carcinoma. Latent membrane protein 1 (LMP1) is considered the EBV oncogene as it is necessary for EBV-induced transformation of B lymphocytes and is able to transform Rat-1 fibroblasts. LMP1 can activate a wide array of signaling pathways, including phosphatidylinositol 3-kinase (PI3K)-Akt and NF-κB. Six sequence variants of LMP1, termed Alaskan, China 1, China 2, Med+, Med−, and NC, have been identified, and individuals can be infected with multiple variants. The frequencies of detection of these variants differ for various EBV-associated malignancies from different geographic regions. In this study, the biological and signaling properties of the LMP1 variants have been characterized. All of the LMP1 variants transformed Rat-1 fibroblasts, induced increased motility of HFK cells, and induced increased homotypic adhesion of BJAB cells. While all the variants activated the PI3K-Akt signaling pathway to similar extents, the Alaskan, China 1, and Med+ variants had limited binding to the E3 ubiquitin ligase component homologue of Slimb and had slightly enhanced NF-κB signaling. These findings indicate that the signature amino acid changes of the LMP1 variants do not hinder or enhance their in vitro transforming potentials or affect their signaling properties.

Epstein-Barr virus (EBV) is a ubiquitous herpesvirus that infects more than 90% of the world’s population. EBV infection is associated with a wide array of malignancies, including nasopharyngeal carcinoma (NPC), Hodgkin’s lymphoma, post-transplant lymphoma, hairy leukoplaikia, Burkitt’s lymphoma, and others (24, 45, 62). EBV primary infection is usually asymptomatic, although at times it results in the benign lymphoproliferative disease infectious mononucleosis (40). Viral reactivation may occur in latently infected memory B cells, and virus frequently can be detected in saliva (3, 49, 50). Viral infection is considered latent in EBV malignancies, and in NPC and Hodgkin’s lymphoma, viral expression is restricted to EBN1, latent membrane protein 1 (LMP1), LMP2, and the EBER and BART RNAs (24).

LMP1 is considered the EBV oncogene, as it transforms NIH 3T3 and Rat-1 fibroblasts. It is essential for EBV-induced B-lymphocyte transformation (22, 24, 32, 52, 58) and is frequently expressed in EBV malignancies (15, 62). LMP1 consists of 386 amino acids with a short 23-amino-terminal cytoplasmic tail, six transmembrane domains, and a long carboxyl-terminal tail that contains the two signaling domains carboxyl-terminal activating region 1 (CTAR1) and CTAR2. LMP1 self-associates in the plasma membrane due to its hydrophobic transmembrane domains and thus acts as a constitutively active growth factor receptor that does not require a ligand for its activation. The tumor necrosis factor receptor-associated factor associates with LMP1, which signals similarly to CD40 and to the type II tumor necrosis factor receptor (9, 21, 39). As such, LMP1 activates a wide array of signaling pathways through CTAR1 and CTAR2, including the NF-κB, mitogen-activated protein kinase, c-Jun N-terminal kinase, and phosphatidylinositol 3-kinase (PI3K)-Akt pathways (7, 12, 14, 20, 32, 42, 46). LMP1 also induces the deregulation of the cell cycle through manipulation of various cell cycle-regulatory molecules, including the cyclin-dependent kinase inhibitor (CDKI) p27Kip1, p16, inhibitor of differentiation 1 (Id1), Id3, retinoblastoma (Rb), and cyclin-dependent kinase 2 (CDK2) (14, 27, 41).

Six LMP1 sequence variants, termed Alaskan, China 1, China 2, Mediterranean+ (Med+), Med−, and North Carolina (NC), have been isolated from clinical specimens (10, 35). These variants are distinguished by various signature amino acid changes from the prototypic LMP1 (B95.8), although changes are not found in CTAR1 or CTAR2. In addition, China 1 and Med+ have a 10-amino-acid deletion between CTAR1 and CTAR2 (10). Although this 10-amino-acid deletion has been linked to augmented transforming ability (28), several studies have indicated that LMP1-containing CTAR1 is essential and sufficient for Rat-1 fibroblast and Madin-Darby canine kidney (MDCK) cell transformation (25, 32). The abilities to induce NF-κB and the transforming properties of the B95, Med+, and CAO variants have been compared (4, 6, 16, 17, 29, 34). Slight enhancements in the abilities of the CAO variant, which is the most similar to China 1, and the Med+ variant to induce NF-κB signaling were mapped to the transmembrane domains. LMP1 also interacts with the E3 ubiquitin ligase component homologue of Slimb (HOS), a member of the β-TrCP/Fbw1 subfamily of F-box proteins which regulates the ubiquitin-dependent destruction of IκBα and β-catenin (34, 47, 53, 60).

Several studies have revealed that individuals can be infected with multiple EBV variants, that the LMP1 variants differ in abundance between throat wash samples and peripheral blood samples, and that their detectabilities and abundances vary over time (49–51). It is possible that these differences in abundance and detectability reflect different biological properties or cellular tropism. Recently, computational analy-
sis of the variants revealed key amino acid changes in potential human leukocyte antigen (HLA)-restricted epitopes, suggesting a potential immune-modulated selection mechanism (11). Although all variants have been detected in various malignancies, the CAO/China 1 LMP1 variant is significantly more prevalent in NPC tumors from the region of endemcity in southern China and is predicted not to be presented by individuals who are A2 or A24, the most common HLA types found in patients with NPC from this region (11).

In this report, the biological properties of the LMP1 variants were characterized through their abilities to transform Rat-1 fibroblasts, affect epithelial cell motility, and induce homotypic adhesion of B lymphocytes. The LMP1 variants also were evaluated for their potentials to activate the PI3K-Akt and NF-κB signaling pathways as well as their abilities to alter cell cycle markers that have been associated with G1/S-phase progression. All the variants were capable of transforming Rat-1 fibroblasts, as measured by blockage of contact-inhibited and anchorage-independent growth, and induced homotypic adhesion in BJAB cells. The PI3K-Akt signaling cascade was activated by all variants, and this activation was necessary for LMP1-induced Rat-1 transformation. Changes in cellular markers that are frequently associated with cell cycle deregulation were also induced by all variants. Finally, the Alaskan, China 1, and Med+ variants had increased NF-κB reporter activity compared to the other variants, in conjunction with decreased binding to HOS. These similarities in biological and molecular properties suggest that all of the variants have equal pathological potentials, in agreement with their detection in multiple types of cancer.

### MATERIALS AND METHODS

**Plasmids.** LMP1 variants were subcloned into the myc-tagged expression vector M3-pcDNA3-hygromycin (26) from pGEM32. The Alaskan, China 1, and China 2 variants were PCR amplified with the 5′ primer (CGACGATCCATGGAACACGACCT) and the 3′ primer (ATCACGAGTCTGAAAGATGTTGGCTTTTCAGCCTAG), which was followed by restriction enzyme digestion and insertion into the BamHI and XbaI sites of M3-pcDNA3-Hyg. The B95.8, Med+, Med−, and NC variants were PCR amplified with the 5′ primer (CGA CGTCCATATGGGACGCCAAGACGCT) and the 3′ primer (ATCACGAGTCTGAAAGATGTTGGCTTTTCAGCCTAG) and subsequently cloned by the above-described procedure.

LMP1 variants were then subcloned into the pBabe-puromycin (pBabe) vector without the myc tag but with a hemagglutinin (HA) tag added. B95.8, Med+, and NC were PCR amplified with LMP1-HA5′ and LMP1-3′ (16). NCde93C was isolated during the cloning of NC as a spontaneous mutation, glutamine to stop, resulting in the deletion of the last 93 amino acids of NC. Med− was PCR amplified with LMP1-HA5′ and LMP1-SAL3′ (ATCACGAGTCTGAAAGATGTTGGCTTTTCAGCCTAG). China 1 and China 2 were PCR amplified with LMP1-C12AL5′ (GCCCGGATCCATGGAACACGACCTGAGAGG) and LMP1-SAL3′ (16). NCde93C was PCR amplified with LMP1-C12AL5′ and LMP1-SAL3′. PCR amplification was followed by restriction enzyme digestion and insertion into the BamHI and EcoRI sites of pBabe. The HA-tagged pcDNA3-HOS plasmid was a gift of Serge Fuchs, University of Pennsylvania (53).

**Retrovirus production and transduction.** Retrovirus production was carried out as described previously (14). Briefly, subconfluent 293T cells were transduced using Fugene 6 (Roche) according to the manufacturer’s directions with 5 μg of each pBabe-HA/pcDNA3-HOS plasmid, 5 μg pVSVG, and 5 μg pGag/Pol. Following overnight incubation at 37°C, media were replaced with fresh media, Dulbecco’s modified Eagle’s medium (DMEM) for BJAB and Rat-1 cells and keratinocyte-SFM ( Gibco) medium following a gentle wash with phosphate-buffered saline (PBS) for HFK cells, and cells were incubated at 33°C overnight. The cell supernatant containing the retrovirus was then cleared of cellular debris by centrifugation at 1,000 × g for 5 min. Rat-1 or HFK cells were then transduced with clarified supernatant with 4 μg/ml polybrene overnight. BJAB cells were transduced through spinoculation of 3.0 × 10^6 cells with 1 ml of retrovirus and 4 μg/ml polybrene for 3 h at 2,000 × g, and then spent media were removed and the pellet was resuspended in RPMI.

**Cell culture and stable cell lines.** Rat-1 fibroblasts and human embryo kidney 293T cells were maintained in DMEM (Gibco) supplemented with heat-inactivated 10% fetal bovine serum (FBS; Sigma) and antibiotic/antimycotic (Gibco). Rat-1 cells were prepared fresh for each experiment and were maintained for no more than four passages. Human foreskin keratinoctyes (HFK) cells, immortalized with hTERT, were maintained in keratinocyte-SFM (Gibco) medium supplemented with 15 ng bovine pituitary extract (Gibco), 100 ng epidermal growth factor (Gibco), and antibiotic/antimycotic. BJAB cells were maintained in RPMI 1640 medium with Penicillin 5 units/ml, 10% FBS and antibiotic/antimycotic. Stable cell lines expressing pBabe or the variants in pBabe were established by transduction with recombinant retrovirus, followed by selection with 5 μg/ml puromycin (Sigma) for Rat-1 cells and with 2.5 μg/ml puromycin for HFK and BJAB cells.

**Homotypic adhesion assay.** BJAB cells were transduced by spinoculation and placed under puromycin selection, a procedure followed by separation through lymphocyte separation medium (ICN) gradient and amplification of live cells using puromycin selection. Equal cell numbers (∼1 × 10^6 cells/ml) were placed in a six-well plate and observed every 24 h for clumping with phase-contrast microscopy.

**Cell harvesting and Western blotting.** Cell lines were grown to confluence and harvested as described previously (14). Briefly, after being harvested, cells were washed with ice-cold PBS (Gibco), centrifuged at 1,000 × g, and lysed with radioimmunoprecipitation assay buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, and protease and phosphatase inhibitors; Sigma). Lysates were clarified by centrifugation and protein concentration was determined using the Bio-Rad DC protein assay system. Lysates were boiled in SDS sample buffer for 5 min. 12.5 to 25 μg of protein was separated by SDS-polyacrylamide gel electrophoresis and transferred to an Optitran membrane (Schleicher and Schuell) for Western blot analysis. An efficient transfer was measured with Ponceau S stain (0.1% FBS Europe Ltd.). Primary antibodies used included HA probe (Santa Cruz), c-myc tag (Santa Cruz), β-catenin (BD Biosciences), CS1-4 (Dako), an LMP1 rat monoclonal pool (CAO 7E10, CAO 8G3, LMP 1G6, and 7G8; Ascension), β-actin (Santa Cruz), total GSK3 (Upstate Biotechnology), p21/KIP (Calbiochem), Id1 (ZS, Santa Cruz), phospho-Rb (T37; Oncogene), Rb (BD Biosciences), p21/WAF (Oncongene), CDK2 (BD Biosciences), phospho-CDK2 (T160), phospho-Akt (Ser473), Akt, phospho-ASKJ (Ser9), phospho-c-Jun (extracellular signal-regulated kinase) (Ser244), phospho-PTEN (Ser400), total IκBα, total PTEN, and E-cadherin (Cell Signaling). Densitometry analysis was done with Image J software.

**Focus formation and soft agar.** Subconfluent Rat-1 cells were transduced with recombinant retrovirus in six-well plates overnight. Cells were then maintained by changing the media every other day for 10 to 14 days. To assay focus formation in the presence of LY294002 (LY; Calbiochem), stable cell lines were established as described previously and seeded in six-well plates. Upon the reaching of confluence and every day thereafter for 10 to 14 days, fresh medium containing 25 μM LY or a vehicle control (dimethyl sulfoxide [DMSO]; Sigma) at a dilution of 1:1,000 was added to the cells. Cells were then stained with 1% crystal violet in 50% ethanol and imaged with a stereomicroscope. Soft agar assays were carried out as described previously (32, 48). Briefly, Bacto agar medium (5% Bacto agar into DMEM with 10% FBS and antibiotics) was prepared in Bacto agar medium, laid over the initial layer of Bacto agar medium, and allowed to solidify. Medium was added on top of the solidified Bacto agar medium and changed every other day for 14 to 21 days. Cells were imaged with a phase-contrast microscope.

**Migration assay.** Stable HFK cells were established as described previously and seeded in six-well plates. Upon reaching confluence, cells were washed with PBS, the medium was replaced with keratinocyte-SFM medium without epidermal growth factor or bovine pituitary extract, and the cells were left in this medium for 24 to 48 h. Cells were then scratched with a 200-μl tip, and the medium was immediately changed and subsequently incubated at 37°C and imaged at 24 and 48 h with phase-contrast microscopy. Prior to imaging, cell medium was replaced to remove cell debris.

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Immunofluorescent labeling. Rat-1 cells expressing pBabe and pBabe-HA-tagged LMP1 variants were maintained in a six-well plate for 10 to 14 days, with the medium being changed every other day. Cells were then washed once with PBS, fixed with methanol for 5 min at room temperature, and washed three more times with PBS. Cells were blocked for 20 min with PBS containing 20% normal goat serum (NGS; Jackson Laboratories), incubated with primary antibody in PBS-20% NGS for 1 h, washed three times with PBS, incubated with rhodamine-conjugated secondary (Jackson Laboratories) in PBS-20% NGS for 1 h, and washed three more times with PBS. Stained cells were observed with phase-contrast and fluorescence microscopy.

Coimmunoprecipitations and NF-κB reporter assays. HA-tagged HOS and myc-tagged LMP1 variants were immunoprecipitated using Pierce mammalian ProFound HA and c-myc tag coimmunoprecipitation (coIP) kits as indicated by the manufacturer’s directions. Briefly, 293T cells were transfected with HA-tagged HOS and either pcDNA3 or the myc-tagged LMP1 variants. Cells were harvested 48 h posttransfection with radioimmunoprecipitation assay buffer, and protein concentration was determined as described previously. Immunoprecipitations were carried out with 500 μg of protein and 10 μl of the provided myc- or HA-agarose slurry with overnight nutation at 4°C according to the manufacturer’s instructions. colPs were then separated by SDS-polyacrylamide gel electrophoresis, and Western blotting was carried out as previously described.

NF-κB reporter assays were carried out by transfecting subconfluent 293T cells in a 12-well plate with Fugene 6 containing 0.2 μg pcDNA3 (vector control) or 0.2 μg pcDNA3-LMP1 variants, 0.2 μg pRL-SV40 (Promega), and 0.2 μg pGL3-Basic or 0.2 μg pGL3-3NF-κB (3xB), a gift from Albert Baldwin’s laboratory (43). Cells were harvested between 36 and 48 h posttransfection, and luciferase activity was determined as described previously (14).

Statistical analysis. P values were calculated using a Tukey-Krammer multiple comparisons test using the triplicate relative luciferase values from the NF-κB reporter. Statistical analysis was done using GraphPad Instat 3 software.

RESULTS

LMP1 variants transform Rat-1 fibroblasts. LMP1 is considered the EBV oncogene as it blocks contact-inhibited growth and induces anchorage-independent growth in Rat-1 fibroblasts (14, 32, 58). To determine the abilities of the LMP1 variants to block contact inhibited growth, Rat-1 fibroblasts were transduced with pBabe and pBabe-LMP1 variants, maintained for 10 to 14 days in full serum, fixed, stained with the anti-LMP1 antibody CS1-4, and subsequently imaged with phase-contrast and immunofluorescent microscopy for focus formation. Alaskan immunofluorescent staining is not provided.

FIG. 1. LMP1 variants block contact-inhibited growth of Rat-1 cells. Rat-1 fibroblasts were transduced with pBabe and pBabe-LMP1 variants, maintained for 10 to 14 days in full serum, fixed, stained with the anti-LMP1 antibody CS1-4, and subsequently imaged with phase-contrast and immunofluorescent microscopy for focus formation. Alaskan immunofluorescent staining is not provided.
variants was restricted to the foci, indicating that all cells that expressed LMP1 formed foci. Immunofluorescent staining for the Alaskan variant is not included, as the CS1-4 antibody does not recognize the Alaskan variant. There was some variability in the sizes and morphologies of the foci induced by all the LMP1 variants. Equivalent amounts of DNA used to make retrovirus and serial dilutions of the retrovirus for each variant yielded similar numbers of foci (data not shown). These data suggest that all variants are equally efficient in focus formation.

To further determine the transforming abilities of the LMP1 variants, Rat-1 cells stably expressing the LMP1 variants were assayed for anchorage-independent growth by growth in soft agar. As in the observations of focus formation, all LMP1 variants were able to induce anchorage-independent growth (Fig. 2). Equivalent numbers of cells stably expressing the variants induced equal numbers of colonies of various sizes and morphologies, indicating that all LMP1 variants are able to induce anchorage-independent growth with equivalent efficiencies. The size variation between colonies may be due to variable LMP1 expression within each colony. These data in conjunction with the ability of all the variants to block contact-inhibited growth suggest that the signature amino acid changes in the LMP1 variants do not impede or enhance their transforming potentials.

**LMP1 variants induce increased motility.** It has been shown previously that expression of LMP1 in the nasopharyngeal carcinoma cell line NP69, which expresses SV40 T antigen, or in the MDCK canine cell line induces cellular motility (25, 29). In addition, LMP1 induces actin stress fiber and filopodium formation (44). To determine the abilities of the LMP1 variants to induce motility in normal human epithelial cells, a scratch wound healing assay was performed using telomerase-immortalized human keratinocytes. Monolayers of HFK cells stably expressing the LMP1 variants or the vector control (pBabe) were scratched and assayed for increased motility 24 and 48 h postscratch by phase-contrast microscopy. White lines delineate approximate locations of scratch boundaries.

**LMP1 variants activate the PI3K-Akt signaling pathway.** A recent study indicated that LMP1 transformation of Rat-1 fibroblasts is dependent on activation of the PI3K-Akt pathway (7, 32). To assess the abilities of the LMP1 strains to activate Akt, phosphorylation of Akt as well as various targets of Akt was assessed in the stably LMP1-expressing Rat-1 cells that were analyzed as shown in Fig. 2 (Fig. 4A). Although the total levels of Akt were not changed, various levels of activated Akt above that of vector control were detected with all the LMP1 variants (Fig. 4B). Increased phosphorylation of the Akt target GSK3β also was detected with all the LMP1 variants (Fig. 4A). Interestingly, phosphorylation of another Akt target, mTOR,
was not affected. Total Akt and total GSK3β levels in conjunction with Ponceau S staining and an actin blot (not shown) confirmed equal loading on all lanes. These findings indicate that activation of Akt by LMP1 affects a subset of Akt-regulated pathways.

Expression of the LMP1 variants was assessed using a polyclonal antibody to HA and the CS1-4 monoclonal antibody pool (Fig. 4A). Detection by HA suggested similar levels of expression of Alaskan, B95.8, China 1, and Med+/− and lower levels of China 2, Med−, and NC (Fig. 4A). In contrast, detection by CS1-4 suggested that B95.8, China 1, and China 2 were expressed at very high levels with low levels of Med− and NC and nearly undetectable Med+. Alaskan is not detected by CS1-4. These differences clearly indicate different affinities of CS1-4 antibody for the different LMP1 variants. Surprisingly, the HA epitope tag apparently was recognized with differing affinities for the tagged variants. CS1-4 also detects consistent cleavage products for each LMP1 variant that are not observed with HA. This suggests that there are differences in preferred proteolytic cleavage sites between the variants that eliminate the amino-terminal end of LMP1, thus removing the HA tag. These differences in cleavage products may account for the differences in the detection of the LMP1 variants by the HA antibody.

The clear differences in reactivity to the antibodies indicate that the expression levels of the LMP1 variants cannot be directly compared. However, densitometry analysis of LMP1 expression levels as well as of total levels of phospho-Akt, phospho-GSK3β, and E-cadherin was performed (Fig. 4B). LMP1 expression was normalized to the B95.8 variant using the HA Western blot data, whereas the other markers were normalized to the vector control (pBabe). Expression of the LMP1 variants, as estimated by comparing HA reactivities, ranged from 19% for China 2 to 117% for Med+ compared to the B95.8 variant. Akt phosphorylation was increased from 127% to 223% of the control. In Alaskan, B95.8, China 1, and Med+, which seem to be roughly equally expressed based on HA reactivity, normalization of the induction of Akt to LMP1 expression ranged from 1.4- to 1.9-fold. In cells expressing China 2, Med−, and NC, which seem to be poorly detected by the HA antibody, normalization to LMP1 suggested induction that ranged from 4.5- to 9.2-fold. Phosphorylation of GSK3β ranged from 257% to 453% of the vector control and normalization to LMP1 indicated increases from 2.9- to 3.8-fold for variants detected similarly by anti-HA and from 4.5- to 9.2-fold for the poorly detected variants. The disproportionately high ratios for the China 1, Med−, and NC variants also suggest that the expression levels of LMP1 are underestimated for these variants.

It has been previously reported that LMP1 induces the methylation of the E-cadherin promoter, resulting in the overall decrease of E-cadherin protein levels in LMP1-expressing NPC cell lines (56, 57). To determine the effects of the LMP1 variants on E-cadherin expression, Western blot analysis was performed with Rat-1 cells stably expressing the LMP1 variants (Fig. 4A). E-cadherin levels were decreased with all variants and detected at levels from 19% in the Med− variant to 46% in the B95.8 variant compared to pBabe. Similar to what was seen for Akt and GSK3β, the overall reduction of E-cadherin levels did not correlate with the estimated expression levels of the LMP1 variants as determined by HA. The China 1 and China 2 variants, which are very differently detected by anti-HA, were expressed at similar levels as detected by CS1-4, and the stable cell lines expressing China 1 and China 2 had 40% and 44% of the E-cadherin expressed in the pBabe control. These data suggest that China 1 and China 2 have equivalent effects on E-cadherin.

The PTEN phosphatase regulates PI3K-Akt signaling through its ability to regulate the phosphorylation status of phosphoinositides. Loss-of-function mutations of PTEN have been identified for several cancers, including endometrial carcinomas and lymphomas (31). To determine if the activation of PI3K by LMP1 is mediated by affecting PTEN expression or its activation status, Western blot analysis for the phosphorylated, inactive form of PTEN as well as for total levels of PTEN was performed (Fig. 4A). Expression of the LMP1 variants did not...
affect the levels of phosphorylated PTEN and resulted in slight increases in total PTEN. These results suggest that PI3K-Akt activation by LMP1 is not mediated by PTEN inactivation or reduced PTEN protein levels.

These findings indicate that all LMP1 variants can activate the PI3K-Akt signaling pathway as well as reduce E-cadherin protein levels. However, none of the variants alter the activation status of mTOR or PTEN.

**LY294002 blocks focus formation by LMP1 variants.** A previous study indicated that transformation of Rat-1 fibroblasts is dependent on PI3K-Akt activation (32). To determine if the requirement of the LMP1 variants to block contact-inhibited growth is dependent on the PI3K-Akt signaling pathway, Rat-1 cells stably expressing the LMP1 variants were treated with the vehicle control (DMSO) or the PI3K chemical inhibitor LY for 10 to 14 days and were observed for focus formation (Fig. 5). As previously observed for the B95.8 strain of LMP1 (32), LY treatment abolished focus formation for all LMP1 variants, indicating that activation of the PI3K-Akt pathway is required to suppress contact inhibition. LMP1 expression and inhibition of PI3K-Akt signaling, through analysis of phosphorylated Akt levels, was confirmed by Western blot analysis (data not shown).

**LMP1 variants regulate cell cycle markers.** The ability to induce cell cycle progression is a key step in oncogenesis. Recent studies indicate that LMP1, through CTAR1, increases the levels of Id1 and Id3 as well as of the procellular growth markers ppRb and CDK2 while reducing levels of the CDKI p27kip1 (14, 27). To determine whether the LMP1 variants could equally affect these cellular markers involved in cell cycle progression, Rat-1 cells were assayed by Western blot analysis. Confirming previous data for the B95.8 strain of LMP1, all variants induced high levels of Id1 in Rat-1 cells compared to the pBabe control cells with reduced protein levels of the CDKI p27kip1 (Fig. 6A).

Densitometry analysis of LMP1 expression as well as of protein levels of p27 and Id1 from the experiment whose results are shown in Fig. 6A was performed (Fig. 6B); the LMP1 bands for each variant that were used for densitometry analysis are shown. LMP1 expression as detected by anti-HA was normalized to the B95.8 variant, whereas levels of p27 and Id1 were normalized to the vector control (pBabe). Expression levels of the LMP1 variants ranged from 289% to 106% of that for the B95.8 variant. Protein levels of Id1 in cells expressing the LMP1 variants ranged from 143% to 250% of that for the vector control, whereas protein levels of p27 ranged from 24% to 59% of that for the vector control. Expression levels of the LMP1 variants did not directly correlate with higher Id1 levels or lower p27 levels. These data suggest that LMP1 can induce or repress Id1 and p27 only to a certain level and that overexpression of LMP1 does not necessarily lead to an increase or a decrease in these cellular markers.

To determine the effects of LMP1 on cell cycle markers in normal epithelial cells, ppRb and CDK2 phosphorylation as well as total Rb levels were assessed for HFK cells expressing the LMP1 variants. As was the case for Rat-1 cells, higher levels of ppRb, total Rb, and phosphorylated CDK2 were detected (Fig. 6C). In contrast to the effects on p27, the CDKI p21WAF1 levels were unchanged, which suggested specific effects of LMP1 on p27. However, all LMP1 variants induced phosphorylation of Rb and CDK2 as well as an induction in overall Rb protein levels. These data reveal that all LMP1 variants regulate a variety of cell cycle markers in a manner similar to that of the well-characterized B95.8 LMP1 strain.

**LMP1 variants induce homotypic adhesion of BJAB cells.** It has been established previously that expression of LMP1 in...
BJAB cells results in increased homotypic adhesion, possibly due to increased LFA-1 or ICAM-1 levels (16, 33, 59). Stable BJAB cell lines expressing the LMP1 variants were established as described previously and were observed for homotypic adhesion (Fig. 7A). All LMP1 variants induced a level of homotypic adhesion similar to that previously observed for the B95.8 strain (59). Although there was a small variation in the sizes and numbers of the cell clumps, overall there appeared to be no significant difference between the variants in terms of their abilities to induce homotypic adhesion.

LMP1 expression in the BJAB cells was confirmed by Western blot analysis (Fig. 6). Rat-1 cells stably expressing pBabe or the LMP1 variants were assayed for LMP1 expression (asterisks indicate LMP1 variants) and p27 and Id1 protein levels by Western blot analysis. Equal loading was confirmed with an actin Western blot. The quantitative analysis of LMP1 (white bars), p27 (black bars), and Id1 (striped bars) protein levels showed that LMP1 expression was normalized to B95.8, whereas p27 and Id1 levels were normalized to pBabe.

HFK cells stably expressing pBabe or the LMP1 variants were assayed for LMP1 expression, phosphorylated and total levels of Rb and CDK2, and p21/Waf1. Equal loading was confirmed with the actin blot.

FIG. 7. Homotypic adhesion of BJAB cells is induced by LMP1 variants. (A) Equal numbers of BJAB cells stably expressing pBabe or the LMP1 variants were seeded and observed for homotypic adhesion every 24 h by phase-contrast microscopy. Representative fields at 3 days post seeding are shown. (B) LMP1 variants activate Akt in BJAB cells. BJAB cells stably expressing pBabe or the LMP1 variants were assayed for LMP1 expression and phosphorylated and total levels of Akt. Equal loading was confirmed with an actin blot.
changes not only in epithelial cells but also in lymphocytes. This indicates that the LMP1 variants are capable of inducing phenotypic changes not only in epithelial cells but also in lymphocytes.

Abrogated HOS binding enhances LMP1 NF-κB activity. The E3 ubiquitin ligase component HOS, a member of the β-TrCP/Fbw1 subfamily of F-box proteins which targets β-catenin and IκBa, has been shown to bind to the B95.8 strain of LMP1 but not to the CAO strain. This binding requires a destruction box motif close to CTAR1 and two serines at amino acids 350 and 366 (53). There are multiple changes in these required motifs in the LMP1 variants. The Alaskan, China 1, China 2, and NC variants of LMP1 have a G212S mutation in the destruction box, and China 1 and Med+ have a 10-amino-acid deletion that eliminates serine 350; and all the variants but B95.8 have a mutation that changes the serine at position 366 to threonine (Fig. 8A) (10).

The ability of HOS to bind to the LMP1 variants was tested using transfection of 293T cells and colPs of HA-tagged HOS and myc-tagged LMP1 (Fig. 8B). Immunoblotting with HA and myc revealed that HOS and the LMP1 variants were efficiently expressed. Immunoprecipitation with HA and blotting with myc indicated that the Alaskan, China 1, and Med+ variants of LMP1 bound HOS weakly compared to the B95.8, China 2, Med−, and NC variants. The low binding ability of China 1 to HOS is similar to that observed previously for the CAO strain. The strong binding of Med−, which contains S350, compared to that of Med+, which is deleted for S350, indicates that this is an important element in binding HOS. The conservative change S366T in Alaskan, China 1, China 2, Med−, and NC did not correlate with HOS binding, as Alaskan bound HOS very poorly in comparison to Med− and NC, which bound strongly. These differences in binding suggest that multiple factors contribute to the HOS-LMP1 interaction.

To determine the abilities of the LMP1 variants to induce NF-κB activity, dual luciferase reporter assays were performed. All of the BJAB cell lines expressed detectable levels of LMP1, as determined by use of CS1-4 (Fig. 7B), as did the Alaskan strain, as determined by use of a pool of rat monoclonal antibodies for LMP1 (data not shown). All of the LMP1-expressing BJAB cells had higher levels of phosphorylated Akt, indicating that activation of Akt by LMP1 occurs in both epithelial and lymphoid cells. These data demonstrate that the LMP1 variants are capable of inducing phenotypic changes not only in epithelial cells but also in lymphocytes.

FIG. 8. Differential binding of HOS by LMP1 variants. (A) Sequence alignment of the LMP1 variants from amino acids 207 to 370 of LMP1 B95.8 showing residues important for HOS-LMP1 binding, including the destruction box, a serine residue at amino acid 350, and a serine residue at amino acid 366. The 10-amino-acid deletion found in the China 1 and Med+ variants is represented by del. Asterisks indicate amino acids important for HOS-LMP1 binding. B95.8 contains all residues required for HOS-LMP1 binding. (B) 293T cells cotransfected with pcDNA3 (vector control), with the LMP1 variants and HOS, or with HOS alone were harvested 48 h posttransfection and analyzed for HOS-LMP1 binding through coIP. HOS was immunoprecipitated with anti-HA-conjugated beads, and Western blot analysis for LMP1 was carried out with anti-c-myc monoclonal antibodies for LMP1 (data not shown). All of the BJAB cell lines expressed detectable levels of LMP1, as determined by use of CS1-4 (Fig. 7B), as did the Alaskan strain, as determined by use of a pool of rat monoclonal antibodies for LMP1 (data not shown). All of the LMP1-expressing BJAB cells had higher levels of phosphorylated Akt, indicating that activation of Akt by LMP1 occurs in both epithelial and lymphoid cells. These data demonstrate that the LMP1 variants are capable of inducing phenotypic changes not only in epithelial cells but also in lymphocytes.

Abrogated HOS binding enhances LMP1 NF-κB activity. The E3 ubiquitin ligase component HOS, a member of the β-TrCP/Fbw1 subfamily of F-box proteins which targets β-catenin and IκBa, has been shown to bind to the B95.8 strain of LMP1 but not to the CAO strain. This binding requires a destruction box motif close to CTAR1 and two serines at amino acids 350 and 366 (53). There are multiple changes in these required motifs in the LMP1 variants. The Alaskan, China 1, China 2, and NC variants of LMP1 have a G212S mutation in the destruction box, and China 1 and Med+ have a 10-amino-acid deletion that eliminates serine 350; and all the variants but B95.8 have a mutation that changes the serine at position 366 to threonine (Fig. 8A) (10).

The ability of HOS to bind to the LMP1 variants was tested using transfection of 293T cells and colPs of HA-tagged HOS and myc-tagged LMP1 (Fig. 8B). Immunoblotting with HA and myc revealed that HOS and the LMP1 variants were efficiently expressed. Immunoprecipitation with HA and blotting with myc indicated that the Alaskan, China 1, and Med+ variants of LMP1 bound HOS weakly compared to the B95.8, China 2, Med−, and NC variants. The low binding ability of China 1 to HOS is similar to that observed previously for the CAO strain. The strong binding of Med−, which contains S350, compared to that of Med+, which is deleted for S350, indicates that this is an important element in binding HOS. The conservative change S366T in Alaskan, China 1, China 2, Med−, and NC did not correlate with HOS binding, as Alaskan bound HOS very poorly in comparison to Med− and NC, which bound strongly. These differences in binding suggest that multiple factors contribute to the HOS-LMP1 interaction.

To determine the effects of this binding on HOS targets, the levels of β-catenin and IκBa were determined. The level of β-catenin was unchanged, corroborating previous data indicating that the LMP1-HOS interaction does not affect β-catenin levels (53). The total levels of IκBa did not fully correlate with the levels of HOS-LMP1 binding. China 1, which bound HOS very poorly, had the least IκBa, confirming an earlier report (53), and most LMP1 variants had lower IκBa levels than the vector control. Interestingly, the B95.8 LMP1, which bound HOS well, had more IκBa than the other variants, including the pcDNA3 vector control. This is in contrast to results for the Med− and NC variants, which also bound HOS very well but had lower levels of IκBa. IκBa levels can be affected by multiple factors in addition to HOS binding, including transcriptional regulation by NF-κB and the activity of the IκB kinases. Thus, the absolute level of IκBa may not be reflective of its activity.

To determine the abilities of the LMP1 variants to induce NF-κB activation, dual luciferase reporter assays were per-
formed with the LMP1 variants in 293T cells (Fig. 9). Interestingly, activation of NF-κB correlated with the ability of HOS to bind LMP1. The Alaskan, China 1, and Med+ variants, which bound HOS poorly, induced higher NF-κB reporter activity than the B95.8, China 2, Med−, and NC variants, which bound HOS well and induced nearly equivalent levels of NF-κB activity. The differences in reporter activity between all the LMP1 variants and pcDNA3 had a P value of less than 0.001 by the Tukey-Kramer multiple comparisons test, indicating that they are statistically significant. Furthermore, the increased reporter activity observed for the Alaskan, China 1, and Med+ variants in comparison to that for the B95.8 variant also had a P value of less than 0.001. Transfected HOS without LMP1 induced 2.4-fold more NF-κB reporter activity, suggesting that HOS can marginally induce NF-κB reporter activity compared to LMP1. In addition, protein levels of the NF-κB subunits p65/RelA and p50 were higher in the nuclear fractions of the LMP1 variants than in pcDNA3 and correlated with the enhanced NF-κB reporter activity (data not shown). These data indicate that while all of the LMP1 variants can activate NF-κB signaling, those variants that do not bind HOS have enhanced NF-κB activation.

**DISCUSSION**

The data presented in this study indicate that all LMP1 variants transform Rat-1 fibroblasts at equal efficiencies and activate the PI3K-Akt signaling pathway, which is required for Rat-1 fibroblast transformation. These data demonstrate that the signature amino acid changes in the LMP1 variants do not hinder or enhance their transforming potentials in vitro. The ability of all the LMP1 variants to activate the PI3K-Akt signaling was not unexpected, as the ability for LMP1 to transform Rat-1 fibroblasts and activate PI3K-Akt signaling has been mapped to CTAR1 (7, 32), which is highly conserved among all the variants (10, 35). The activation of the PI3K-Akt signaling pathway in BJAB cells also reveals that activation of this pathway is not restricted to epithelial cells but also occurs in B lymphocytes. Interestingly, activation of Akt had differential effects on known Akt targets, as the phosphorylation and inactivation of GSK3β were significantly affected in comparison to the unchanged levels of phosphorylated mTOR. The subcellular localization of activated Akt and/or availability of Akt targets likely contribute to these variable effects on regulation. The association of LMP1 with lipid rafts (19) may target activation of Akt by limiting LMP1 to specific regions in the cell.

PTEN is a tumor suppressor gene with mutation frequencies in human cancer approaching that of p53 (5). PTEN, a phosphatase, regulates PI3K signaling by reversing the PI3K-driven phosphorylation of phosphoinositides that are necessary for the downstream activation of Akt. The LMP1 variants, including the B95.8 strain, did not affect the phosphorylation levels of PTEN, and LMP1-expressing cells had slightly increased levels of PTEN. These findings suggest that while LMP1 induces increased PI3K-Akt signaling, it does not regulate this pathway through inhibiting the activity of PTEN.

Cadherin molecules are a group of calcium-dependent transmembrane proteins that aid in cell-cell adhesion (18). LMP1 has been shown to downregulate E-cadherin levels in NPC cell lines through activation of DNA methyltransferases (56). Similar to what has been observed with LMP1 B95.8, all LMP1 variants downregulated E-cadherin protein levels in Rat-1 cells. These findings are congruent with the increased motility observed with the LMP1 variants.

The Wnt/β-catenin signaling pathway is regulated by GSK3β, which can phosphorylate β-catenin and induces β-catenin ubiquitination and proteasome-mediated degradation (37). Inactivation of GSK3β through phosphorylation by Akt can lead to the accumulation of β-catenin in the cytoplasm followed by nuclear translocation, where it interacts with TCF/LEF transcription factors to drive transcription of several genes (54). The EBV protein LMP2A also activates the PI3K-Akt pathway and induces GSKβ phosphorylation and β-catenin accumulation in the nucleus (38). The data in this study reveal that LMP1 expression activated Akt and induced phosphorylation of GSKβ but did not affect nuclear β-catenin signaling.
protein levels (reference 13 and data not shown). This indicates that in contrast to LMP2, which promotes β-catenin accumulation and nuclear translocation (38), LMP1 does not affect β-catenin levels in epithelial cells.

The regulation of the cell cycle is an important step in oncogenesis, and LMP1, through CTAR1, affects expression of several proteins that regulate G1/S-phase progression, including Rb, CDK2, Id1, Id3, and CDKI p27Kip1 (14). All of the LMP1 variants had similar effects on the expression of these critical proteins, further suggesting that the signature amino acid changes do not enhance or detract from the ability to deregulate the cell cycle. Interestingly, the regulation of the CDKI proteins by LMP1 appears to be limited to p27, as p21 levels were not changed in HFK cells. Also, hyperphosphorylated and total protein levels of Rb were increased in LMP1-expressing HFK cells. Although hyperphosphorylated Rb can be targeted for proteasomal degradation (30), LMP1-expressing cells also had increased total levels of Rb. Higher total and hyperphosphorylated levels of Rb also were observed in BL41 cells expressing the B95.8 strain of LMP1 (1), suggesting that LMP1 may either block Rb degradation or induce increased Rb expression while also inducing its hyperphosphorylation.

It has been documented that LMP1 activates the NF-κB signaling pathway through both CTAR1 and CTAR2 (2, 8, 21, 34, 36, 55). It has also been demonstrated that the CAO strain, which is the most similar to China 1, and Med+ activate NF-κB signaling to a larger extent (4, 34). Subsequently, the increased NF-κB activity observed with CAO was reported to be due to its lack of binding of HO5; an E3 ubiquitin ligase component that regulates IκBα turnover (47, 53, 60). The data presented here confirm that link, as increased NF-κB reporter activity was observed with the Alaskan, China 1, and Med+ variants compared to that for B95.8 and the other variants that bound HOS. However, the proposed motifs required for HOS binding did not correctly predict HO5-LMP1 binding, indicating that HO5-LMP1 binding is more complex than previously described. Furthermore, IκBα protein levels did not correlate with HO5-LMP1 binding, suggesting that IκBα turnover is regulated by factors other than HO5.

Enhanced NF-κB signaling of the LMP1 strain Med+ was previously mapped to the transmembrane domains (34). Other data indicate that the first and sixth transmembrane domains of LMP1 are important for proper signaling. Six leucine repeats that may confer a specific structure when LMP1 self-assembles in the cellular membrane (23) and an FWLY motif in the first transmembrane domain that possibly mediates interactions between LMP1 and other molecules in lipid rafts (61) have been identified. The first transmembrane domains of the Alaskan and China 2 variants contain two isoleucine mutations in the six-leucine repeat, although the isoleucine substitutions do not disrupt LMP1 signaling with the B95.8 strain (23). In addition, the FWLY motif is unchanged in all the variants. These data suggest that multiple properties of LMP1 which may be affected by the leucine repeats and the FWLY motif, including HOS binding and self-aggregation, contribute to the activation of NF-κB signaling.

However, it should be noted that all LMP1 variants induce robust NF-κB reporter activity. Activation of NF-κB is required for continued growth of EBV-transformed lymphocytes but is not required for rodent fibroblast transformation. This study reveals that all of the LMP1 variants activate both NF-κB and PI-3K/Akt signaling and have equivalent effects on cell growth and transformation. It has previously been shown that the sequence changes that distinguish the LMP1 variants are preferentially located within known and predicted class I HLA epitopes (11). The similar biological and biochemical properties of the variants described in this study may suggest that these variants arose and are maintained through immune selection.

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