Epstein-Barr Virus Latent Membrane Protein 2 Effects on Epithelial Acinus Development Reveal Distinct Requirements for the PY and YEEA motifs

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Epstein-Barr virus (EBV) is a gammaherpesvirus associated with numerous cancers, including the epithelial cancers nasopharyngeal carcinoma (NPC) and gastric carcinoma. The latent membrane protein 2 (LMP2) encoded by EBV is consistently detected in NPC tumors and promotes a malignant phenotype when expressed in epithelial cells by inducing transformation and migration and inhibiting differentiation. Grown in three dimensions (3D) on Matrigel, the nontumorigenic mammary epithelial cell line MCF10A forms hollow, spherical acinar structures that maintain normal glandular features. Expression of oncogenes in these cells allows for the study of multiple aspects of tumor development in a 3D culture system. This study sought to examine the effects of LMP2 on the generation of MCF10A acini. LMP2 expression induced abnormal acini that were large, misshapen, and filled, indicating that LMP2 induced proliferation, impaired cellular polarization, and induced resistance to cell death, leading to luminal filling. Induction of cell death resistance required the PY, immunoreceptor tyrosine activation motif (ITAM), and YEEA signaling domains of LMP2 and activation of the Src and Akt signaling pathways. The PY domain was required for the inhibition of anoikis and also the delayed proliferative arrest of the LMP2-expressing cells. In addition to directly altering acinus formation, expression of LMP2 also induced morphological and protein expression changes consistent with epithelial-mesenchymal transition (EMT) in a manner that required only the YEEA signaling motif of LMP2. These findings indicate that LMP2 has considerable transforming properties that are not evident in standard tissue culture and requires the ability of LMP2A to bind ubiquitin ligases and Src family kinases.

Epstein-Barr virus (EBV) is a highly successful gammaherpesvirus that infects the majority of people worldwide and establishes lifelong latency with B cells with sporadic reactivation and shedding at the oropharynx and other mucosal surfaces. EBV is also linked to the development of multiple cancers, including lymphomas that can occur in immunocompromised individuals, Hodgkin lymphoma (HD) and Burkitt lymphoma, and the epithelial cancers nasopharyngeal carcinoma (NPC) and gastric carcinoma (1–7). Within the malignant cells, viral expression is restricted; most cells do not produce virus, and the infection is considered nonpermissive or latent. However, several viral proteins and RNAs are expressed and are thought to contribute to the development of malignancy. One important latent protein is latent membrane protein 2 (LMP2), which is expressed at high levels in HD (8) and is also detected in NPC tumors (2).

LMP2A is localized within membranes and contains 12 transmembrane domains and a long cytoplasmic domain at the amino terminus. The N-terminal cytoplasmic domain contains multiple tyrosines, and at least three major signaling motifs have been identified. One domain, YEEA, including tyrosine 112, has been shown to bind members of the Src family tyrosine kinases, and in B cells, this domain recruits tyrosine kinases, particularly Lyn (3, 9–11). This initiates the tyrosine phosphorylation of a domain analogous to the immunoreceptor tyrosine activation motif (ITAM) found within the alpha and beta chains of the B-cell receptor (BCR). Phosphorylation at the ITAM signaling domain at tyrosine 74 and 85 recruits additional tyrosine kinases, including Syk (3, 9). This activation prevents BCR-induced viral replication but in transgenic mice has been shown to also contribute to cell development and survival through the constitutive activation of

phosphoinositide kinase 3 (PI3) (3). Signaling by LMP2A is also regulated by the recruitment of Nedd4 ubiquitin ligases, in particular Itch, to the two N-terminal PY domains of LMP2A. PY regulates the ubiquitination and potential degradation of LMP2A-associated proteins and in B cells enhances the turnover of the Src family kinase, Lyn (3, 11–15). In epithelial cell lines, expression of LMP2A has been shown to inhibit differentiation and increase migration, and it may induce anchorage-independent growth dependent on Ras activation (16–20). These changes in epithelial growth regulation are dependent on the activation of the Ras/PI3 kinase/Akt pathway, and inhibition of Akt or Syk activation impairs LMP2-induced migration. The induction of migration has been linked to LMP2A effects on integrin expression and location with subsequent activation of Src and focal adhesion kinase (FAK) (16). LMP2A also induces stabilized expression of the cellular p53 homolog, ΔNp63, and this induction is required for LMP2-mediated inhibition of differentiation (17). Additionally, in epithelial cell lines, LMP2 stabilizes β-catenin and induces its nuclear translocation (20, 21). Interestingly, despite these intriguing properties, in transgenic mice with LMP2A expressed under the control of the keratin 14 (K14) promoter, there is no detectable effect on epithelial growth and the skin is not sensitized to tumor promo-

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tion after exposure to carcinogens and tumor promoters (22). However, in doubly transgenic mice and in combination with LMP1, the expression of LMP2A promotes the development of carcinoma (22, 23). It is likely that other intriguing biologic and molecular properties will be identified for LMP2.

To further define the functional effects of LMP2A expression on epithelial cell growth regulation, LMP2A was expressed in the normal mammary epithelial cell line MCF10A. These cells have been used to identify multiple steps in epithelial cell differentiation when induced to differentiate by growth by exposure to extracellular matrix (ECM) as produced in Matrigel. After initial proliferation, the cells begin to develop apical/basolateral polarization, followed by a stop in growth and cell cycle arrest. Interior cells do not receive ECM stimulation, and this anchorage-independent growth induces anoikis with caspase-mediated death of the interior cells (24–26). Thus, the cells form round acini characterized by a hollow lumen within a sphere of polarized cells. The effects of multiple cellular oncogenes and requirements for specific signaling pathways involved in the sequential cell proliferation, apoptosis, and potential malignant progression have been identified in this system. Deregulation of the cell cycle through overexpression of cyclin D1 or inactivation of the retinoblastoma tumor suppressor gene by the human papillomavirus (HPV) E7 protein resulted in continued proliferation of apical cells, resulting in enlarged acini; however, the apoptotic process that forms the hollow lumen was not impaired (25). Induction of activated Akt repressed the formation of acinar lumen and, in combination with E7, induced larger, filled structures (27). Expression of the oncogene ErbB2 also inhibited cell death within the acinar lumen, preventing hollowing, disrupted the polarity of cells surrounding the lumen, and induced proliferation, leading to spheroids that were large, filled, and irregular, all features of tumors *in vivo* (25). Additional studies have identified specific effects linked to the expression of Δ Np63, Notch, and Ras (28–31).

Many of the same pathways and properties are also modulated by LMP2A, which can activate Ras and Akt, induce proliferation, impair differentiation, and modulate protein levels through its interaction with ubiquitin (12, 15–17, 19, 32). Therefore, this study focused on determining how LMP2A expression in MCF10A would affect the formation and structure of acini and which signaling domains of LMP2A and pathways affected by LMP2 contributed to potential changes in acinar development.

MATERIALS AND METHODS

Cell culture. MCF10A breast epithelial cells were obtained from American Type Culture Collection and were cultured in Dulbecco's modified Eagle medium (DMEM)/F12 medium (Gibco, Grand Island, NY) supplemented with 5% horse serum, 20 ng/ml epidermal growth factor (EGF), 100 ng/ml cholera toxin, 500 μ g/ml hydrocortisone, 10 μ g/ml insulin, and 1% penicillin/streptomycin in a humidified growth chamber with 5% CO₂. Cells were transduced with retroviral vectors to stably express LMP2A, the LMP2A mutants of PY, ITAM, and YEEA, and the pBabe vector control as previously described (17, 19, 21). Stably transduced cells were selected and maintained in DMEM/F12 medium containing puromycin (0.5 μ g/ml). Cell lines were generated a minimum of three separate times for this study.

Growth of mammary epithelial acini. To induce formation of acini, MCF10A cells were seeded on layers of growth factor-reduced Matrigel (BD Biosciences, San Jose, CA) in 8-well chamber slides. Seeding densities were 2×10^4 cells per well in DMEM/F12 medium supplemented with 100 ng/ml cholera toxin, 500 μ g/ml hydrocortisone, 10 μ g/ml insulin, 5 ng/ml

EGF, 2% horse serum, 2% growth factor-reduced Matrigel, and 0.5 μ g/ml puromycin. Bright-field images were obtained with 10 $\times$ and 20 $\times$ objectives on an inverted microscope at the Tissue Culture Facility, Lineberger Comprehensive Cancer Center, University of North Carolina. The size of day 20 acini was determined by measuring the area of 4 acini per field using ImageJ software. Each acinus was measured 3 times, and the averages from 4 acini per field were determined relative to those for pBabe for each experiment. The average size ratio relative to the pBabe results from 4 experiments was expressed graphically, and statistics were calculated using the Student *t* test.

Immunofluorescence staining. Mammary epithelial acini were fixed and subjected to immunofluorescence staining as described previously (26). Medium was removed from slides, and acini were fixed with cold acetone-methanol (1:1) or with 2% paraformaldehyde for 20 min. For acini fixed with paraformaldehyde, cells were permeabilized following fixation with 0.5% Triton X-100 prepared in phosphate-buffered saline (PBS) for 10 min at 4°C. Matrigel was washed 3 times with PBS-glycine (100 mM glycine prepared in PBS) and blocked for 90 min at room temperature with blocking buffer (130 mM NaCl, 7 mM Na₂HPO₄, 3.5 mM NaH₂PO₄, 7.7 mM NaN₃, 0.1% bovine serum albumin [BSA], 0.2% Triton X-100, 0.05% Tween 20, 10% goat serum). Primary antibody was incubated overnight at room temperature using 1:100 dilutions of anti- β -catenin (Cell Signaling Technology, Danvers, MA), anti-Ki67 (Millipore, Billerica, MA), anti-total Akt (Cell Signaling Technology, Danvers, MA), or anti-caspase 3 (cleaved; Cell Signaling Technology, Danvers, MA) antibodies prepared in blocking buffer. Following primary antibody incubations, acini were washed 3 times with PBS-glycine and incubated for 60 min with 1:100 dilutions of goat anti-mouse 488 or goat anti-rabbit 594 (Invitrogen, Grand Island, NY). For immunofluorescence of 2-dimensional cell culture, MCF10A cells were seeded on 8-well chamber slides and allowed to adhere overnight. Cells were fixed with ice-cold acetone-methanol, blocked with 3% BSA, and incubated with primary antibody (anti-N-cadherin and anti-E-cadherin [Cell Signaling Technology, Danvers, MA], anti-vimentin [Abcam, Cambridge, MA], anti-LMP2A [Thermo Scientific, Rockford, IL]) for 2 h at room temperature. Slides were incubated with secondary antibody, stained with 4',6'-diamidino-2-phenylindole (DAPI), and sealed with anti-fade mounting medium. Images were acquired at the Microscopy Services Laboratory, University of North Carolina at Chapel Hill, using a Zeiss 710 confocal laser scanning microscope. Brightness and contrast were adjusted equally across images.

Protein lysates and Western blots. Whole-cell lysates from MCF10A cells were prepared by incubating cell pellets on ice with radioimmuno-precipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100). Lysates were centrifuged for 15 min at 4°C at 16,000 $\times$ g, and supernatants were subjected to SDS-PAGE. To prepare acinar lysates, cells were extracted from Matrigel using cell recovery solution (BD Biosciences, San Jose, CA) according to the manufacturer's directions. Cell pellets were lysed in RIPA buffer as described for whole-cell lysates, and supernatants were subjected to SDS-PAGE. Gels were transferred to nitrocellulose membranes, blocked with 5% milk prepared in Tris-buffered saline (TBS)-Tween and incubated overnight with primary antibody at 4°C (1:1,000 anti-Bim [Cell Signaling Technology, Danvers, MA], 1:5,000 anti-HSC70 [Santa Cruz Biotechnology, Santa Cruz, CA], 1:1,000 anti-E-cadherin [Cell Signaling Technology, Danvers, MA], 1:1,000 anti- β -catenin [Cell Signaling Technology, Danvers, MA], 1:1,000 anti-phospho-Akt [Ser 473 and Thr 308; Cell Signaling Technology, Danvers, MA], 1:1,000 anti-N-cadherin [Cell Signaling Technology, Danvers, MA], 1:2,000 anti-vimentin [Abcam, Cambridge, MA], 1:1,000 anti-fibronectin [BD Biosciences, San Jose, CA], 1:1,000 anti-LMP2A [Thermo Scientific, Rockford, IL]). Membranes were washed 3 times with TBS-Tween and incubated in horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h (1:1,000 anti-mouse, 1:1,000 anti-rabbit, 1:1,000 anti-rat).

Bands were visualized with enhanced chemiluminescence (Thermo Scientific, Rockford, IL).

polyHEMA anoikis assay. Tissue culture plates (96 well) were treated with 10 mg/ml poly(2-hydroxyethyl methacrylate) (polyHEMA) dissolved in 95% ethanol and were allowed to dry overnight in sterile conditions. MCF10A cells were seeded on treated culture plates, and proliferation was determined at the time points indicated in Fig. 4B using a CellTiter 96 AQueous One Solution cell proliferation assay (Promega, Madison, WI) according to the manufacturer's instructions.

RESULTS

LMP2A is consistently detected in the epithelial cancer NPC, and multiple effects have been identified in 2-dimensional (2D) tissue culture, including increased migration and motility, transformation, and inhibition of differentiation, that are characteristic of tumor growth and development (16–19, 21). The MCF10A cells are spontaneously immortalized mammary epithelial cells that form acini with hollow lumen when cultured on Matrigel. This *in vitro* acinus model provides an opportunity to study the effects of oncogenes on the complex differentiation and growth processes that occur in the formation of the 3-dimensional (3D) acini. This study examined the effects of LMP2A expression on 3D acinus growth to further understand how LMP2A affects epithelial cell growth and differentiation.

MCF10A cells were transduced to stably express the retroviral vector pBabe, LMP2A, and the LMP2A mutants within the PY, ITAM, and YEEA motifs. pBabe and LMP2A expressing MCF10A cells were seeded on growth factor-reduced Matrigel and maintained for 20 days, and growth of acini was documented with bright-field images. By day 8 in culture, pBabe acini appeared organized, uniform, and round, whereas LMP2A acini were larger and somewhat misshapen (Fig. 1A). By day 20 (Fig. 1A), pBabe acini were slightly larger than on day 8, indicative of the previously described growth arrest, and they appeared polarized, uniform, and round. The LMP2A acini were larger than those of pBabe, misshapen, and lobular, and some appeared to have formed multiacinar structures, similar to what has been observed for ErbB2-expressing MCF10A cells (24, 25, 33). Confocal images of pBabe- and LMP2A-expressing acini at day 20 were stained with DAPI to visualize nuclei and confirmed these findings (Fig. 1B). pBabe acini were round, most contained a hollow lumen, and the organized outer layer of cells suggested polarization (Fig. 1B). LMP2A-expressing acini were large, filled, occasionally lobular, misshapen, and lacked any organization suggestive of polarization (Fig. 1B). The large size and filled lumen indicated that both growth arrest and anoikis were impaired.

To identify the properties of LMP2A that were required to induce the large, misshapen, and multiacinar phenotype in MCF10A acini, MCF10A cells expressing mutants of the PY, ITAM, or YEEA signaling motifs of LMP2A were generated. At day 20, pBabe acini had the organized, round, hollow phenotype consistent with those shown in Fig. 1, and β -catenin was localized to the plasma membrane, indicating membrane organization and the presence of junctions in the cells of the outer ring (Fig. 2A). LMP2A-expressing acini were large, misshapen, and filled, showing disorganized expression of β -catenin, with both junctional expression and increased detection in the cytoplasm of some cells. These findings confirmed those from the DAPI stains and indicated that LMP2A cells did not form the hollow lumen characteristic of acini (Fig. 2A). Analysis of LMP2 mutants revealed that mutation of either the ITAM or the YEEA domain eliminated the

effects of LMP2A on growth, polarization, and lumen formation. The cells formed acini that were similar in size and shape to pBabe acini, with lumen hollowing and junctional staining of β -catenin (Fig. 2A). These results suggest that both the induction of proliferation and the resistance to cell death induced by LMP2A required both the ITAM and YEEA motifs. Surprisingly, cells expressing LMP2 with mutation of the PY ubiquitin ligase binding motif formed acini that were as large as or larger than the LMP2A spheres. The formation of large acini by cells expressing LMP2A with the mutated PY signaling domain revealed that this domain was not required for enhanced proliferation but was essential for the resistance to anoikis and cell death that resulted in filled acini or spheroids with LMP2A-expressing cells. The size of the acini was calculated by measuring the area using ImageJ software. Four acini per field were measured 3 times, and the average size relative to that of the pBabe control is expressed graphically in Fig. 2B. This analysis confirmed that the consistent effects of LMP2A on acinar size were statistically significant and that these effects required the ITAM and YEEA signaling domains.

To further evaluate the effect of LMP2A expression on MCF10A cell proliferation during acinus formation, acini from pBabe-, LMP2A-, and PY mutant-expressing cells were grown for 8 days and stained with DAPI to visualize nuclei and, for Ki67 staining, to identify proliferating cells. DAPI staining confirmed the formation of round acini with the pBabe control cells and a partially hollow lumen at day 8 (Fig. 2C). The proliferating cells detected by Ki67 staining were primarily in the outer layer, with no staining detected within the acinar lumen. These data confirm previous studies showing decreased proliferation by day 8 restricted to the outer ring of polarized cells (Fig. 2C). In contrast, Ki67-positive cells were detected throughout the LMP2A-expressing acini, including the outer layer of cells and cells within the filled lumen, indicating that LMP2A induced resistance to anoikis-mediated cell death, leading to lumen filling, and induced cell proliferation (Fig. 2C). The acini expressing the PY mutant were large, round, and partially hollow, and they were similar to pBabe in structure but larger than pBabe (Fig. 2C). Ki67 staining identified proliferating cells that were restricted to the outer layer, with little staining detected within the partially hollow lumen (Fig. 2C).

The formation of large, filled, misshapen acini by cells expressing LMP2A indicated that the growth arrest, polarization, and induction of cell death required to form hollow lumen were affected. LMP2A has been shown to activate PI3 kinase and Akt, and this activation was required for its ability to inhibit migration (16). In MCF10A cells, previous studies have shown that Akt activation induces the formation of misshapen colonies with filled lumen in part through its abilities to block apoptosis and caspase 3 activation (27). To determine the requirement for Akt activation in the inhibition of lumen hollowing by LMP2A, pBabe- and LMP2A-expressing acini were treated with an Akt inhibitor, triciribine, from day 6 to day 8, and examined for changes in size, lumen hollowing, and caspase 3 cleavage. Treatment with a dimethyl sulfoxide (DMSO) vehicle control for 48 h did not affect the growth of pBabe or LMP2A acini, and both acinus types grew noticeably, as seen by bright-field imaging, from day 6 to day 8 (Fig. 3A). Inhibition of Akt with triciribine decreased the size of pBabe acini and completely blocked the growth of LMP2A acini (Fig. 3A), indicating that Akt signaling was important for the growth of pBabe acini and the increased growth of LMP2A acini. At day 8, following 48 h of treatment with the DMSO vehicle control or the

FIG 1 LMP2A expression promotes formation of large, filled, multiacinar structures in MCF10A cells grown in Matrigel. MCF10A cells stably expressing the pBabe vector control or LMP2A were grown on Matrigel for 20 days. (A) Bright-field images were acquired at day 1, 4, 8, and 20 after seeding with a 20 $\times$ objective. Bars, 100 pixels. (B) Acini were grown for 20 days on Matrigel, fixed, and stained with DAPI to visualize nuclei. Images were acquired with a 63 $\times$ objective and are representative of 8 total experiments performed using 3 sets of stable cell lines. DIC, differential interference contrast.

Akt inhibitor triciribine, acini were stained with DAPI to visualize nuclei and with antibodies specific for cleaved caspase 3, to identify apoptotic cells within the lumen, and total Akt or Ki67 (Fig. 3B). Akt was detected predominantly on the outer membranes of the outer ring of cells in both the control and LMP2-expressing cells. Cleaved caspase 3 was readily detected within the lumen of the DMSO-treated pBabe control cells but was not detected in the DMSO-treated LMP2-expressing cells. These findings confirmed that the luminal cells in pBabe acini were undergoing cell death to promote luminal hollowing and that the luminal cells in LMP2A acini were protected from cell death (Fig. 3B). Following treat-

ment with triciribine to inhibit Akt, cleaved caspase 3 was still present within the luminal cells of pBabe acini, indicating that luminal hollowing was progressing in the presence of Akt inhibition (Fig. 3B). LMP2A-expressing acini were smaller after treatment with triciribine, and they had positive staining for cleaved caspase 3, indicating that Akt inhibition decreased the size and promoted luminal cell death of LMP2A-expressing acini (Fig. 3B). Cell proliferation induced by LMP2A was also impaired by triciribine treatment. In DMSO vehicle control-treated cultures, Ki67-positive proliferating cells were detected in the outer layer of the control cells and in the luminal cells of LMP2A-expressing

FIG 2 Induction of filled, multiacinar structures by LMP2A requires PY, ITAM, and YEEA signaling domains. MCF10A cells stably expressing the pBabe vector control, wild-type LMP2A, or the LMP2A signaling mutants PY, ITAM, and YEEA were seeded on Matrigel, and acini were grown for 20 days. (A) Bright-field images were acquired with a 20 $\times$ objective and are representative of 5 total experiments performed using 3 sets of stable cell lines. Acini were fixed and stained for β -catenin (green) and with DAPI (blue) to visualize nuclei. Fluorescence images were acquired using a 63 $\times$ oil objective. (B) Acinar area was determined using ImageJ software, and averages from 4 experiments are expressed relative to pBabe. (C) Acini grown for 8 days from cells expressing the pBabe vector control, wild-type LMP2A, and the PY mutant were stained with DAPI (blue) to visualize nuclei and for Ki67 (green) to visualize dividing cells by immunofluorescence. Images were acquired using a 63 $\times$ oil objective and are representative of 2 experiments performed with 1 set of stable cell lines.

acini. Following triciribine treatment, rare cells stained positive for Ki67, indicating that proliferation was impaired (Fig. 3B). In whole-cell lysates generated from acini recovered from Matrigel at day 8, immunoblotting for two phosphorylated residues of Akt indicated that Akt was activated in LMP2A-expressing acini compared with pBabe at the serine 473 residue, but not at the threonine 308 residue (Fig. 3C). This implicates mTOR, rather than PDK1, as a pathway activating Akt in acinus cultures when LMP2A was expressed. The phosphorylation of Akt at S473 by LMP2A did not occur in acini expressing the PY signaling mutant, indicating that activation of Akt by LMP2A during acinus formation required the PY domain (Fig. 3C).

To further examine the resistance to cell death and luminal filling induced by LMP2A, cells expressing the pBabe vector control, LMP2A, and the specific domain mutants were seeded on Matrigel and cultured for 8 days. Following 8 days in culture, acini were fixed and stained with DAPI to visualize nuclei and stained for cleaved caspase 3 to identify cells within the acinar lumen that were undergoing cell death. At day 8, pBabe acini exhibited clear staining for cleaved caspase 3, indicating that cells were undergoing cell death and the lumen was hollowing (Fig. 4A). LMP2A acini did not contain detectable cleaved caspase 3 and had the characteristic disorganized, filled morphology (Fig. 4A), suggesting that the luminal cells were protected from cell death. The

FIG 3 LMP2A induction of filled, multiacinar structures requires Akt signaling. MCF10A cells stably expressing the pBabe vector control or wild-type LMP2A were seeded on Matrigel to induce formation of acini. Six days after seeding, acini were treated for 48 h with the Akt inhibitor triciribine (TCN; 1 μ M). (A) Bright-field images were acquired with a 20 $\times$ objective in acini before treatment on day 6 with triciribine or the DMSO vehicle control and at day 8, 48 h following treatment with triciribine or the DMSO vehicle control. Bars, 100 pixels. (B) Acini were fixed and costained at day 8 with DAPI and for cleaved caspase 3 and total Akt. Day 8 acini were also costained with DAPI and for cleaved caspase 3 and Ki67. Fluorescent confocal images were obtained with a 63 $\times$ oil objective. Bright-field and fluorescent images are representatives of 3 total experiments performed using 2 sets of stable cell lines. (C) Protein lysates were prepared from acini recovered from Matrigel, and Western blotting of 7.5 μ g protein lysate was used to determine the expression of phospho-Akt (serine 473 and threonine 308) and total Akt. Band intensity was calculated with ImageJ software, and fold changes relative to pBabe are expressed graphically.

LMP2 PY-, ITAM-, and YEEA-expressing acini clearly expressed cleaved caspase 3 within the lumen. Acini form at slightly variable rates; thus, Fig. 4A includes two representative fields for each cell line. These findings indicate that the resistance to cell death that prevents lumen hollowing required the PY, ITAM, and YEEA signaling motifs.

The cells within the lumen lack contact or attachment to matrix proteins from the Matrigel, and detachment-induced cell death, known as anoikis, contributes to the death of luminal cells and to lumen hollowing. Anoikis can be studied with an assay using tissue culture plates coated with polyHEMA that prevents epithelial cells from adhering. MCF10A cells cultured on polyHEMA plates remain in suspension and subsequently undergo

detachment-induced cell death, or anoikis. MCF10A cells expressing the pBabe vector control, LMP2A, and the LMP2A mutants PY, ITAM, and YEEA were seeded on polyHEMA-coated plates and maintained for 7 days. Cell viability was determined by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay at day 0 to evaluate the baseline, and thereafter at day 2 and day 7. LMP2A-expressing cells maintained MTS values throughout the assay, whereas pBabe values dropped 22.13% by day 7. MTS levels from LMP2A cells at day 7 were partially inhibited by each mutant tested (Fig. 4B), suggesting that all signaling motifs partially contributed to the maintenance of cell viability by LMP2A in the *in vitro* anoikis assay.

The abnormal acinus morphology induced by LMP2A was in-

FIG 4 LMP2A expression impairs caspase 3 cleavage to prevent lumen hollowing of MCF10A acini. MCF10A cells stably expressing the pBabe vector control, wild-type LMP2A, and the PY, ITAM, and YEEA mutants of LMP2A were seeded on Matrigel, and acini were grown for 8 days. (A) Bright-field images were acquired using a 20 $\times$ objective prior to fixation of the Matrigel. Acini on Matrigel were fixed and stained with DAPI to visualize nuclei and with an antibody to cleaved caspase 3, followed by a secondary antibody conjugated with Alexa Fluor 594 dye (Molecular Probes) (red). Images from 2 different fields are presented. Bars, 100 pixels. (B) MCF10A cells expressing the pBabe vector control, wild-type LMP2A, and the PY, ITAM, and YEEA mutants of LMP2A were seeded on polyHEMA-coated 96-well plates. Cell viability was determined by MTS assay at day 0, 2, and 7 and is expressed graphically relative to that on day 0. Both caspase 3 staining and polyHEMA cell viability experiments were performed 3 times using 2 sets of stable cell lines.

hibited in MCF10A cells expressing the ITAM and YEEA signaling motif mutants (Fig. 2A). The YEEA motif is thought to contribute to the activation of the ITAM and subsequent binding and activation of the Syk kinase (10). The Src tyrosine kinase has been shown to be important for LMP2A-induced epithelial migration and to contribute to activation of Akt in HaCaT epithelial cells (16). To test the contribution of Src kinase activity in LMP2A-induced acinus morphology, acini expressing pBabe and LMP2A were treated with the Src inhibitor PP2 for 48 h from day 6 to day 8.

Bright-field images at day 8 indicated that PP2 did not change the appearance or size of pBabe acini but did reduce the size of enlarged LMP2A acini (Fig. 5A). Day 8 acini were also stained with DAPI to examine nuclei and acinar structure, with antibody for cleaved caspase 3 to identify dying luminal cells and for Ki67 to visualize dividing cells. DAPI staining confirmed the bright-field images, showing that the size of pBabe acini was unaffected by PP2 but that the size of LMP2A acini was reduced, suggesting that increased proliferation by LMP2A required Src signaling

FIG 5 LMP2A-induced resistance to lumen hollowing can be reversed with an Src kinase inhibitor. MCF10A cells stably expressing pBabe vector control and wild-type LMP2A were seeded on Matrigel, and acini were grown for 6 days. On day 6, acini were treated with DMSO vehicle control or with the Src kinase inhibitor PP2 (500 nM) until day 8. (A) Following 48 h of treatment with DMSO and PP2, bright-field images were obtained using a 20 $\times$ objective. Bars, 100 pixels. (B) Acini were fixed and stained with DAPI to visualize nuclei, for cleaved caspase 3 (red) to visualize dying cells within the acinar lumen, and for Ki67 (green) to visualize dividing cells. Images are representative of 2 experiments performed with 1 stable cell line.

(Fig. 5B). Additionally, in the presence of PP2, the LMP2A colonies began to show evidence of lumen formation with detection of cleaved caspase 3. Identification of proliferating cells by Ki67 staining detected proliferating cells restricted to the outer ring of polarized cells in both pBabe- and LMP2A-expressing colonies after treatment with PP2. These findings indicate that Src activation contributes both to LMP2A-induced proliferation and to resistance to cell death (Fig. 5B).

Loss of epithelial polarity is a feature of several tumors *in vivo* and is a characteristic of acini impaired by expression of LMP2A. Loss of polarity is also a prominent early feature in epithelial-mesenchymal transition (EMT), and EMT has been implicated as a contributing factor in tumor growth and development. Other factors that induce EMT, such as treatment with transforming growth factor beta (TGF- β), also inhibit proper acinus formation. To determine if LMP2A expression in MCF10A cells induced EMT as a possible mechanism contributing to abnormal acinus formation, potential changes in morphology were assessed and changes in expression levels of EMT markers were determined by Western blotting and immunofluorescence in cells expressing the pBabe vector control, wild-type LMP2A, and the LMP2A signaling mutants. Bright-field images of cells growing in 2D tissue culture revealed that the pBabe vector control cells grew in tight clumps and had a cobblestone appearance typical of epithelial cells. The edges of the clumps of cells were smooth, as indicated by the small black arrows in Fig. 6A. LMP2A-expressing cells also grew in clumps, although the clumps were edged by cells with a feathery appearance and lacking the cuboidal appearance of epi-

FIG 6 LMP2A promotes an epithelial-mesenchymal transition phenotype in MCF10A cells. Bright-field images show morphology of MCF10A cells expressing the pBabe vector control, wild-type LMP2A, or the LMP2A signaling mutants PY, ITAM, and YEEA growing in 2 dimensions. Images are representative of 3 sets of stable cell lines. Bars, 100 pixels.

thelial cells (Fig. 6A). LMP2A-expressing cells grew with more space between them, extending outward toward each other until they reached confluence. Confluent LMP2A cells lost the mesenchymal morphology they had at subconfluence and acquired the typical cobblestone epithelial appearance (data not shown). MCF10A cells expressing the PY and ITAM signaling motif mutants of LMP2A partially reversed the mesenchymal phenotype induced by LMP2A in that the cells at the edges of the clumps still appeared feathery and lacked a cobblestone appearance; however, they did not have pronounced cellular extensions with areas of individual cells growing and extending toward each other (Fig. 6). Mutation of the YEEA signaling domain, however, completely reversed the mesenchymal phenotype induced by LMP2A, and the cells resembled the tight, cobblestone patches of cells expressing the pBabe control vector (Fig. 6).

To further investigate whether LMP2A expression induced a mesenchymal phenotype in MCF10A cells, markers of EMT were detected by immunofluorescence in MCF10A cells. Compared to results for pBabe, LMP2A-expressing cells expressed high levels of vimentin and N-cadherin, both of which indicate induction of EMT (Fig. 7). N-Cadherin was upregulated by LMP2A and was expressed predominantly at the cell membrane. E-Cadherin staining was strongly detected at the cell membrane in pBabe cells, and at cell-cell junctions, whereas expression of E-cadherin in LMP2A cells was decreased, with localization at cell membranes and some in the perinuclear region (Fig. 7). LMP2A was detected in LMP2A-expressing cells and not in vector control cells (Fig. 7). The up-regulation of vimentin and N-cadherin expression and down-regulation of E-cadherin expression suggest that LMP2 affects

FIG 7 LMP2A expression induces changes in protein expression consistent with epithelial-mesenchymal transition. MCF10A cells expressing the pBabe vector control and wild-type LMP2A were subjected to immunofluorescence to visualize expression of N-cadherin, vimentin, E-cadherin, and LMP2A. Secondary antibody only controls are also shown for antibodies conjugated to Alexa Fluor 488 and 647 dyes (Molecular Probes) and FITC. Confocal images were acquired with a $63\times$ oil objective. Images are representative of 3 total experiments performed using 2 sets of stable cell lines.

components of EMT transition and support the morphological changes observed with cells grown in 2D culture.

To confirm these findings, changes in EMT marker expression were also determined by immunoblotting. Consistent with the immunofluorescent staining, E-cadherin was decreased by LMP2A, N-cadherin was increased, and vimentin demonstrated a modest increase (Fig. 8A). These results were confirmed by quantitation of band intensity, and averages from 3 experiments are presented graphically and expressed as the change relative to pBabe values (Fig. 8B). Mutation of the YEEA signaling domain eliminated the LMP2-mediated effects on levels of E-cadherin, N-cadherin, and vimentin expression, indicating that Src tyrosine kinase signaling was required for EMT-related changes (Fig. 8A and B). The MCF10A cells expressing LMP2A also had increased levels of Akt phosphorylated at the serine 473 residue, the same phosphorylation site which was increased in the LMP2A acini, and this increase in Akt phosphorylation was blocked by mutation of

the ITAM motif and also impaired by mutation of the YEEA signaling domain (Fig. 8A). LMP2A expression in MCF10A cells was confirmed and revealed that the LMP2A PY mutant was consistently expressed at higher levels than that of the wild type or the PY and YEEA mutants (Fig. 8A), consistent with LMP2A associating with ubiquitin ligases at the PY domain (23). Signaling through the PY domain of LMP2A contributed to some of the changes in protein expression, in particular the reduction of E-cadherin levels, consistent with EMT. These findings indicate that activation of Akt requires the ITAM motif as previously shown (16, 34) and the YEEA motif, while the effects on EMT are dependent on the YEEA motif.

DISCUSSION

During the development of carcinoma, many of the normal functions of epithelial cells, including proliferation, cell survival and motility, cell death, and differentiation, are affected. The develop-

FIG 8 LMP2A induction of epithelial-mesenchymal transition requires the YEEA signaling motif. Whole-cell lysates from MCF10A cells expressing the pBabe vector control, wild-type LMP2A, or the LMP2A signaling mutants PY, ITAM, and YEEA were subjected to Western blotting to detect expression of markers for epithelial-mesenchymal transition. (A) Representative Western blots show expression changes of E-cadherin ($n = 5$), N-cadherin ($n = 3$), fibronectin ($n = 3$), vimentin ($n = 5$), β -catenin ($n = 5$), and phospho-Akt (Ser 473; $n = 4$). LMP2A expression is also detected, with HSC70 used as a loading control. (B) Western blots were quantitated with ImageJ software, were normalized to the HSC70 loading control, and are expressed graphically relative to pBabe for each protein.

ment of acini *in vitro* by normal epithelial MCF10A cells allows for the examination of many of these processes. These include changes in proliferation, cellular polarity, and anoikis-mediated cell death that occur during acinus formation (26). These are important factors in tumorigenesis but are difficult to evaluate using 2-dimensional culture. The 2D culture system has been used to determine the properties of multiple cellular and viral genes, including assessing the effects of cyclin D1, Bcl2, Ras, and ErbB2 (24–26, 28, 33). Additional contributions of p53, Notch, and Δ N63 have been identified (29–31, 35). In the present study, the effect of LMP2A, a protein expressed in EBV-infected carcinoma, on the development of epithelial acini was determined. The con-

tribution of specific domains of LMP2A that activate distinct signaling pathways was assessed using LMP2A mutants and chemical inhibitors.

MCF10A acini are generated by the formation of a cluster of cells that proliferates and forms a polarized ring of outer cells with enhanced survival. The luminal cells that lack contact with the ECM undergo anoikis, forming a clear lumen, and eventually undergo proliferation arrest (24–26). Expression of LMP2A disrupts each stage of acinus development. LMP2A-expressing cells form colonies that are larger than the pBabe controls, lack the polarized outer ring of cells, and lack the anoikis-induced lumens and expression of activated caspase 3. The LMP2A colonies express the

FIG 9 LMP2A induced abnormal, filled, multiacinar structures through ITAM- and YEEA-dependent activation of Src tyrosine kinases and Akt to induce proliferation, impair polarization, and impair luminal cell death in MCF10A cells grown in a 3D culture system. LMP2A acini were larger than those for pBabe, indicating that proliferation was induced, but acini were still susceptible to a delayed proliferative arrest. Mutating the PY domain induced acini that were round, polarized, and hollow but significantly larger than those for LMP2A, indicating that the PY domain of LMP2A was required for the delayed susceptibility to proliferative arrest. Changes in the stages of acinar development were consistent with reports on the oncogenes indicated (24–28, 33, 35, 36). LMP2A expression also induced changes in morphology and protein expression patterns consistent with epithelial-mesenchymal transition, and these changes required the YEEA signaling domain of LMP2A.

proliferation marker Ki67 both in outer ring and luminal cells, confirming the enhanced proliferation induced by LMP2A. The adherens junction protein β -catenin was translocated from being predominantly junctional in pBabe acini to being diffuse and expressed throughout the cytoplasm in LMP2A-expressing colonies, indicating impaired polarization, which could contribute to cell death resistance. Overexpression of ErbB2 or mutant Src results in colonies that are similar to LMP2A, with increased proliferation and impaired polarization, luminal cell death, and proliferative arrest (24, 25, 33, 36). Constitutively active Akt increased colony size and enhanced proliferation, while E7 produced acini similar to PY that were large, polarized, and hollow and bypassed growth arrest (24, 25, 27).

Cells expressing LMP2A developed large, filled, and irregularly shaped colonies that lacked an outer layer of polarized cells, with some forming multiacinar structures. In the absence of the putative Src kinase YEEA motif or the ITAM, LMP2A did not induce cell proliferation, resulting in smaller colony size. Nor did LMP2A lacking the YEEA or ITAM motifs inhibit anoikis, as revealed by the formation of hollow lumen. Consistent with the hypothesis that YEEA-dependent signaling was required, the Src family ki-

nase inhibitor PP2 reversed the inhibition by LMP2A of luminal cell death and the induction of hyperproliferation. The survival pathway Akt was also important for controlling hyperproliferation and luminal filling induced by LMP2A. Inhibition of Akt activation by triciribine reduced the size of acini and increased the detection of caspase 3 in LMP2A acinar lumen, indicating that LMP2A-induced Akt signaling was important for proliferation and, at least partially, for resistance to cell death (Fig. 9). Acini expressing activated Akt have previously been shown to have filled, misshapen structures similar in morphology to those induced by LMP2A; however, Akt activation induced only a partial protection from luminal cell death, which was ascribed to the tight localization of Akt in the outer cells interacting with the ECM (25, 27). Consistent with this finding, LMP2-expressing cells had elevated total Akt that was detected in the outer ring of cells in LMP2A-expressing acini. LMP2A expression induced an increase in serine 473 phospho-Akt levels (Fig. 8), which required the YEEA signaling domains and is consistent with a role of ITAM/YEEA-activated Akt as a contributor to proliferation and partially to cell death resistance in acini. These findings are also consistent with previous studies that indicated that ITAM-mediated Akt ac-

tivation was required for migration (16) and that Akt signaling contributed to LMP2A-induced cell survival (32). In addition, LMP2A-induced Akt activation has been shown to involve ITAM and YEEA signaling in B cells (34).

Intriguingly, cells expressing the PY mutant established large, round, luminal acini that maintained a regular shape. Indeed, the impaired PY signaling appeared to further increase proliferation beyond that of wild-type LMP2A. Ki67 staining of PY acini was restricted to the outer cell layer, and more cells were positive for the proliferation marker than was the case for pBabe. Quantitation of the acinar area as an estimate of size indicated that LMP2A and PY induced a similar increase at day 20; however, PY acini were typically large and spherical, whereas those of LMP2A were lobular. Measurements of acinar area indicated that their areas were similar even though the PY acini appeared larger due to differences in their surface structure. Perhaps the PY domain functions to modulate LMP2A-induced proliferation. Most importantly, this domain was required for the ability of LMP2A to block cell death and caspase induction. This finding indicates that the PY mutant, which retains both the YEEA and ITAM motifs, is required for LMP1 inhibition of anoikis and for the delayed proliferative arrest that occurs by day 20. As the PY domain binds ubiquitin ligases, the inhibition of anoikis and delayed proliferative arrest are likely affected through modulation of the levels of proteins critical to anoikis and proliferation. This is the first identification of specific impairment of LMP2 effects on growth regulation that are mediated through the PY domain. Interestingly, this phenotype mimics that of expression of the human papillomavirus E7 gene (25). These findings reveal that LMP2 affects multiple aspects of acinus development with distinct requirements for the signaling motifs of LMP2A and subsequent activation of required pathways.

In addition to being an important feature of epithelial tumor development, disruption of cell polarity is also an early factor in epithelial-mesenchymal transition. EMT is thought to contribute to tumor metastasis by promoting a mesenchymal phenotype with increased capacity for motility and invasion. In 2D culture, markers of EMT were apparent in that the epithelial protein E-cadherin was decreased and the mesenchymal markers N-cadherin and vimentin were increased. The slight feathery appearance detectable at subconfluence was distinct from the epithelial cobblestone morphology of pBabe-expressing cells. Similar changes in the morphology of MCF10A cells at subconfluence have been previously identified, and this phenotype can be induced by irregular passage (26). However, the changes in EMT markers and the slight effects on appearance were consistent and, interestingly, required only the YEEA signaling domain. This result suggests that in addition to being important for each feature of altered acinus development, YEEA and Src family tyrosine kinase signaling are required for inducing EMT in MCF10A cells *in vitro*.

These findings reveal that LMP2A has many of the properties of cellular oncogenes and, similarly to other viral oncogenes, has combined multiple functional domains. Previous studies have shown that overexpression of 14-3-3 ζ also impaired luminal cell death, resulting in filled lumen, and that mutant RAS impaired polarization and luminal cell death (28, 37). Knockdown of an RNA binding protein, HuR, also impaired the formation of acinar lumen, and this resistance to anoikis correlated with increased expression of Δ Np63, a protein previously reported to be increased and stabilized by LMP2A (38). The effects on Δ Np63 may

provide an additional mechanism for anoikis resistance in LMP2A acini.

In summary, the activation of multiple pathways by LMP2 induced a highly transformed, proliferative phenotype evidenced in the acinus cultures. The cells lack normal cellular regulation both in cessation of growth and in the induction of cell death during differentiation. The identification of the pathways that are critical to the LMP2A-mediated effects may provide new therapeutic targets. Additionally, it is likely the mechanisms through which LMP2A activates these pathways and induces these changes will point to novel mechanisms of regulation. It is intriguing that the ability to bind ubiquitin ligases is an essential component in the LMP2A-induced uncontrolled proliferation and protection from cell death in the absence of ECM signaling. It will be important to identify the cellular proteins that are targeted by this domain.

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