The Role of LCV and EBV Latent Membrane Protein 2A in Epithelial Cells

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ABSTRACT

Catherine A. Siler: The Role of LCV and EBV Latent Membrane Protein 2A in Epithelial Cells

(Under the direction of Nancy Raab-Traub)

Epstein-Barr Virus (EBV) is a ubiquitous human herpesvirus that is associated with malignancies of both lymphoid and epithelial origin including Burkitt's lymphoma and nasopharyngeal carcinoma (NPC) among others. The focus of this study was to investigate both EBV and rhesus EBV (LCV) latent membrane protein 2A (LMP2A) signaling and biological functions in epithelial cells and their contribution to EBV-mediated carcinogenesis.

Rhesus LCV LMP2A (Rh-LMP2A) has an overall sequence homology of 62% to EBV LMP2A. The 12 transmembrane domains and the N-terminal cytoplasmic domain containing an immunoreceptor tyrosine-based activation motif (ITAM) and PY motifs are conserved in Rh-LMP2A. In this study, Rh-LMP2A expression in human foreskin keratinocytes (HFK) activated Akt and inactivated GSK3β. This led to the subsequent accumulation and nuclear translocation of β-catenin which was found to be Akt dependent. Rh-LMP2A also inhibited epithelial cell differentiation. A mutant form of Rh-LMP2A lacking the last six transmembrane domains was able to function similarly to full-length Rh-LMP2A.

Since EBV LMP2A is consistently expressed in metastatic NPC, the potential role of LMP2A in metastasis was investigated. EBV LMP2A expression in HFK cells led to increased expression and activity of several matrix metalloproteinases (MMPs), which are

involved in basement membrane and extracellular matrix degradation. The activity of MMP-7 and MMP-13 was dependent on Akt activation, whereas MMP-2 activity was dependent on MAPK activation. These results identify a mechanism by which EBV LMP2A could contribute to NPC metastasis.

Through the activation of the PI3K/Akt and β-catenin pathways that impact cell proliferation and survival in addition to upregulating MMP expression and activity, LMP2A likely contributes to carcinogenesis and metastasis. Lastly, the similarity of rhesus LCV to EBV further validates the rhesus macaque model for the study of EBV pathogenesis.

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LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
BART	*Bam*HIA rightward transcripts
BCR	B cell receptor
BL	Burkitt's Lymphoma
Btk	Bruton's tyrosine kinase
CAEBV	Chronic active EBV infection
Csk	Carboxy-terminal Src kinase
CTAR1	Carboxy-terminal activating region 1
CTAR2	Carboxy-terminal activating region 2
CTL	Cytotoxic T lymphocyte
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBER	Epstein-Barr virus encoded RNAs
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
GSK3β	Glycogen synthase kinase 3 beta
HFK	Human foreskin keratinocyte
HIV	Human immune deficiency virus
HL	Hodgkin lymphoma

HLA	Human leukocyte antigen
HRS	Hodgkin/Reed-Sternberg
hTERT	Human telomerase reverse transcriptase
Id	Inhibitor of differentiation
IE	Immediate early
IFN	Interferon
Ig	Immunoglobulin
IGF-1	Insulin like growth factor 1
IL	Interleukin
IM	Infectious mononucleosis
ITAM	Immunoreceptor tyrosine based activation motif
JCV	JC virus
JNK	c-Jun N-terminal kinase
LCV	Lymphocryptovirus
LEF	Lymphoid enhancer factor
LMP	Latent membrane protein
LCL	Lymphoblastoid cell line
MAPK	Mitogen activated protein kinase
MEK	Mitogen activated ERK kinase
MHC	Major histocompatibility complex
miRNA	Micro RNA
MMP	Matrix metalloproteinase
NFκB	Nuclear factor kappa B

NK	Natural killer cell
NotchIC	Notch intracellular domain
NPC	Nasopharyngeal carcinoma
OHL	Oral hairy leukoplakia
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3'OH kinase
PKC	Protein kinase c
PKR	Double stranded RNA activated protein kinase
PTLD	Post-transplant lymphoproliferative disease
RACK	Receptor for activated protein kinase c
Rb	Retinoblastoma protein
RBPJκ	Recombination signal binding protein J kappa
RIP	Receptor interacting protein
RNA	Ribonucleic acid
RNAi	RNA interference
RT-PCR	Reverse Transcriptase PCR
SAP	SLAM associated protein
SDS	Sodium dodecyl sulfate
siRNA	small interfering RNA
SLAM	Signaling lymphocyte activation molecule
STAT	Signal transducers and activators of transcription

TCF	T cell factor
TCN	Triciribine
TGF-β1	Transforming growth factor beta 1
TNFR	Tumor necrosis factor receptor
TRADD	TNF receptor associated death domain
TRAF	TNF receptor associated factor
VAHS	Virus associated hemophagocytic syndrome
VCA	Viral capsid antigen
XLPS	X-linked lymphoproliferative syndrome

CHAPTER ONE

Introduction

Epstein-Barr Virus

Denis Burkitt first identified a novel extranodal lymphoma occurring in children in equatorial Africa where malaria was also endemic in the 1940s (24-28). Burkitt hypothesized the involvement of an additional infectious agent due to the atypical epidemiological and clinical presentation of the lymphomas (24, 27, 28). Epstein-Barr virus (EBV) was first identified by Epstein, Achong, and Barr in 1964 using electron micrographs of Burkitt's lymphoma cells grown in culture showing a herpesvirus-like particle that did not react to antibodies from other known herpesviruses and was unable to replicate in cultured cells(71).

EBV, also known as human herpesvirus 4, is a ubiquitous gamma herpesvirus within the *lymphocryptovirus* genus (275). It is an enveloped virus with a large double-stranded DNA genome of 184 kilobase pairs (12). EBV is a successful human pathogen as over 90% of the global adult human population is infected with the virus (127). As with all herpesviruses, EBV infection remains for the life of the host and humans are the only known host for EBV (275). EBV infection is typically asymptomatic and does not induce disease in the host, however, in certain contexts such as immunosuppression, EBV is associated with several pathologic conditions. The virus exhibits tropism for both B lymphocytes and epithelial cells (7, 275). Of note, the defining characteristic of lymphocryptoviruses is the ability to immortalize B lymphocytes *in vitro* which are known as lymphoblastoid cell lines (LCLs) in the case of EBV (128). In B lymphocytes, the virus can enter a latent state to enable its persistence and evade immune detection as well as undergo a productive infection with lytic replication resulting in virion assembly and release (10). Both lytic and latent infection of epithelial cells can occur with latent infection contributing to the development of

EBV-associated epithelial cancers. In addition to B lymphocytes and epithelial cells, numerous reports have shown infection in other cell types such as natural killer (NK) cells, T cells, monocytes, and neutrophils (114, 147, 151, 290, 306, 326, 338).

EBV Life Cycle

EBV is transmitted through saliva that contains both cell-free virus as well as infected cells that ultimately leads to the infection of the oral epithelium (279). One early study suggested that EBV infection of a host commenced with the fusion of EBV positive B lymphocytes to epithelial targets (15). Although this study had not been confirmed nor refuted to date, infection of epithelial cells is more efficient when in close proximity with virus producing B lymphocytes than cell-free virus (146, 332). Infection of the oral epithelium can lead to progeny production through lytic replication in oral epithelial cells (187, 188, 299). Released virions can subsequently infect circulating B lymphocytes in the oral epithelium or in various lymphoid organs (10, 230, 243). Naïve and memory B cells are equally susceptible to EBV infection *in vitro* (355). Notably, viral attachment and entry differs between epithelial cells and B lymphocytes.

EBV attachment to B lymphocytes involves high affinity binding between the gp350/220 viral envelope glycoprotein and the CD21 cellular receptor to initiate endocytosis of the virus into the host cell (80, 86, 234, 242, 317). This action leads to viral fusion with the endosomal membrane at a low pH (226). Conversely, the role of CD21 in epithelial cell EBV infection remains unclear and indicates a different mechanism is involved in EBV attachment and entry of epithelial cells. EBV lacking the gH and gL viral proteins is unable to bind several epithelial cell lines, although the cellular receptor has not yet been identified

(232, 245). A recent report showed the EBV glycoprotein BMRF2 interacts with the α5β1 integrin on epithelial cells and antibodies against the integrin and BMRF2 partially blocked viral binding (332). In contrast to EBV infection B lymphocytes, endocytosis is not required for epithelial cell entry and fusion occurs at a neutral pH (279). Notably, EBV produced from human leukocyte antigen (HLA) class II negative epithelial cells can infect B lymphocytes more readily than virus produced from HLA class II positive B lymphocytes. In addition, virus produced in B lymphocytes can infect epithelial cells more readily than virus produced in epithelial cells (17, 279).

Once EBV enters B lymphocytes, the viral genome circularizes to form a viral episome and initiates a limited latency program through transcription from the viral Wp promoter using cellular RNA polymerase II (289). Latently infected B lymphocytes have a restricted repertoire of viral gene products which promotes evasion from the immune system (11, 230, 320, 323). Latently infected B lymphocytes periodically undergo reactivation which leads to the production of new viral progeny (7, 10). The newly formed virus is released and can infect surrounding B lymphocytes or oral epithelial cells. Additionally, the virus can be shed through saliva, allowing for transmission to the next host. A schematic illustration of the EBV life cycle can be seen in Figure 1.

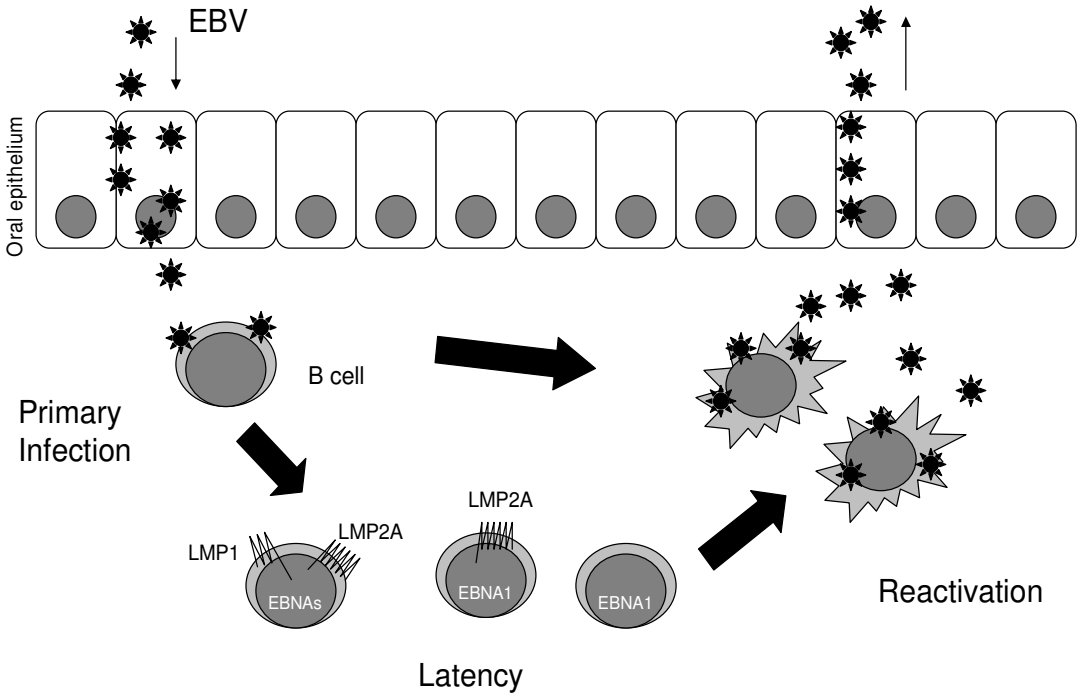

Figure 1. The EBV Life Cycle. Virus enters the oral cavity through saliva and may lytically infect cells of the oral epithelium. Resulting progeny access the B cell compartment by infecting B lymphocytes circulating nearby. Once B cells are infected, the virus can enter a latent form with limited expression of viral genes. Periodically, these latently infected B cells can reactivate and the lytic program results in the release of progeny. Reactivation of B cells near oral mucosal sites may lead to lytic infection of the oropharyngeal epithelial cells, thus allowing progeny virus to be shed back out into the saliva, allowing for horizontal transmission to a new host.

Lytic Infection

In order to study EBV lytic replication, latently infected cells must be induced to enter the lytic program. Studies in vitro have focused on inducing lytic replication in latently infected cells through the use of phorbol esters. Phorbol esters induce lytic replication by activating the protein kinase c (PKC) pathway. The PKC pathway induces lytic replication via the activation of AP-1 sites located upstream of the viral immediate-early (IE) genes and through the activation of the BZLF1 protein (8, 83, 84). Lytic replication can also be induced through the activation of the B cell receptor (BCR) with soluble immunoglobulin (315). Once induced, cells undergo cytopathic changes prior to virion release including margination of nuclear chromatin, inhibition of host molecular synthesis, replication of viral DNA, assembly of nucleocapsids, nucleation of nucleocapsids, and envelopment of the virus through the inner nuclear membrane (56, 102, 107, 168, 254, 275, 290). Lytic EBV infection, as with other herpesviruses, follows a temporal and sequential order and can be divided into the immediate early, early, and late phases of gene expression.

Immediate Early Genes

The immediate early proteins BZLF1 and BRLF1, designated as such by their expression in the presence of protein synthesis inhibitors such as cycloheximide, serve as viral transactivators to initiate the events of lytic infection and are necessarily located in the nucleus (85, 271, 315, 357). With this, expression of either protein during latency will lead to the induction of the other and the virus will enter the lytic cycle (50, 111, 121, 272, 302). Both BZLF1 and BRLF1 activate transcription of early gene promoters to secure progression of the lytic cycle and BZLF1 also downregulates the Cp promoter controlling expression of

the latent EBNA genes, thereby ensuring the effective switch from latency to the lytic cycle (23, 41, 121, 135, 171, 198, 200). Additionally, BZLF1 is required for viral DNA replication and BRLF1 enhances viral replication efficiency (81, 291). BZLF1 also has important activities which allow the virus to evade the immune system such as the downregulation of tumor necrosis factor receptor 1 and interferon gamma receptor, making the host cell unresponsive to immunostimulatory cytokines (238, 239).

Early Genes

The EBV early genes are designated as such because their expression requires protein synthesis, but viral replication is not required for their expression. The early genes encode a myriad of proteins involved in the modulation of gene transcription and viral DNA replication, among others (82). The EBV genome is replicated during the lytic cycle by a viral DNA polymerase encoded by BALF5 (161). Notably, another early protein, BHRF1, is highly homologous to cellular Bcl-2 (125, 354). Cellular Bcl-2 is located in the mitochondria and plays a critical role in protecting the cell from apoptosis. Interestingly, the *bcl-2* locus is activated by chromosomal translocation which is characteristic of several types of follicular B-cell lymphomas (329-331). This suggests the EBV Bcl-2 homolog, BHRF1, may act to protect infected cells from undergoing apoptosis until the replication cycle including virion assembly and release is complete.

Late Genes

Late genes typically encode structural components of the virion, such as tegument proteins and glycoproteins. The primary EBV glycoproteins, gp350/220 and gp42, are encoded by the late *BLLF1* and *BZLF2* genes, respectively. They serve as binding partners for the CD21 cellular receptor and major histocompatibility complex (MHC) class II receptor on B lymphocytes mediating viral entry (80, 192, 275). In the case of epithelial cells, the cellular receptor(s) responsible have not yet been identified. However, a recent report has implicated integrins in viral entry of epithelial cells (332). Another late gene, *BCRF1*, encodes a viral homolog to human IL-10 (140, 233). As with human IL-10, BCRF1 has negative immunomodulatory functions by decreasing responses from NK cells, T cells, and macrophages (287, 309). The late phase of the lytic cycle concludes with virion assembly and egress, typically about four days after productive infection or the onset of reactivation.

Establishment of Latency

EBV latency is characterized by the constitutive expression of specific subsets of viral genes. These latent genes include the six EBV nuclear antigens (EBNAs 1, 2, 3, 3A, 3B, 3C, and LP) and three latent membrane proteins (LMPs 1, 2A, and 2B), as well as a group of spliced 3´ transcripts, BamHIA transcripts known as BARTs, and small non-polyadenylated RNAs known as EBER1 and EBER2. As seen in Table 1, latency is categorized into three different types depending on gene expression patterns. During Type I latency, viral gene expression is quite limited. Only EBNA1 is expressed at the protein level along with transcripts for the BARTs and EBERs. This type of latency is characteristic of viral expression in endemic Burkitt's lymphoma. Type II latency, which is characteristic of

EBV-associated Hodgkin lymphoma and nasopharyngeal carcinoma (NPC), exhibits expression of EBNA1, LMP1, 2A, and 2B, and transcription of the EBERs and BARTs. In Type III latency, EBNA 1, 2, 3A-C, and LP, as well as LMP1, 2A, 2B, the EBERS and BARTs are all expressed. Type III latency is typical of LCLs established by *in vitro* infection of primary B cells with EBV and of post-transplant lymphoproliferative disease (PTLD) *in vivo* (20). EBV-mediated transformation of B lymphocytes is dependent on a group of latent proteins including EBNA1, 2, 3A, 3C and LMP1 (47, 270).

Latency Type	Viral products expressed	Associated Diseases
Type I	EBNA1, BARTs, EBERs	Burkitt's Lymphoma
Type II	EBNA1, BARTs, EBERs, LMP1, LMP2A, LMP2B, +/- BARF1	Hodgkin Lymphoma, Nasopharyngeal Carcinoma, Gastric Carcinoma
Type III	EBNA1, 2, 3A-C, and LP, BARTs, EBERs, LMP1, LMP2A and 2B	Lymphoblastoid cell lines, post-transplant lymphoproliferative disease

Table 1. EBV Latency Expression Patterns

EBNA1

EBNA1 is a nuclear protein that is expressed in each type of latency with its principal role being the maintenance and replication of the viral genome through specific binding to the origin of plasmid replication known as oriP (273, 355). Through the tethering of the viral episome to mitotic chromosomes in dividing cells, each daughter cell retains the viral

genome (246, 350, 351). EBNA1 can also regulate the transcription of itself and the other EBNAs, as well as LMP1 through its interaction with viral promoters (355). EBNA1 contains glycine-alanine repeats between the amino- and carboxy-terminal ends which stabilizes the protein by preventing proteasomal degradation (190). EBNA1 expression in murine cells does not elicit a CTL response as a result of its lack of proteasomal breakdown which is required for antigen processing and presentation by MHC class I (190, 327). These unique characteristics of EBNA1 allow latently infected cells, particularly those with a Type I expression pattern, to avoid immune recognition by the host.

EBNA2 and EBNA-LP

Although both EBNA2 and EBNA-LP are expressed during Type III latency, only EBNA2 is essential for the transformation of B lymphocytes by EBV. EBNA-LP is not essential, but is assists in the outgrowth of LCLs (4, 48, 212). EBNA-LP has two runs of basic amino acids that facilitate its nuclear translocation where is coactivates EBNA2-mediated transcription from the Cp and LMP1 promoters (120, 244, 259). Moreover, EBNA-LP is a phosphoprotein, and its phosphorylation status and function may be cell cycle dependent (167, 175). EBNA-LP can also interact with several cellular proteins such as Rb, p53, and p14ARF (165, 313). The role of EBNA-LP in cell cycle progression may occur through these interactions with tumor suppressors, but the importance of these interactions in LCLs remains undetermined. Additionally, EBNA-LP interacts with the viral Bcl-2 homolog, BHRF1, indicating a possible role in modulating apoptosis (119, 216). These functions of EBNA-LP underscore the importance of EBNA-LP in the establishment of viral latency and cellular transformation.

EBNA2 is similar to the cellular Notch protein and is a potent viral transactivator of both viral and cellular promoters via its interaction with recombination signal sequence-binding protein (RBP)-Jκ in the nucleus (112, 139, 158). Through this interaction, EBNA2 induces cellular genes such as CD21, CD23, *c-fgr* and *c-myc* in addition to EBV LMP1 and LMP2A and is critical for EBV transformation of B lymphocytes (1, 49, 75, 76, 337, 348).

EBNA3A, EBNA3B, and EBNA3C

As with EBNA2 and EBNA-LP, the EBNA3s are only expressed in Type III latency. EBNA3B is not necessary for B lymphocyte transformation and not required for lytic replication, LCL outgrowth, or cell survival (324). In contrast, EBNA3A and 3C along with LMP1 are essential for EBV-mediated B lymphocyte transformation (325). Even though the EBNA3 mRNAs are present at very low levels, the protein products exhibit a long half life (173).

The EBNA3s negatively regulate the transcriptional activation of EBNA2 and EBNA-LP through competition for Notch and RBP-Jκ (159, 179, 213, 278, 280, 333, 359). EBNA3A can repress the viral Cp promoter in both epithelial cells and B lymphocytes (45). EBNA3C cooperates with RAS to disrupt cell cycle checkpoints and induces rodent fibroblast transformation (255, 256). In conjunction with EBNA2, EBNA3C can induce LMP1 transcription through the binding of the PU.1 site within the LMP1 promoter (213, 360).

BARTs

BamHIA rightward transcripts (BARTs) are a group of abundantly expressed and highly spliced RNAs that can be detected in all EBV-associated cancers and in all types of latency as well as in the lytic cycle (20, 37, 104, 105, 133, 164, 221, 257, 258). Although the BARTs are ubiquitously expressed in all types of latency, they are not necessary for EBV-mediated transformation of B lymphocytes in vitro (281). Three BART transcripts have been of interest as they appear to encode open reading frames, *rkbarf0*, *rb2*, and *rk103*, with RK-BARF0 and RB2 mRNAs being the most plentiful in NPC (285).

RK-BARF0 binds Notch 1 and Notch 4 as well as induces the nuclear translocation of unprocessed Notch (183). Notably, RK-BARF0 also induces the proteasomal degradation of Notch which correlates with reduced levels of Notch in EBV positive cell lines (321). RK103 binds RBP-Jκ and inhibits the transactivating activities of both Notch1C and EBNA2, and RB2 can bind the receptor for activated protein kinase C (RACK) (301, 358). BARF1, another BART product, is a secreted protein and exhibits oncogenic activity when expressed in rodent fibroblasts (57, 298).

Recent reports have shown that EBV encodes at least 17 micro RNAs (miRNAs) with 14 of them found in BARTs (31, 113, 260). The BART miRNAs are expressed in Type III latency with high levels detected in infected epithelial cells and reduced levels in infected B lymphocytes and virtually undetectable in epithelial cells and B cells in Type I or Type II latency (31, 176). Notably, the induction of lytic replication enhances the expression of many viral miRNAs. These novel findings indicate a potential mechanism by which EBV could regulate host gene expression without viral gene expression, thereby further eluding immune surveillance.

EBERs

EBER1 and EBER2 are small non-polyadenylated RNAs that are the most copious RNAs in latently infected cells. Although present in all types of latency, they are not required for EBV-mediated transformation of B lymphocytes (310). Since the EBERs are expressed in most EBV-associated cancers, they form the basis of an in situ hybridization assay to detect EBV infection in tissues (14, 116, 138, 257, 258). Currently, the function of the EBERs remains unclear, however, one report details EBER-mediated induction of insulin-like growth factor 1 (IGF-1) leading to enhanced proliferation of EBV positive gastric carcinoma cells (153). EBERs have also displayed oncogenic characteristics when expressed in EBV-negative Burkitt's lymphoma cell and rodent fibroblasts and confer properties such as anchorage-independent growth and tumorigenicity in immunodeficient mice (178, 194, 349). Additionally, the EBERs can also confer resistance to apoptosis in Burkitt's lymphoma and other B cell lines through binding and inhibiting the double-stranded RNA-activated protein kinase (PKR), a known protein synthesis inhibitor and putative tumor suppressor (241, 296, 349).

LMP1

LMP1, which is expressed in latency Types II and III, is the primary EBV oncogene as it can transform rodent fibroblasts *in vitro* and is essential for EBV-mediated transformation of B lymphocytes (169, 335). LMP1 expression in epithelial cells drastically alters cell growth, induces morphologic changes, and inhibits differentiation (53, 76). LMP1 expression in cells has a multitude of effects resulting in the induction of a variety of cellular

proteins such as cell adhesion molecules (ICAM1 and E-cadherin), anti-apoptotic proteins (Bcl-2 and A20), and growth factors (EGFR) (67, 68, 90, 126, 228, 336).

LMP1 contains two major signaling domains at the carboxy-terminus known as carboxy-terminal activating region 1 (CTAR1) and carboxy-terminal activating region 2 (CTAR2). Although LMP1 is not particularly homologous to any other known protein, it functions in a similar manner to the tumor necrosis factor receptor (TNFR) and CD40 (240). Both CD40 and LMP1 signal by recruiting adapter proteins called TNFR associated factors (TRAFs) to their respective PxQxT motifs (60, 89). While CTAR1 recruits TRAFs 1, 2, 3, and 5 directly through its PxQxT motif, CTAR2 indirectly recruits TRAFs 2 and 6 by binding adapter proteins such as the TNFR associated death domain (TRADD), the receptor interacting protein (RIP), and BS69 (154, 155, 334). Unlike TNFR, LMP1 does not require a ligand to associate with the TRAFs, thereby allowing LMP1 to be constitutively active (60, 89, 227, 240).

LMP1 can activate a multitude of signaling pathways including NFκB, Cdc42, p38, c-jun N-terminal kinase (JNK), phophoinositol 3-kinase (PI3K), and the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated (ERK) 1 (MEK1) pathway (54, 69, 70, 118, 199, 251, 277, 286). LMP1 also upregulates cell surface expression of a myriad of markers including CD23, CD39, CD40, CD44, ICAM1, MHC class II, and LFA3. Additionally, it can also induce cellular secretion of IL-10 and downregulate CD10 levels (173). LMP1 inhibits apoptosis in B lymphocytes as well as protects epithelial cells from p53-mediated apoptosis by increasing levels of A20 and Bcl-2 in the cell (90, 91, 110, 126, 214, 284).

LMP1 can induce expression the inhibitor of differentiation (Id) family of proteins as well as deregulate an array of markers associated with cell cycle progression (73, 191, 279). LMP1 expression in epithelial cells blocks cellular differentiation (53). In transgenic mice, LMP1 expression in the skin induces epidermal hyperplasia and when expressed under the control of the immunoglobulin heavy chain promoter, increases the incidence of B cell lymphomas (180, 345).

LMP2A and 2B

LMP2A and 2B are splice variants that are transcribed across the fuses terminal repeats of the EBV episome. Therefore, latent infection and genome circularization are required for their expression. Transcription of LMP2A and 2B are initiated from two different promoters, with differences in the first exons of the two proteins. LMP2A is a 12 pass transmembrane protein with a 119 amino acid cytoplasmic N-terminus and a short 27 amino acid C-terminus tail. LMP2B lacks the N-terminal 119 amino acids present in LMP2A, however, the remainder of LMP2B is identical to LMP2A. In EBV infected B lymphocytes, LMP2A and 2B are expressed in the plasma membrane and colocalize with LMP1 in lipid rafts (63, 131, 203). In epithelial cells, LMP2A and 2B expression exhibits perinuclear localization and colocalizes with the endoplasmic reticulum, golgi complex, lysosome, and late endosome (52, 209). With these disparate expression patterns, it is likely LMP2A signaling may have different mechanisms and outcomes in B lymphocytes in comparison with epithelial cells. In a similar fashion to LMP1, LMP2A in the plasma membrane can oligomerize, and the clustering signal required for this is located at its C-terminus (215). Currently, the function of LMP2B is unknown, however, it is possible that

LMP2B may serve to limit LMP2A signaling by complexing with LMP2A at the plasma membrane and reducing the LMP2A contacts needed for signaling (202). LMP2B is conserved in the rhesus homolog, rhesus lymphocryptovirus, suggesting LMP2B has an important, but currently unknown function (276).

LMP2A expression is not required for EBV-mediated immortalization of B lymphocytes *in vitro*, but *in vivo* infection efficiency may be enhanced by the presence of LMP2A (19, 174, 204-206, 282). In latently infected B lymphocytes, LMP2A expression is critical for the maintenance of latency. It blocks the B cell receptor (BCR) activation and signaling through several approaches (222-225). This BCR block is important in maintaining latency as events downstream of B lymphocyte activation lead to the reactivation of the EBV lytic cycle (222, 224).

LMP2A blocks BCR signaling by several different methods. In a normal cell, antigen binds and the BCR translocates into lipid rafts where the necessary signaling molecules needed for its function are located. However, in an EBV infected cell, LMP2A blocks this antigen-stimulated relocation of the BCR to lipid rafts (63). In addition, LMP2A blocks antigen processing downstream of binding to the BCR by an other unknown mechanism (63). LMP2A also absorbs molecules typically utilized by the BCR such as Lyn and Syk kinases and mediates their turnover so they are unavailable for BCR signaling (93, 94, 143, 222, 223, 225).

The N-terminus cytoplasmic domain of LMP2A contains several important motifs that are critical for its signaling functions in both B lymphocytes and epithelial cells. The N-terminus cytoplasmic domain contains eight tyrosine residues, of which five have roles in LMP2A signaling. Tyrosines at residues 74 and 85 are part of an immunoreceptor tyrosine-

based activation motif (ITAM). The Igα/β chains of the BCR also have ITAM motifs, and it is through these ITAMs that the BCR is able to bind and activate Syk, a kinase that can then recruit and activate other effector molecules. Phosphorylation of the ITAM tyrosine residues is important for BCR activity, and other kinases such as Lyn are able to phosphorylate these residues upon BCR translocation to lipid rafts. The phosphorylated ITAM of LMP2A can also bind the SH2 domains of Syk, and tyrosine 112 of LMP2A binds Lyn. Lyn phosphorylates the ITAM of LMP2A, and through the binding of Syk, LMP2A uses its ITAM to control downstream signaling in B lymphocytes. Ultimately, LMP2A renders B lymphocytes less responsive to antigen stimulation.

LMP2A can also manipulate the turnover of BCR signaling molecules through its association with ubiquitin ligases. LMP2A contains two PPPPY motifs involving tyrosines 60 and 101 that facilitate binding to members of the Nedd4 ubiquitin ligase family through the WW domains of the ligases (143, 346). This association in B lymphocytes leads to increased degradation of LMP2A, Lyn, and potentially Syk (143, 144, 346). Interestingly, the PY motifs of LMP2A are not required for its block of B cell signal transduction (144, 311). However, the LMP2A ITAM motif and Lyn binding site at tyrosine 112 are critical for the inhibition of BCR activation and signaling (92-94).

In addition to blocking the BCR, LMP2A has other important functions in B lymphocytes as seen with transgenic mouse model studies (33). Transgenic mice were generated to direct LMP2A expression to cells of a B lymphocyte lineage by inserting the LMP2A transgene under the control of the immunoglobulin heavy chain promoter and enhancer. In the B lymphocytes of these mice, LMP2A interferes with normal B cell development processes. For example, LMP2A blocks immunoglobulin heavy chain

rearrangement and leads to changes in cell surface markers (33). Notably, LMP2A expressing B lymphocytes from these transgenic mice are able to circumvent normal B cell developmental checkpoints, and immunoglobulin negative B cells are then able to leave the bone marrow and colonize the periphery (32, 33). The LMP2A ITAM motif is crucial for its ability to manipulate these developmental and survival signals in the transgenic B lymphocytes, and the activation of Bruton's tyrosine kinase (Btk) has been associated with some of these effects (219, 220). Additionally, microarray analysis revealed that the transgenic B cells have global downregulation of genes involved in B cell development and the transcription factors that regulate them, such as E2A and its target Pax-5 (265).

In addition to affecting B lymphocyte signaling, LMP2A has also been shown to affect signaling in epithelial cells. In epithelial cells, LMP2A phosphorylation is triggered by cell adhesion to extracellular matrix proteins such as fibronectin, and the Src family kinase inhibitor C-terminal Src kinase (Csk) has been implicated in this event (293). Furthermore, a putative Csk binding motif has been identified which involved tyrosine 101 of the LMP2A N-terminal (293). LMP2A has also been shown to activate PI3K kinase and its target Akt in both B cells and epithelial cells (237, 292, 312). Notably, some epithelial cell lines expressing LMP2A exhibit oncogenic properties. LMP2A expression in the HaCaT human keratinocyte cell line conferred anchorage-independent growth in soft agar and gave rise to poorly differentiated, highly metastatic tumors when injected into nude mice (292). Colony formation in soft agar was PI3K signaling dependent, as it was inhibited by treatment with LY294002, a PI3K inhibitor.

LMP2A can also activate ERK, JNK and Syk in epithelial cells (39). Activation of these pathways resulted in enhanced migration in LMP2A expressing epithelial cells, and

treatment with ERK or Syk inhibitors abolished the enhanced migration (39, 207). Additionally, LMP2A led to increased stability of the target transcription factor, c-Jun, and was shown to be hyperphosphorylated in LMP2A expressing cells (39). Notably, an earlier study revealed that ERK could interact with and phosphorylate LMP2A at serine 15 and serine 102 *in vitro* (253). One recent study showed that LMP2A can inhibit transforming growth factor-β1 (TGF-β1) in gastric carcinoma cells as well as in an EBV-negative B cell line (98). The study showed that LMP2A could partially block apoptosis triggered by treatment with TGF-β1 via PI3K/Akt pathway activation. The diverse signaling capabilities of LMP2A in both B lymphocytes and epithelial cells highlight its importance in EBV latency as well as EBV-associated diseases.

EBV-Associated Diseases

Fortunately, the infection and lifetime persistence of EBV is typically asymptomatic. However, viral infection can result in disease. Host factors such as age, genetic background, immune status, and behaviors can affect the development of EBV-associated diseases. Notably, EBV-associated disease varies in severity from self-limited episodes to fatal outcomes.

Infectious Mononucleosis

Although primary infection with EBV is usually asymptomatic, delay of primary infection until adolescence or early adulthood can result in infectious mononucleosis (IM). Symptoms of IM include fever, pharyngitis, lymphadenopathy, hepatomegaly, splenomegaly, and malaise (46, 72, 275). Occasionally, complications including splenic rupture, anemia,

and hepatitis arise (46). The symptoms associated with IM are generally due to the massive response of the immune system to infection of the B cell compartment. IM results in an increase of pro-inflammatory cytokines such as IL-1, IFN-γ, and TNF-α released by T lymphocytes in response to lytic and latent viral antigens (2, 243). The CD8 positive T-cell memory response is maintained for the lifetime of the host and typically constitutes up to 5% of the total circulating CD8 positive T-cell population (132). During IM, individuals shed high titers of virus in the saliva from lytic infection in the oropharynx in addition to maintaining large numbers of latently infected, EBNA2 and LMP1 positive, lymphoblasts in the tonsillar lymph nodes (7, 182). The disease generally resolves itself over the course of weeks to months, and an asymptomatic, latent carrier state in the host is established.

Oral Hairy Leukoplakia

Oral Hairy Leukoplakia (OHL) typically develops when an EBV-infected individual become immunocomprimised, as in the case of acquired immune deficiency syndrome (AIDS). OHL appears as a raised, white, corrugated lesion on the oral epithelium, typically on the lateral borders of the tongue (109) . It is a focus of EBV lytic infection, with viral lytic proteins and linear EBV genomes present (103, 252) . Additionally, some latent viral proteins such as EBNA1, EBNA2 and LMP1 can also be detected (339). Typically, OHL lesions respond well to treatment with acyclovir, a nucleoside analog that inhibits the viral DNA polymerase (274).

X-linked Lymphoproliferative Syndrome

X-linked lymphoproliferative syndrome (XLPS) is a disease in males who inherit a mutation in the gene encoding the signaling lymphocyte activation molecule (SLAM)-associated protein (235, 236, 300). These individuals have impaired interactions between B and T cells, thereby making them extraordinarily sensitive to EBV infection (13, 266). Upon primary EBV infection, their immune systems are unable to control the proliferation of EBV-infected B lymphocytes. The majority of patients present with hyperacute IM followed by hepatic failure and subsequent death (275). If the patient survives the episode of IM, they then may succumb to lymphoma or hypogammaglobulinemia (46, 275).

Chronic Active EBV Infection

Typical IM infections generally result in the resolution of the infection with the host returning to an asymptomatic state. In some instances, patients present with chronic fatigue, fever, and other symptoms for several years after primary EBV infection. Diagnosis of chronic active EBV infection (CAEBV) is dependent on three criteria: severe progressive illness that is associated with abnormally high EBV-specific antibody titers, major organ involvement, and detection of EBV proteins, RNA, or DNA in the affected tissues (3, 129, 247, 294). Although the exact cause of CAEBV is unknown, studies have speculated that the defective cytotoxic activity of NK cells and CTLs in conjunction with genetic factors may be responsible for or contribute to the development of CAEBV (95, 160, 166, 328).

Virus Associated Hemophagocytic Syndrome

Virus associated hemophagocytic syndrome (VAHS) is a T cell lymphoma primarily found in Southeast Asian populations. VAHS can occur alone or in conjunction with IM, CAEBV, XLPS, or lymphoproliferative disease (9, 40, 43, 136, 150). VAHS typically affects children and adolescents, and symptoms include persistent fever, liver dysfunction, cytopenia, hepatosplenomegaly, and hemophagocytosis in various tissues often resulting in death (148).

EBV-Associated Malignancies

Although most EBV infected individuals remain asymptomatic for the course of their lives, a subset of people will develop an EBV-associated malignancy. Both geographical location and immune status are linked to the risk of tumor development. Since EBV infection is associated with B cells and epithelial cells, the common EBV-associated malignancies are of lymphoid and epithelial origin. EBV-associated tumors are strongly linked to latent infection as the genomes located in them are episomal and clonal and viral gene expression falls into one of the three latency categories (268). Notably, expression of lytic EBV proteins has been observed in some tumors and may partly contribute to tumorigenesis (137, 295).

Burkitt's Lymphoma

Burkitt's lymphoma (BL) is divided into three categories: endemic, sporadic, and AIDS-associated. All EBV-associated BL has a Type I latency expression profile, with the viral products limited to EBNA1, the EBERs, and the BARTs (275). Endemic BL is the most

common form and is the leading childhood cancer in equatorial Africa and New Guinea with a high incidence and a marked geographic distribution that correlates with endemic malaria (24, 25, 27, 211, 262). Notably, endemic BL tumors are always EBV positive (16). Endemic BL typically develops at extranodal sites in the jaw during molar eruption (275). Tumors are homogeneous, monoclonal, and composed of B lymphocytes that are similar to germinal center B lymphocytes (66, 268). Both endemic and sporadic BL are characterized by a chromosomal translocation that places the c-myc oncogene under the control of the Ig heavy or light chain promoter, leading to its aberrant expression in B lymphocytes (51).

Sporadic BL does not have a specific geographical distribution and its association with EBV varies greatly. The incidence of sporadic BL is much lower than endemic BL and the disease typically strikes at a later age. Additionally, sporadic BL presents differently as it is usually associated with an abdominal mass and can appear as a leukemia (211).

In HIV infected individuals, a small B cell lymphoma similar to BL appears in the early stages of AIDS when the T cell population is largely unaffected. This type of BL has a 30-40% association with EBV. This lymphoma type lacks EBNA2 and LMP1 expression and often heralds the onset of AIDS (34, 99, 117, 172).

Hodgkin Lymphoma

In the United States, Hodgkin lymphoma (HL) is one of the most commonly occurring cancers in young adults. Approximately 40% of HL cases in industrialized nations are EBV-positive and nearly 100% are EBV-positive in developing nations (141). Incidence of HL peaks in early adulthood, typically in the 30s and then peaks again in older adults. Additionally, males are more affected by HL than women, particularly as age increases

(156). Persons with a history of IM have a three-to-four-fold risk of developing HL (106). Notably, HL in patients with a history of IM is less likely to be EBV-associated. HL is divided into several subtypes that vary with EBV status, histology, incidence, and prognosis.

The malignant cells of HL, known at Hodgkin/Reed-Sternberg cells (HRS), arise from germinal center B cells, are clonal, and multinucleated with abundant cytoplasm (36, 149). HRS cells stain positive for EBV DNA and exhibit a Type II latency gene expression pattern with no chromosomal abnormalities (6, 18, 181, 343, 344). HRS cells are associated with increased NFκB activity as well as increased levels of Bcl-2, p53, and IL-10 (263). Notably, a study has shown that LMP2A expression in the B cells of transgenic mice leads to a myriad of changes in B cell transcription similar to the transcription patterns seen in HL HRS cells (264).

Post-Transplant Lymphoproliferative Disorders

Chronic, pharmacologic-induced immunosuppression in transplant patients allows for uncontrolled proliferation of EBV-infected B lymphocytes and can lead to the development of post-transplant lymphoproliferative disorder (PTLD). PTLD occurs with variable incidence in renal, liver, heart, lung and bone marrow transplants (275). Since bone marrow transplants typically require the total elimination of host lymphoid cells, PTLD likely stems from EBV-infected donor B lymphocytes in the transplanted marrow (108, 115). Furthermore, patients who are EBV negative before transplantation have a greater risk of developing PTLD (134). Both the reduction of immunosuppressive therapy and the depletion of graft B- and T-lymphocytes can reduce the incidence of PTLD (115, 303).

PTLD typically occurs within two years post transplantation. Approximately 90% of lesions are associated with EBV and exhibit a Type III latency expression pattern with EBNA1, EBNA2, and LMP1 found in the majority of EBV-associated lesions (195, 318, 352). In some instances, *p53* and *c-myc* mutations are associated with PTLD (195, 318, 352). Of note, EBNA2 is not always detected in AIDS-associated PTLD, suggesting a Type II latency gene expression pattern (58, 177, 221).

Nasopharyngeal Carcinoma

Undifferentiated nasopharyngeal carcinoma (NPC) is intimately associated with EBV and is a result of EBV infection of the epithelium (267). In all cases, this undifferentiated form of NPC consists of monoclonal EBV-infected epithelial cells of nasopharyngeal origin and exhibits a Type II latency gene expression pattern (22, 38, 74, 268, 269, 347, 353). Although uncommon in the United States and Europe, NPC is endemic to parts of Southeast Asia and Northern Africa (356). The incidence of NPC is related to cultural and environmental factors such as salted fish consumption and exposure to chemical carcinogens, as well as genetic factors such as HLA type (267, 275, 356).

NPC is comprised of malignant epithelial cells surrounded and infiltrated by lymphocytes and granulocytes (267). NPC is a highly invasive and metastatic tumor, and over 50% of patients have metastases at the time of diagnosis. Loss of the p16 tumor suppressor has been linked to NPC development and progression by allowing cells to bypass a critical cell cycle checkpoint (201). One study detected specific activation of NFκB p50 homodimers by LMP1 in NPC, indicating this pathway may be involved in pathogenesis

(322). The activation of the NFκB pathway may be responsible for the upregulation of epidermal growth factor receptor (EGFR) seen in NPC (228, 229, 308).

HLA haplotype also contributes to NPC pathogenesis as genes closely related to HLA are associated with an increased risk for NPC development (64, 208). HLA haplotypes HLA-A2, BW46, A19, B17 class I, and HLA-DR β10803 class II are associated with an increased risk for NPC, whereas HLA A11 and B13 class I have a decreased risk (64, 275). Although the majority of the high risk haplotypes are prevalent in China, Chinese immigrants and their children have a reduced risk for NPC, indicating the importance of environmental factors (64, 275).

Overall, the role of EBV in initiating NPC is poorly understood. EBV cannot transform epithelial cells, but it is readily detectable in pre-malignant lesions of the nasopharynx, suggesting that is may play a part in the early stages of NPC development (258, 345). LMP2A is abundantly transcribed in NPC, and NPC patients have high titers of antibodies against LMP2A, suggesting it is also expressed at the protein level (22, 29, 189). In one report, LMP2A protein expression was detected by immunohistochemistry in approximately 46% of NPC tumors examined (130). Futhermore, LMP2A has been shown to activate the PI3K/Akt signaling pathway in epithelial cells (237, 292). Activation of this pathway could facilitate transformation and growth of EBV infected cells through the activation of cell survival and proliferation pathways.

Gastric Carcinoma

Research has shown that approximately 10% of gastric carcinomas are comprised of monoclonal, EBV infected cells (145, 314). EBV-associated gastric carcinoma deviates from

the standard latency type classifications. They have a modified Type II latency expression pattern with the additional expression of the latent *BARF1* gene in addition to several lytic genes such as *BZLF1*, *BRLF1*, and *BLLF1* in some instances (137, 361). Notably, expression of LMP1 and LMP2B is frequently absent in these tumors, which also deviates from classical Type II latency (307).

The lack of LMP1 expression in these tumors suggests a more critical role for LMP2A in gastric carcinogenesis. LMP2A has previously been shown to have oncogenic properties in the HaCaT human epithelial cell line (292). In addition to LMP2A, expression of the BARF1 gene has been detected in some gastric carcinomas (361). BARF1 was first described as a lytic protein, however, some studies have described its expression in epithelial malignancies with a modified Type II latency expression pattern (57, 122, 221, 361). BARF1 is the secreted viral homolog to human colony stimulating factor 1 and has immortalizing effects in primary primate epithelial cells and transforming effects in human B lymphocytes and rodent fibroblasts (248, 297, 305, 319, 340-342). Additionally, one study showed that the addition to BARF1 to serum starved cultures of rodent fibroblasts, primate epithelial cells, and human B cells resulted in cell cycle activation and progression (288). This potential role for BARF1 as a growth factor could contribute to the establishment and progression of EBV-associated gastric carcinomas.

Other Associated Malignancies

In addition to the previously described malignancies, EBV has also been associated with several other malignancies. T cell and NK cell lymphomas as well as some non-Hodgkin B cell lymphomas are infected with EBV (55, 142, 163, 218, 249). Additionally,

central nervous system lymphomas arising in HIV patients have a strong connection to EBV (210). There have also been several reports of EBV-associated breast cancers, but these findings have been, and remain controversial (21, 44, 185). Lastly, leiomyosarcoma, a rare smooth muscle tumor, is strongly associated with EBV in the context of immunosuppression (186, 217).

Therapeutics

Drugs that induce the EBV lytic cycle in conjunction with the nucleoside analog gancyclovir as well as demethylating agents such as 5-azacytidine, have been used to directly target the EBV life cycle (5, 79, 152). Chemical compounds targeting individual EBV proteins through RNA interference (RNAi), single-chain antibodies, or by inhibiting signal transduction pathways used by the virus, such as NFκB, have had minimal success (30, 78, 170, 261). A more effective approach involved the infusion of effector T cells prepared from the bone marrow donor by autologous LCL stimulation and expansion *in vitro*. This method has been successful in bone marrow transplants and now a similar adoptive transfer approach is being used for PTLD cases and solid organ transplants (283, 355).

Rhesus Lymphocryptovirus

Infection of Old World primates with EBV related lymphocryptoviruses (LCV) was first recognized in the early 1970s when investigators developed and immunoflourescence assay to detect antibodies cross-reactive to the EBV viral capsid antigen (VCA) in the serum of Old World primates (62, 162). Subsequently, LCV infected cell lines were established from both healthy and diseased Old World primates. Simian LCV infected cell lines

produced virus that could immortalize B cells from autologous and closely related species *in vitro* (77, 97, 100). These cell lines also expressed latent infection nuclear antigens similar to EBV EBNAs, but these antigens did not cross react well with EBV immune human sera (61, 101, 193, 246). More recent work has shown that rhesus LCV can also infect epithelial cells in immunosuppressed macaques and induce epithelial lesions resembling oral hairy leukoplakia in AIDS patients (184).

In vivo, LCV infection in primates resembles EBV infection in humans. Newborn animals are sero-positive for VCA antibodies due to maternal antibody transfer. They become sero-negative within four to six months after birth, and then most sero convert again within a year, indicating a high prevalence of infection (87, 96, 157). Lifelong antibody responses, the ability to recover LCV infected B cell lines from the peripheral blood of healthy animals, and the ability to detect virus shed from the oropharynx indicate that a similar host-LCV relationship exists in primates as with EBV and humans (231).

Studies into the LCV genome revealed a similar DNA organization to EBV (123, 124). Notably, EBV DNA cross-reacts with viral DNA from simian LCVs with the exception of the EBNA3s, LMP1, and LMP2 (123, 124). Rhesus LCV homologs for most of the EBV latent genes have been described (42). In virtually all aspects, these rhesus LCV latent genes are functionally interchangeable with the EBV genes despite varying degrees of homology.

The rhesus LCV homolog for EBV LMP2A was readily identified by the conservation of the characteristic ITAM motif (88, 276). The full-length rhesus LCV LMP2A was deduced from the genomic sequence and confirmed by RT-PCR analysis. The 12 transmembranes are well conserved, and the cytoplasmic domain associated with

signaling is somewhat divergent. Despite this sequence divergence, the ITAM and praline rich domains that are important for interaction with protein tyrosine kinases (PTKs) are conserved and the rhesus LCV LMP2A both induces PTKs and are themselves tyrosine phosphorylated. It remains unclear whether rhesus LCV LMP2A is dispensable for B cell transformation as in the case of EBV. However, it is clear that there is strong selective pressure for LMP2A as it is evolutionarily conserved (276).

Matrix Metalloproteinases

There are currently 24 members of the matrix metalloproteinase (MMP) family which all share a catalytic domain coordinated by zinc, which catalyze the degradation of protein components of the extracellular matrix (ECM) in their own environment, as well as activating latent growth factors, cell surface receptors, and adhesion molecules (35, 250). Cell-ECM interactions trigger cellular signaling that promotes cell differentiation, migration, and mobilization, essential for homeostasis. Accordingly, the balanced and regulated degradation of ECM proteins by MMPs is involved in numerous physiological processes including wound healing, tissue remodeling, angiogenesis, and embryo development (304). Conversely, excess MMP activity plays a role in several diseases including rheumatoid arthritis, osteoarthritis, autoimmune diseases, cardiovascular diseases, and cancer. MMPs have been regarded as critical molecules assisting tumor cells during metastasis (65, 304). From the earliest work on MMPs in cancer, there has been a clear connection between MMPs, ECM degradation, and cancer cell invasion. Numerous studies have linked inhibition of MMPs with a corresponding inhibition of cell invasion. Conversely, upregulation of MMPs usually leads to enhanced tumor cell invasion (59, 196, 197).

With regards to EBV, work has shown that MMP-9 is induced by LMP1 expression in C33A human epithelial cells (316). This report indicated that MMP-9 expression and activity were dependent on LMP1 mediated activation of the NFκB pathway. These results indicate that LMP1 may contribute to NPC invasiveness and metastasis.

OBJECTIVES

EBV LMP2A is a latent protein that is expressed in most EBV-associated malignancies. This broad spectrum of expression indicates important roles for LMP2A in oncogenesis. As previously noted, the importance of LMP2A in B lymphocytes has been well established, but its role in epithelial cells is only beginning to unfold. The goal of these studies is to evaluate the properties EBV LMP2A and rhesus LMP2A in epithelial cells and how these properties can contribute to EBV pathogenesis.

Aim 1. Characterization of rhesus LMP2A expression in epithelial cells

Previous work in our lab has shown that LMP2A activates PI3K/Akt signaling in HFK cells and this signaling pathway was responsible for the translocation of β-catenin to the nucleus. In this study, we assess the activation of this pathway by the rhesus LMP2A homolog in HFK cells to determine if it behaves in a similar fashion to EBV LMP2A.

Aim 2. Microarray analysis of EBV LMP2A expressing HFK cells

The goal of this aim is to identify and assess LMP2A targets that may affect cell migration and adhesion. Here we identify MMP proteins and identify the signaling pathways involved in their LMP2A mediated expression and activity.

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CHAPTER TWO

Rhesus Lymphocryptovirus Latent Membrane Protein 2A Activates β-Catenin Signaling and Inhibits Differentiation in Epithelial Cells

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ABSTRACT

Rhesus lymphocryptovirus (LCV) is a γ-herpesvirus closely related to Epstein-Barr virus (EBV). The rhesus latent membrane protein 2A (LMP2A) is highly homologous to EBV LMP2A. EBV LMP2A activates the phosphatidylinositol 3-kinase (PI3K) and β-catenin signaling pathways in epithelial cells and affects differentiation. In the present study, the biochemical and biological properties of rhesus LMP2A in epithelial cells were investigated. The expression of rhesus LMP2A in epithelial cells induced Akt activation, GSK3β inactivation and accumulation of β-catenin in the cytoplasm and nucleus. The nuclear translocation, but not accumulation of β-catenin was dependent on Akt activation. Rhesus LMP2A also impaired epithelial cell differentiation; however, this process was not dependent upon Akt activation. A mutant rhesus LMP2A lacking six transmembrane domains functioned similarly to wild-type rhesus LMP2A indicating that the full number of transmembrane domains is not required for effects on β-catenin or cell differentiation. These results underscore the similarity of LCV to EBV and the suitability of the macaque as an animal model for studying EBV pathogenesis.

INTRODUCTION

Epstein-Barr virus (EBV) is a γ-herpesvirus within the lymphocryptovirus (LCV) subgroup. EBV is the etiologic agent of infectious mononucleosis and it is closely associated with numerous diseases including nasopharyngeal carcinoma, Burkitt's lymphoma, Hodgkin's disease, and numerous lymphoproliferative disorders, particularly in immunocompromised individuals (20). The majority of Old World primates are naturally infected with simian homologs of EBV. As with EBV infection in humans, infection is ubiquitous in animals raised both in captivity and in the wild. These simian homologs share many genetic, biologic, pathogenic, and epidemiologic properties with human EBV. Rhesus EBV (cercopithicine herpesvirus 15) can immortalize B cells in vitro (14) and has an identical genetic repertoire to EBV with an overall sequence homology of 75% (21). Rhesus EBV can also induce B-cell tumors in animals immunosuppressed due to simian immunodeficiency virus infection similar to the development of EBV induced tumors in AIDS patients (6, 7). Recently, rhesus EBV has been shown to infect epithelial cells in immunosuppressed macaques and can induce epithelial cell lesions that resemble oral hairy leukoplakia in AIDS patients (11). Additionally, a previous study has shown that rhesus macaques experimentally infected with rhesus EBV exhibited acute and persistent infections similar to EBV infection in humans, thus indicating that rhesus macaques are a suitable animal model for EBV (15).

These striking similarities between rhesus LCV and EBV and the 62% amino acid similarity suggest that the molecular properties of rhesus LMP2A (Rh-L2A) would be similar. EBV LMP2A has previously been shown to have striking properties in epithelial cells where its expression in human keratinocyte cell line, HaCaT, induced PI3K activation

and the subsequent phosphorylation and activation of Akt (22). In addition, HaCaT cells expressing EBV LMP2A had impaired differentiation when grown in organotypic raft cultures. In human telomerase immortalized human foreskin keratinocytes, HFK cells, LMP2A expression activated both the PI3K/Akt signaling and Wnt/β-catenin signaling pathways. The activation of these pathways led to the subsequent phosphorylation and inactivation of the Akt target GSK3β. The effects on these pathways also increased the levels and induced the nuclear translocation of β-catenin resulting in activation of TCF mediated transcription (17). In HFK cells, EBV LMP2A also inhibited epithelial cell differentiation in assays where differentiation was induced by suspension in methylcellulose. In these assays, involucrin and keratinocyte transglutaminase increase during differentiation and these increases were blocked by EBV LMP2A (18).

In this study, the effects of Rh-L2A expression were analyzed in the HFK epithelial cells. Additionally, a mutant Rh-L2A (Rh-L2AΔC) lacking the C-terminal six transmembrane domains was examined. The N-terminus of EBV LMP2A contains multiple important signaling motifs including a src binding site, an ITAM motif, and a PY motif that is essential for interactions with Nedd4 ubiquitin ligases. These motifs are retained in Rh-L2A and in Rh-L2AΔC. The data presented here indicate that both Rh-L2A and the truncation mutant induced the phosphorylation and activation of Akt and the phosphorylation of an Akt target, GSK3β. Rh-L2AΔC increased cytoplasmic and nuclear levels of β-catenin and the translocation of β-catenin was blocked by treatment with the Akt inhibitor, triciribine. In a novel differentiation assay, Rh-L2A expressing cells had decreased levels of both involucrin. Interestingly, loss of six transmembrane domains did not impair the properties of Rh-L2AΔC. In addition, inhibition of Akt did not affect the ability of LMP2A

to block differentiation. These data indicate that the rhesus homolog of EBV LMP2A behaves in a similar manner to EBV LMP2A in epithelial cells and further validates the rhesus macaque as an animal model for the study of EBV infection and pathogenesis in epithelial cells.

MATERIALS AND METHODS

Cell culture and retrovirus. Human foreskin keratinocytes (HFK) immortalized with human telomerase (5) were cultured in serum-free keratinocyte media (K-SFM, Gibco) supplemented with 2ng/ml of epidermal growth factor, 30μg/ml bovine pituitary extract, and 1% antibiotic-antimycotic solution (Gibco). 293T cells were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco) supplemented with 10% FBS and 1% antibiotic-antimycotic solution. Both HFK and 293T cells were grown at 37°C in a humidified incubator with 5% CO_2. To create stable cell lines, recombinant retroviruses expressing either vector alone (pBabe) or vector subcloned with FLAG-tagged rhesus LMP2A (pBabe-Rh-L2A) were generated as previously described using FuGene 6 transfection reagent (Roche) according to manufacturer's instructions and used to transduce HFK cells (22). Pools of stable cells expressing either pBabe, pBabe-Rh-L2A, and pBabe-Rh-L2AΔC were selected for in the presence of .5μg/ml puromycin (Sigma).

Lysate preparation and cellular fractionations. Whole cell lysates were prepared using Nonidet P-40 (NP-40) lysis buffer containing 50mM Tris-HCl, 150mM NaCl, 2mM EDTA, 10% glycerol, 1% NP-40, 1mM sodium vanadate (Na_3VO_4), 0.4mM phenylmethylsulfonyl fluoride (PMSF), and protease and phosphatase cocktails (Sigma) at 1:100. Cellular fractionations were performed using OptiPrep (Sigma) according to a modified manufacturer's protocol. In brief, cells were resuspended in buffer A (20mM HEPES, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM dithiothreitol, 1mM Na_3VO_4, 0.5mM PMSF, and protease and phosphatase inhibitor cocktails at 1:100) with 1% NP-40.

Crude nuclei were pelleted at 1000 rpm and cytosolic fractions extracted. Nuclei were purified over an OptiPrep gradient with 25, 30, and 35% layers and then lysed with a hypotonic buffer (20mM Tris-HCl, 400mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 25% glycerol, 1mM Na_3VO_4, 0.5mM PMSF, and protease and phosphatase inhibitor cocktails at 1:100).

Differentiation assays. To set up the assay, approximately $4x10^6$ HFK cells expressing Rh-L2A, Rh-L2AΔC, or vector alone were plated in 100mm Petri dishes containing keratinocyte media and placed at 37°C overnight. The keratinocyte media was then removed and replaced with DMEM and placed again at 37°C for 48h. After the incubation period, cells were harvested and cell pellets were then lysed using Nonidet P-40 (NP-40) lysis buffer containing 50mM Tris-HCl, 150mM NaCl, 2mM EDTA, 10% glycerol, 1% NP-40, 1mM sodium vanadate (Na_3VO_4), 0.4mM phenylmethylsulfonyl fluoride (PMSF), and protease and phosphatase cocktails (Sigma) at 1:100.

Inhibitor experiments. For fractionation and zero time experiments (cells not induced to differentiate), HFK cells were plated in either 100mm or 150mm dishes containing keratinocyte media. Within 24h post-seed, triciribine (TCN) was added to the media at a concentration of 5μM. Control plates were treated with an equivalent volume of DMSO (vehicle control). After 24h of inhibitor treatment, cells were harvested and lysates generated for Western blot analysis. For differentiation experiments, HFK cells were plated in 100mm Petri dishes containing standard DMEM media to induce differentiation. At 24h post-seed, TCN was added to the media at a concentration of 5μM. DMSO was used as a

vehicle control. At 24h post-treatment (48h into the induction of differentiation), cells were harvested and lysates generated for Western blot analysis.

Western blot analysis and antibodies. Protein concentrations were determined using the Bio-Rad DC assay system according to manufacturer's instructions. Lysates were boiled in a protein loading solution containing SDS and β-mercaptoethanol for five minutes and subjected to SDS-10% polyacrylamide gel electrophoresis. Proteins were transferred to an Optitran nitrocellulose membrane (Schleicher & Schuell) and subjected to Western blot analysis. Antibodies used include anti-involucrin antibody from Sigma; anti-Akt, anti-phospho-Akt Ser473 and anti-phospho-GSK3β from Cell Signaling; anti-actin from Santa Cruz; and anti-β-catenin from Transduction Laboratories. Horseradish peroxidase secondary antibodies (Amersham) and SuperSignal West Pico System (Pierce) were used to detect antibody bound proteins.

Immunoflourescence. HFK cells expressing pBabe, Rh-L2A, or Rh-L2AΔC were plated in six well plates containing a coverslip. Approximately 24h post seed, the cells were fixed with 4% paraformaldehyde for 15 minutes at RT. Cells were then washed 3X with PBS and anti-FLAG antibody (Sigma) or DAPI (Molecular Probes) was applied as specified by the manufacturer. Again, cells were washed 3X with PBS and a FITC-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch) was added according to manufacturer's instructions. Cells were then washed 3X with PBS and mounted on coverslips using an anti-fade mounting medium.

RESULTS

Rhesus LMP2A signaling in telomerase immortalized HFK cells activates the PI3K/Akt pathway. EBV LMP2A has been shown to activate Akt via the PI3K pathway in both lymphocytes and epithelial cells (17, 22, 24). To investigate whether rhesus LMP2A (Rh-L2A) and the C-terminus deletion mutant, Rh-L2AΔC, also activate Akt in epithelial cells, RhL2A was expressed in HFK cells. Stable HFK cell lines expressing FLAG-tagged Rh-L2A or Rh-L2AΔC in the pBabe vector or vector alone were developed, and pools of stably expressing cells generated after puromycin selection. Immunoflourescent immunohistochemistry and immunoblotting with anti-FLAG antiabody confirmed the expression of FLAG-tagged Rh-L2A and Rh-L2AΔC in the stable cell lines (Fig.1). Immunohistochemistry staining indicated expression in all cells. DAPI staining of the nucleus and overlay with the FLAG antibody revealed that the majority of the protein was located at the perinuclear membrane. Despite the loss of six transmembrane domains, the Rh-L2AΔC mutant also localized to the perinuclear membrane (Fig. 1A). Expression of the two forms was also identified by immunoblotting with anti-FLAG antibody (Fig.1B). The full-length Rh-L2A migrated with a MW of 55 kDa, while the Rh-L2AΔC had the appropriate MW of 42 kDa.

To detect activated Akt, Western blot analysis was performed using a phosphospecific antibody directed against Ser473 to detect activated Akt (Fig. 2A). Both Rh-L2A and Rh-L2AΔC activated Akt to levels higher than the pBabe control. These data indicated the similarity with EBV LMP2A in that both Rh-L2A and Rh-L2AΔC activated Akt in HFK cells and that the C-terminus of Rh-L2A was not required for this function.

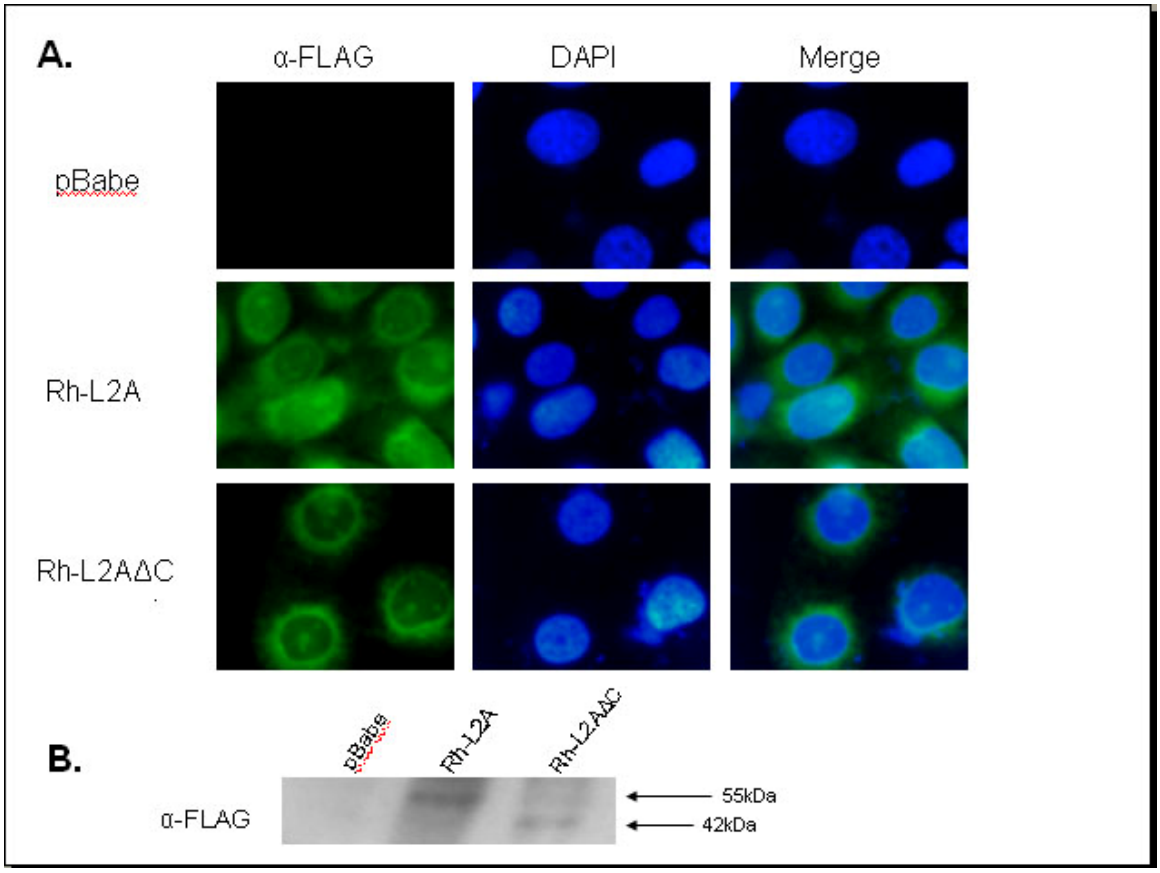

Figure 1: Expression of Rh-L2A in human foreskin keratinocytes. HFK cells were transduced with the pBabe (vector) retrovirus alone or expressing Rh-L2A. Transduced cells were placed under puromycin selection. A) Expression was determined by immunoflourescence using an anti-FLAG antibody. DAPI was used to visualize the nuclei. B) Immunoblot was prepared with equal amounts of protein of the Rh-L2A and Rh-L2AΔC expressing HFK cells and reacted with anti-FLAG antibody.

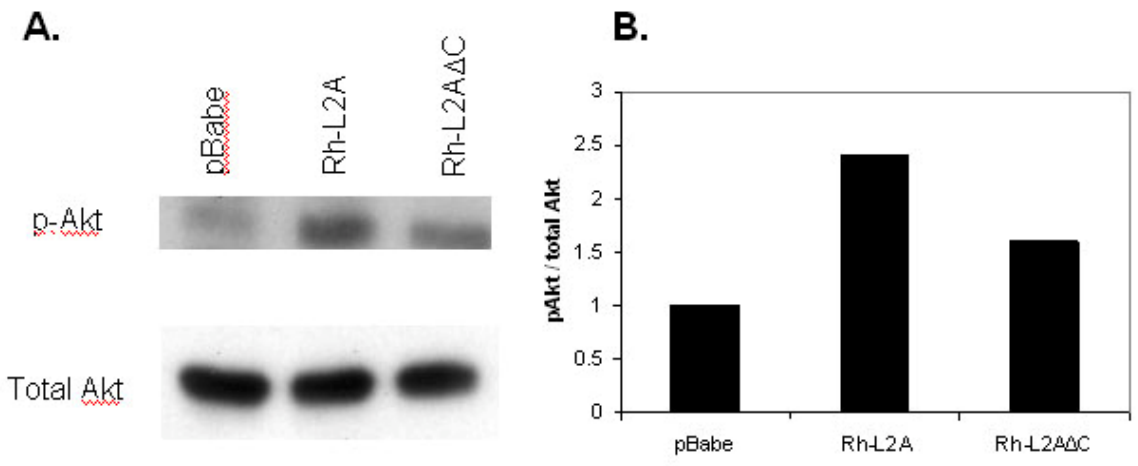

Figure 2: Rh-L2A and Rh-L2AΔC phosphorylate and activate Akt in HFK cells. Cells lines expressing Rh-L2A, Rh-L2AΔC, or vector alone were harvested, lysed, and subjected to Western blot analysis. To detect the activated form of Akt, a phospho specific antibody for Ser473 was used. Total Akt was used for a loading control and densitometry with Image J software was performed to normalize p-Akt levels to the corresponding total Akt levels. Fold increase of the normalized p-Akt values are depicted in panel B. The Fig. shown is a representative experiment from five independent experiments.

Akt can phosphorylate and affect the activity of many proteins including glycogen synthase kinase beta (GSK3β) (2). GSK3β is an important enzyme involved in regulating glycogen storage and wnt signaling. Phosphorylation by Akt at Ser9 inactivates GSK3β, suggesting that Akt is involved in controlling cellular metabolism. This inactivation may affect cellular metabolism, survival and/or cell cycle progression. Western blot analysis with phospho-specific antibodies revealed increased phosphorylation of GSK3β in both Rh-L2A and Rh-L2AΔC expressing cells relative to vector control cells (Fig. 3). These data indicate that Rh-L2A expression in HFK cells leads to the activation of Akt and subsequent phosphorylation and inactivation of the Akt target GSK3β and that the C-terminus of Rh-L2A is not necessary for this function. Interestingly, endogenous levels of β-catenin were increased in the Rh-L2A

expressing cells and levels of involucrin, a terminal differentiation marker, were reduced (Fig. 3). These data further indicated the similarity in the function of Rh-L2A with EBV LMP2A (17).

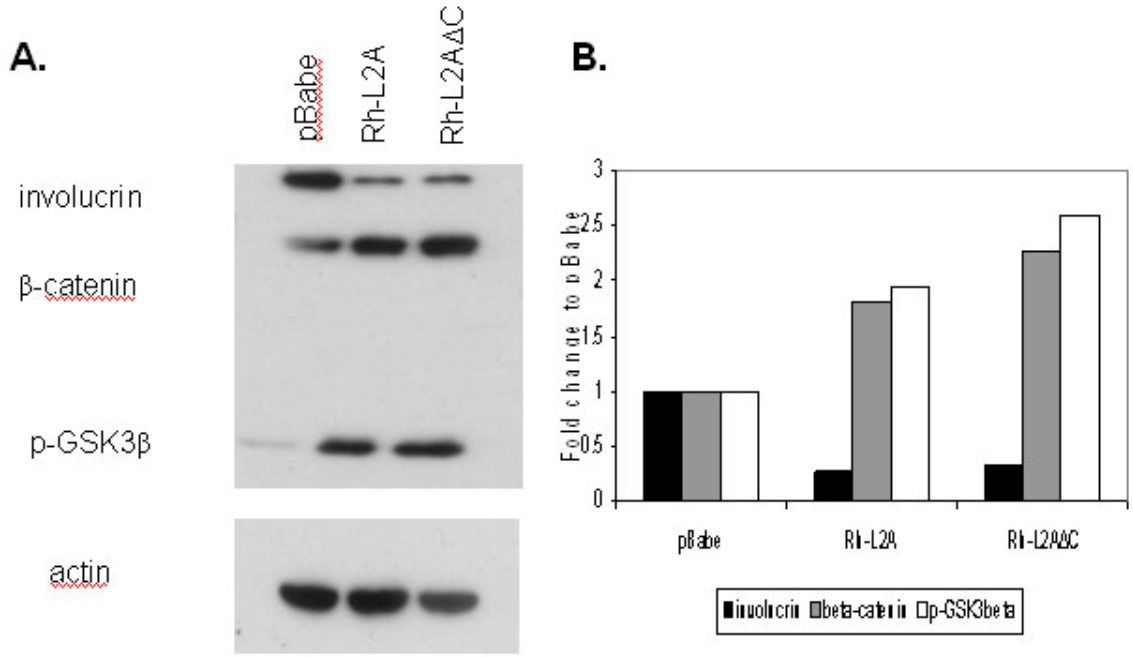

Figure 3: Rh-L2A and Rh-L2AΔC reduce involucrin levels and increase levels of β-catenin and p-GSK3β in HFK cells. HFK cells stably expressing Rh-L2A, Rh-L2AΔC, or vector alone were harvested, lysed and subjected to Western blot analysis. A phospho-specific antibody directed against Ser9 was utilized to detect the inactivated form of GSK3β. Actin served as a loading control. Panel B is a graphical depiction of the quantitation performed on the blot shown in part A and is representative of four independent experiments. Quantitation analysis was performed using ImageJ software.

Rh-L2A signaling results in increased expression and nuclear accumulation of β-catenin in HFK cells. GSK3β can bind to the axin complex and within this complex GSK3β phosphorylates β-catenin thereby targeting it for ubiquitination and degradation via the proteosome (19). Phosphorylation of GSK3β by Akt blocks GSK3β kinase activity, however this GSK3β inactivation does not always affect β-catenin accumulation and localization (3). For example, in 293 cells and CHO cells expressing insulin receptor, GSK3β inactivation due

to insulin signaling did not induce β-catenin accumulation and activation of TCF/LEF transcription factors. However, Wnt signaling activation did induce β–catenin accumulation and transcriptional activation without inducing the phosphorylation of GSK3β (4). Previous work from our laboratory has shown a PI3K activation requirement for the nuclear translocation of β-catenin in HFK cells expressing EBV LMP2A (17). To investigate the effects of Rh-L2A and Rh-L2AΔC on the β-catenin/Wnt pathway and the status of endogenous β-catenin, a fractionation experiment was performed with Rh-L2A and Rh-L2AΔC expressing HFK cells. Stable cell lines expressing Rh-L2A, Rh-L2AΔC or vector alone were fractionated and levels of β-catenin were determined by immunoblotting of nuclear and cytosolic extracts with an antibody directed against β-catenin (Fig. 4). HFK cells express abundant levels of β-catenin such that expression of Rh-L2A or Rh-L2AΔC had a minimal effect on the cytosolic amounts of β-catenin compared with vector alone. However, nuclear β-catenin levels were considerably increased in the HFK cells expressing Rh-L2A and Rh-L2AΔC over cells expressing vector alone (Fig. 4). These results indicated that Rh-L2A and Rh-L2AΔC induce the nuclear translocation of β-catenin similarly to that observed in EBV LMP2A expressing cells.

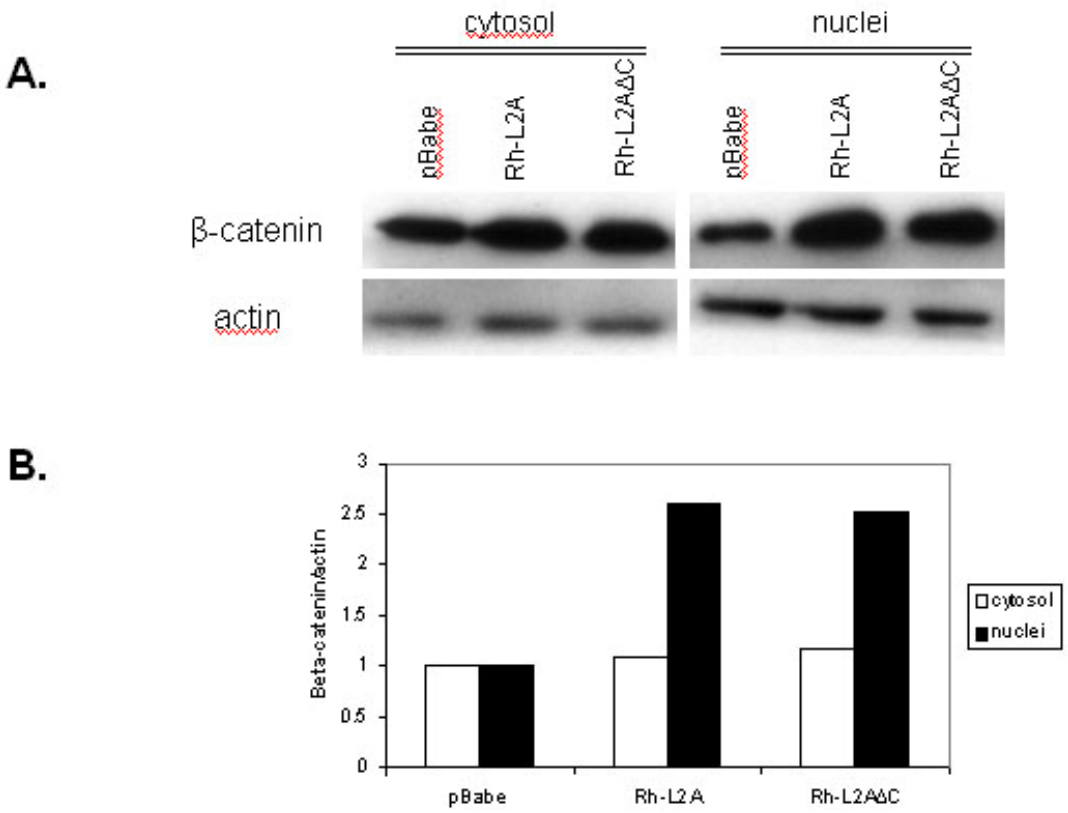

Figure 4: Rh-L2A and Rh-L2AΔC increases levels of nuclear β-catenin in HFK cells. Cells stably expressing Rh-L2A, Rh-L2AΔC, or vector alone were harvested and fractionated into cytosolic and nuclear components. Each component was subjected to Western blot analysis with antibodies to β-catenin, actin as a loading control, and GRP78 to attest to the purity of the fractions (data not shown). Densitometry was performed with Image J software as previously described. The fold increase of the normalized β-catenin values are graphically depicted in panel B and this is representative of three independent experiments.

Inhibition of Akt reduces expression and nuclear accumulation of β-catenin in Rh-L2A expressing cells. Previous studies of EBV LMP2A indicated that accumulation of nuclear β-catenin was inhibited by the PI3K inhibitor, LY294002, however, the specific requirement for Akt activation was not determined. To further delineate a specific requirement for activated Akt in this process and assess the contribution of the intact transmembrane domains, the effects of the Akt inhibitor, triciribine (TCN) were determined. A previous study has shown that TCN is a potent and selective inhibitor of Akt in tumor cells (25). TCN inhibits Akt by inducing a conformational change in the molecule that prevents it from becoming phosphorylated and phosphorylating its targets. To determine the efficacy of TCN in our stable cell lines, cells expressing Rh-L2A, Rh-L2AΔC or vector alone were treated with a 5μM concentration of TCN or DMSO as a vehicle control. Triciribine treatment effectively reduced the levels of phosphorylated Akt and GSK3β in all cells confirming previous work indicating that triciribine is an effective inhibitor of Akt activation (Fig. 5) (25). Triciribine treatment did not affect the total β-catenin levels in Rh-L2A or Rh-L2AΔC expressing cells as compared to the vehicle control (Fig. 5). These data confirm previous studies that indicated the inhibition of PI3K did not significantly affect the accumulation of cytosolic β-catenin (17).

Studies from our laboratory have also shown a requirement for PI3K signaling for inducing the nuclear translocation of β-catenin by EBV LMP2A (17). To further investigate a specific role for Akt activation in this process and the contribution of the intact transmembrane domain, stable HFK cells expressing Rh-L2AΔC or vector alone were treated with TCN or DMSO as a vehicle control for 24h. The cells were harvested, fractionated, and subjected to Western blot analysis. Treatment with TCN considerably decreased the nuclear

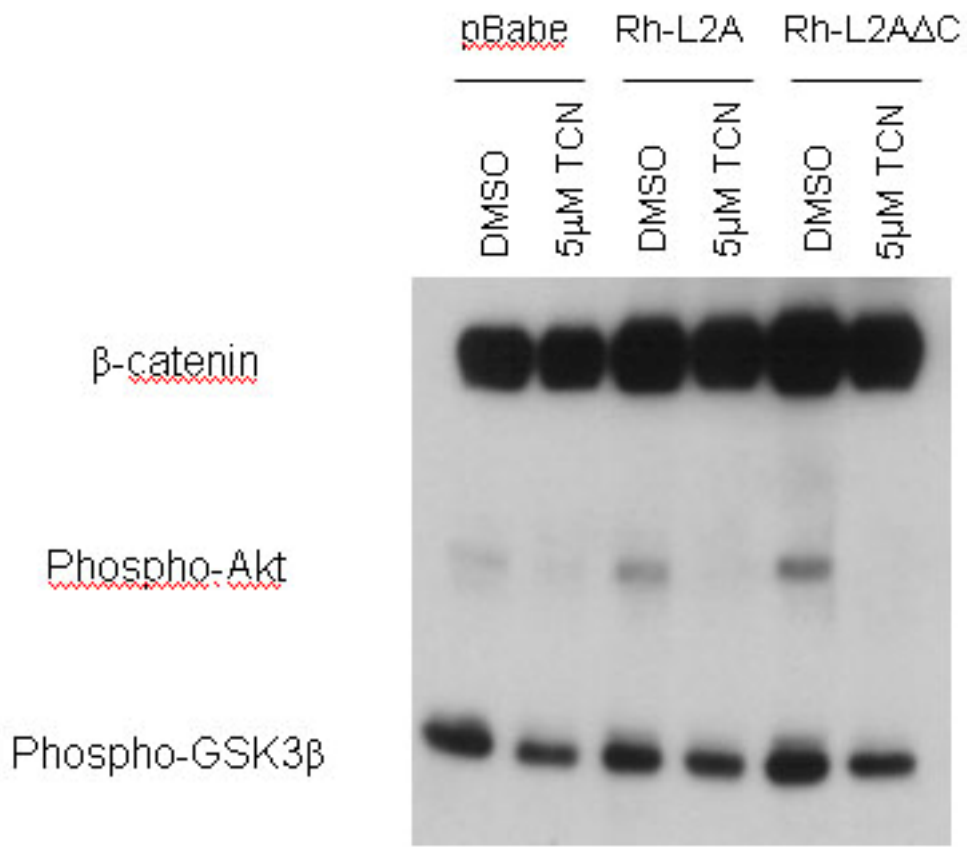

Figure 5: Triciribine inhibits Akt activation in HFK cells. Stable cell lines expressing Rh-L2A, Rh-L2AΔC, or vector alone were treated with 5μM triciribine (TCN) for 24h prior to harvest. Western blot analysis was performed using antibodies to β-catenin, Akt phosphorylated at Ser473, and GSK3β phosphorylated at Ser9.

β-catenin in the Rh-L2AΔC cells and slightly decreased the levels in the vector control (Fig. 6). Similarly, treatment of cells expressing the full length Rh-L2A with triciribine also inhibited nuclear accumulation of β-catenin (data not shown). These results indicated that the deletion of six transmembrane domains in Rh-L2AΔC did not affect this property of Rh-L2A as both full length and Rh-L2AΔC induced nuclear translocation of β-catenin and this process specifically required activation of Akt.

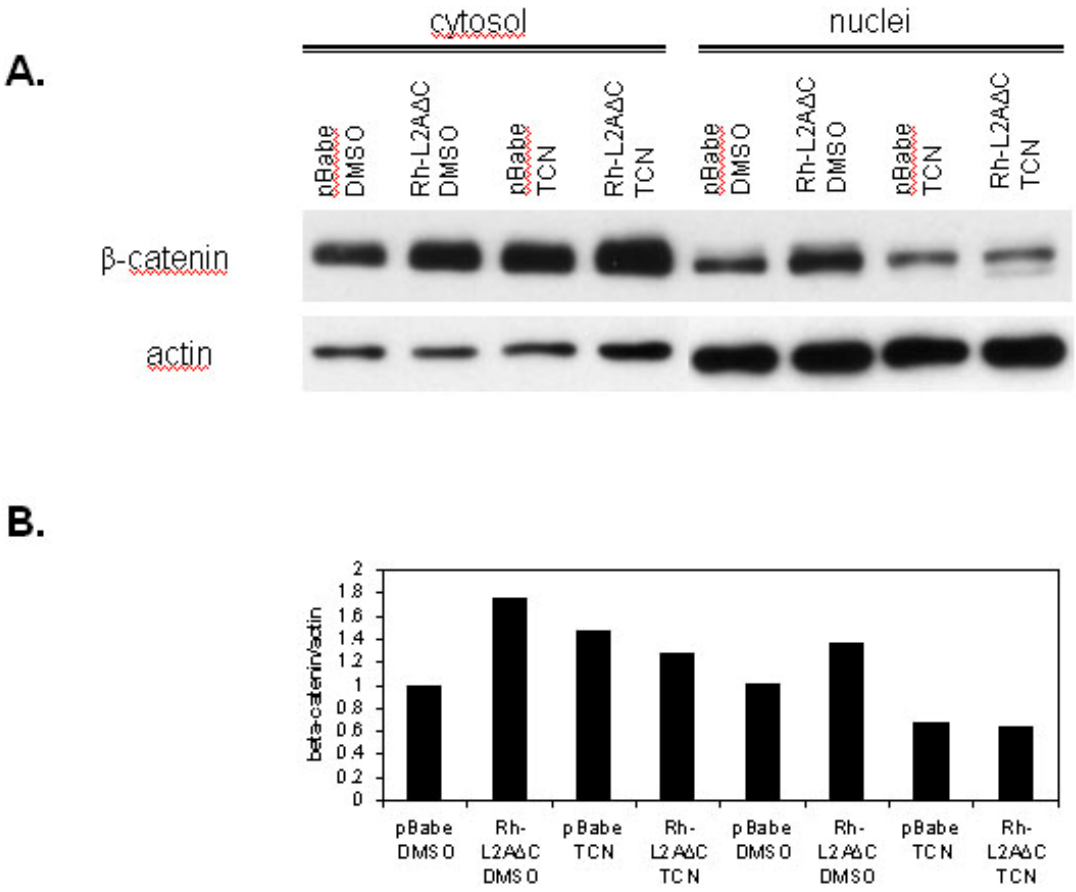

Figure 6: Triciribine inhibits induction of nuclear β-catenin levels in HFK cells. Cells stably expressing Rh-L2AΔC or vector alone were harvested and fractionated into cytosolic and nuclear components 24h post triciribine treatment. Each component was subjected to Western blot analysis with antibodies to β-catenin, actin as a loading control, and GRP78 to attest to the purity of the fractions (data not shown). Densitometry was performed with Image J software as previously described. The fold increase of the normalized β-catenin values are graphically depicted in panel B and this is representative of three independent experiments.

Rh-L2A inhibits differentiation in HFK cells. Previous studies have shown that EBV LMP2A blocks activation of B cells through the B-cell receptor and also can inhibit epithelial cell differentiation that is induced when HaCaT cells are grown in organotypic raft cultures (22). Differentiation of HFK cells induced by growth in methylcellulose was also blocked as evidenced decreased involucrin (18). To test other methods of inducing differentiation, cells

were plated in Petri dishes containing DMEM media instead of regular keratinocyte media. This has been shown to induce differentiation by impairing cell attachment and increasing calcium levels in a similar fashion to previously described methods (1). To further characterize the effects of Rh-L2A and Rh-L2AΔC expression on differentiation and to determine the efficacy of this method for the induction of differentiation, HFK cells expressing Rh-L2A, Rh-L2AΔC or vector alone were grown in Petri dishes with standard DMEM media to induce differentiation and subsequently analyzed for involucrin expression. During growth in regular keratinocyte media, the Rh-L2A and Rh-L2AΔC expressing cells had decreased levels of involucrin as compared to the control cells (Fig. 7A). Upon the induction of differentiation, involucrin expression increased approximately 3 fold in the control cells. In comparison, involucrin levels were remained considerably lower in both Rh-L2A and Rh-L2AΔC expressing cells with an increase in involucrin of approximately 40% (Fig. 7B). These data indicate that Rh-L2A expression negatively regulates differentiation in HFK cells in a similar manner to EBV LMP2A. Furthermore, these data confirm that the C-terminus of Rh-L2A is not required for LMP2A mediated inhibition of differentiation.

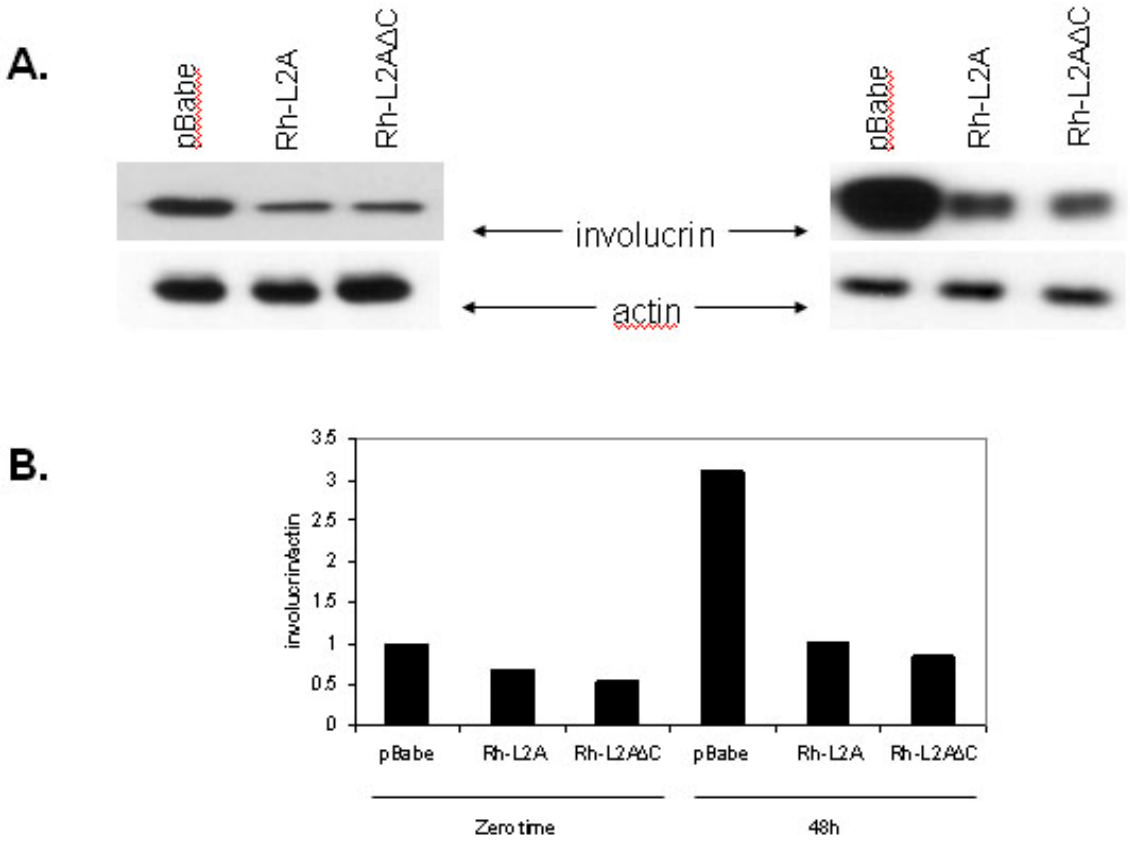

Figure 7: Rh-L2A inhibits differentiation in HFK cells. Stable cells expressing Rh-L2A, Rh-L2AΔC, or vector alone were plated in petri dishes containing standard DMEM media. 48h post seed, cells were harvested and lysates analyzed by Western blot. The induction of differentiation was ascertained using an antibody directed against involucrin, a marker of terminal differentiation. Actin was used as a loading control. Panel B shows the fold increase of involucrin after differentiation normalized to actin and vector control cells at zero time. Involucrin levels were determined by Image J software and are representative of five independent experiments.

As inhibition of Akt effectively blocked the nuclear translocation of β-catenin, it was of interest to assess the contribution of Akt activation to the effects of LMP2A on markers of differentiation. Interestingly, in cells that have not been induced to differentiate, treatment with triciribine did not affect the ability of Rh-L2A or Rh-L2AΔC to inhibit expression of the differentiation marker, involucrin. Triciribine reduced levels of involucrin in both control

and Rh-L2A cells (Fig. 8). These data suggest that the effects of Rh-L2A and Rh-L2AΔC on differentiation markers do not require activation of Akt.

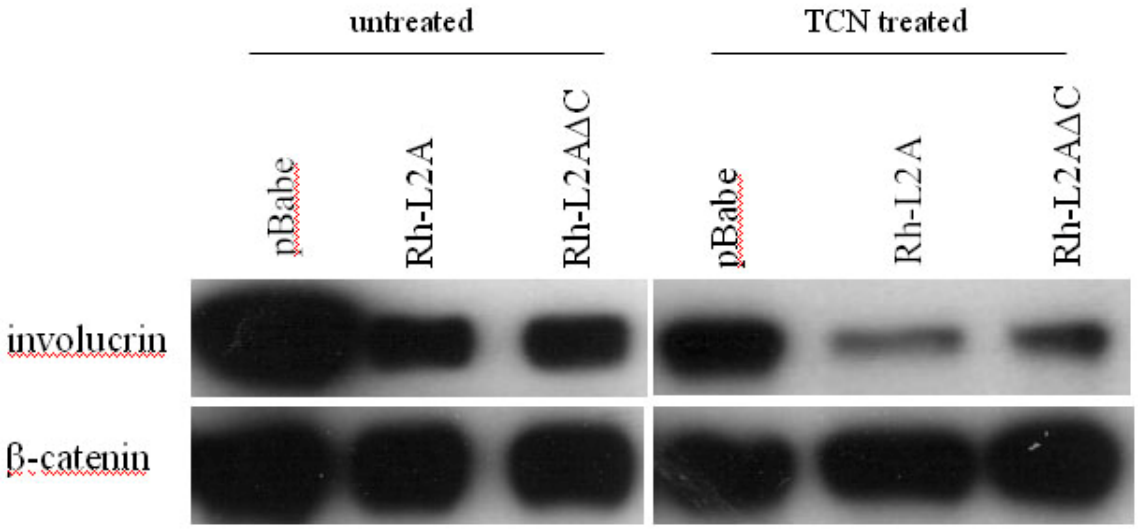

Figure 8: Rh-L2A inhibition of involucrin expression is not Akt dependent. HFK cells stably expressing Rh-L2A or vector alone were untreated or treated with triciribine (TCN) for 24h. Cell lysates were analyzed by Western blotting.

DISCUSSION

This study reveals that the rhesus homolog of the EBV LMP2A protein has similar properties and can affect both the PI3K/Akt and Wnt/β–catenin signaling pathways in telomerase immortalized HFK cells. Expression of both Rh-L2A and Rh-L2AΔC in HFK cells induced the phosphorylation and activation of Akt and subsequent phosphorylation and inactivation of the Akt target, GSK3β. With the inactivation of GSK3β, β-catenin accumulated in the cytoplasm and translocated into the nucleus. The nuclear translocation of β-catenin was dependent on Akt activation and the inhibition of Akt by triciribine blocked this effect (Fig. 6). Previous work in our laboratory had shown a requirement for PI3K activation for the nuclear translocation of β-catenin, but not for cytosolic accumulation (17). The data presented here reveal that the requirement for PI3K is likely mediated by its effects on Akt.

The present study also indicates that Rh-L2A blocks epithelial cell differentiation. Previous work from our laboratory has shown a similar role for EBV LMP2A in both HFK and HaCaT cell lines (17, 22). Importantly, the data presented here clearly indicate that the loss of six of the twelve transmembrane domains does not affect the ability of Rh-L2A to activate the PI3K/Akt pathway or inhibit epithelial cell differentiation. This likely reflects the retention of the signaling motifs within the cytoplasmic N-terminus of both proteins. The N-terminus of both Rh-L2A and EBV LMP2A contain PY and ITAM motifs which are involved in many functions of the protein. The two proline rich PY motifs are known to interact with members of the Nedd4 family of ubiquitin ligases such as AIP4 and Itch in B cells (10). Through the ITAM motif the PI3K/Akt pathway is activated and associates with Syk kinase in B cells (8, 24). Additionally, the ITAM and PY motifs are involved in

PI3K/Akt and β-catenin signaling in epithelial cells (17, 23). The data presented here confirm that these properties of LMP2A require the motifs at the N-terminus but do not require the full complement of transmembrane domains. The transmembrane domains are thought to contribute to oligomerization and constitutive signaling. Previously, the contribution of the LMP2 transmembrane domains to B-lymphocytes immortalization was assessed using genetically engineered EBV. Deletion of the last seven transmembrane or first five transmembrane spanning domains did not impair B-cell immortalization by EBV (12, 13). The data presented here indicate that six transmembrane domains are also sufficient for the effects of Rh-L2A in epithelial cells.

Previous work from our laboratory has shown activated Akt and nuclear accumulation of β-catenin in NPC clinical samples and NPC xenografts (16). Infection of epithelial cells by the rhesus LCV has also been detected in immunosuppressed macaques (11). SIV infected, immunosuppressed LCV infected macaques also develop B cell lymphomas and oral lesions resembling oral hairy leukoplakia (9, 11). The data presented here suggest that Rh-L2A may also contribute to these pathologies.

In summary, these data indicate Rh-L2A can phosphorylate and activate Akt, phosphorylate and inactivate GSK3β, enable accumulation and nuclear translocation of β-catenin, and inhibit differentiation in epithelial cells. The activation of Akt is critical for the nuclear accumulation and translocation of β-catenin but is not required for the inhibition of differentiation by of Rh-L2A and Rh-L2AΔC. Importantly, this study also reveals that at least six transmembrane domains that are deleted in Rh-L2AΔC are not required for these properties. These data prove analogous properties of EBV LMP2A and Rh-L2A and further validate the rhesus macaque model for the study of EBV pathogenesis.

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CHAPTER THREE

EBV Latent membrane protein 2A activates MMP-2, -7, and -13 in epithelial cells

Catherine A. Siler and Nancy Raab-Traub

ABSTRACT

Epstein-Barr virus (EBV) is closely associated with several cancers including the highly metastatic nasopharyngeal carcinoma (NPC). A critical step in tumor metastasis is the degradation of the basement membrane and extracellular matrix which is mediated by a group of proteins known as matrix metalloproteinases (MMPs). This study shows that EBV LMP2A, which is expressed in NPC, increases the expression and activity of MMP-2, MMP-7, and MMP-13 in human epithelial cells. The inhibition of the PI3K/Akt pathway led to reduced RNA levels and decreased activity of both MMP-7 and MMP-13, but not MMP-2. In contrast, the inhibition of the MAPK pathway led to decreased RNA levels and activity of MMP-2, but had no effect on MMP-7 or MMP-13. β-catenin bound the respective Tcf and Lef sites in the MMP-7 and MMP-13 promoters, but did not bind the MMP-2 promoter. These data indicate that EBV LMP2A can activate several MMPs through the activation of both the PI3K/Akt and MAPK pathways and may contribute to NPC invasiveness and metastasis through the induction of MMP-2, -7, and -13 transcription and enzymatic activity.

INTRODUCTION

Epstein-Barr virus (EBV), a γ-herpesvirus, is a ubiquitous pathogen that infects greater than 90% of the adult human population. EBV is a well established tumor virus as it is causally associated with several types of cancer including Hodgkin lymphoma, endemic Burkitt's lymphoma, and nasopharyngeal carcinoma (NPC). EBV is strongly associated with NPC as it is consistently detected in episomal form in tumors (12). Specific viral gene products expressed in NPC include EBNA1, LMP1, and LMP2A. LMP2A is often co-expressed with LMP1 and transcription of both genes is regularly detected in NPC. Additionally, high titers of antibodies against LMP2A have been detected in NPC patients, which suggest its expression at the protein level (6).

EBV LMP2A has previously been shown to have striking properties in epithelial cells where its expression in the human keratinocyte cell line, HaCaT, induced PI3K activation and the subsequent phosphorylation and activation of Akt (13). In addition, HaCaT cells expressing EBV LMP2A had impaired differentiation when grown in organotypic raft cultures. In human telomerase immortalized human foreskin keratinocytes, HFK cells, LMP2A expression activated both the PI3K/Akt signaling and Wnt/β-catenin signaling pathways. The activation of these pathways led to the subsequent phosphorylation and inactivation of the Akt target GSK3β. The effects on these pathways also increased the levels and induced the nuclear translocation of β-catenin resulting in the activation of TCF mediated transcription (8). In HFK cells, EBV LMP2A also inhibited epithelial cell differentiation in assays where differentiation was induced by suspension in methylcellulose. In these assays, involucrin and keratinocyte transglutaminase increase during differentiation and these increases were blocked by EBV LMP2A (9).

NPC is a highly metastatic and invasive tumor associated with EBV. Although NPC ranges from well-differentiated keratinizing squamous cell carcinoma to undifferentiated non-kertatinizing carcinoma, the undifferentiated variety is consistently associated with EBV. Critical steps in tumor invasion and metastasis are the degradation of the basement membrane (BM) and extracellular matrix (ECM). Numerous proteolytic enzymes degrade components of the BM and ECM, including matrix metalloproteinases (MMPs) (10). MMPs are a family of zinc-dependent proteinases that consists of 23 members in humans and taken together, can degrade all components of the ECM. MMPs are translated as zymogens and contain a signal sequence targeting them for secretion. MMPs are classified into five-subgroups, based on their substrate specificity. These groups are the collagenases (MMP-1, -8, -13, -18), gelatinases (MMP-2, -9), stromelysins (MMP-3, -10, -11), matrilysins (MMP-7, -26), and membrane type MMPs (MMPs 14-17 and MMPs 24-25) (16). Increased MMP activity has been demonstrated for several herpes viruses including EBV and KSHV. One study in epithelial cells showed that MMP-9 was induced by the EBV oncoprotein LMP1, while another demonstrated significant correlation between MMP-9 and LMP1 in NPC samples, and this increased expression of MMP-9 was associated with lymph node metastasis (4, 15). For KSHV, it was shown that the K1 protein, the KSHV homolog of LMP1, induced MMP-9 expression and activity in endothelial cells (17).

To determine the effects of LMP2A expression in epithelial cells on MMP expression and activity, LMP2A was expressed in immortalized human foreskin keratinocytes (HFK). In this system, LMP2A expression increased the expression of MMP-2, -7, and -13. This increased expression correlated with increased activity as determined by gelatin zymography. In order to delineate which signal transduction pathways were involved in MMP expression

and activity, cells expressing LMP2A were treated with several chemical inhibitors of the PI3K/Akt and MAPK pathways. The inhibition of PI3K/Akt pathway led to decreased expression and activity of MMP-7 and MMP-13, but not MMP-2. Conversely, the inhibition of the MAPK pathway led to decreased expression and activity of MMP-2, but not MMP-7 or MMP-13. ChIP analysis revealed β-catenin bound the MMP-7 and MMP-13 promoters via their respective Tcf/Lef sites but did not bind the MMP-2 promoter. These data indicate that LMP2A activates several MMPs via two distinct signal transduction pathways. This activation suggests LMP2A plays a critical role in the development of EBV associated nasopharyngeal carcinoma.

RESULTS

LMP2A expression in HFK cells increases MMP gene and protein expression. Although previous work has shown LMP1 and BZLF1 to increase MMP-9 expression in C33A epithelial cells (15, 21), no other publications to date have investigated the potential role of LMP2A in the induction of MMP expression. To determine whether LMP2A expression in HFK cells increased levels of MMPs, we first generated HFK cells stably expressing HA-tagged LMP2A in the pBabe vector or vector alone. Western blot analysis confirmed the expression of LMP2A (Figure 1). Next we performed microarray analysis to determine differences in MMP levels of LMP2A expressing cells compared to cells expressing vector alone. As shown in Table 1, we observed approximately a four-fold increase in the levels of MMP-2, -7, and -13 in the LMP2A expressing cells compared to vector alone.

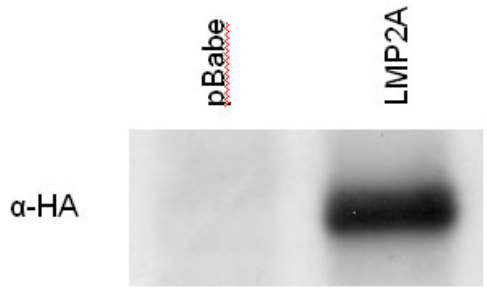

Table 1: MMP expression in LMP2A cells

Name (Accession number)	Fold increase
MMP-2 (NM_004530)	4.477456
MMP-7 (NM_002423)	4.481689
MMP-13 (NM_002427)	4.0804

Figure 1 and Table 1: Expression of EBV LMP2A in human foreskin keratinocytes. HFK cells were transduced with the pBabe (vector) retrovirus alone or expressing HA-tagged LMP2A. Transduced cells were placed under puromycin selection and expression was determined by Western blot using an anti-HA antibody.

To confirm the results observed in the array, we performed quantitative, real-time RT-PCR. As depicted in Figure 2A, MMP-2, -7, and -13 levels were increased in the LMP2A expressing HFK cells compared to vector alone. RNA levels for each MMP were increased approximately four-fold, which correlates with the data obtained from the microarray analysis. In order to determine increased protein levels of each MMP, we performed Western blot analysis on TCA precipitated supernatants from both pBabe and pBabe-LMP2A expressing cells (Figure 2B). In the TCA precipitated supernatants, all three MMPs were detected in the pBabe-LMP2A supernatants. These results indicate that EBV LMP2A induces MMP expression and secretion in epithelial cells.

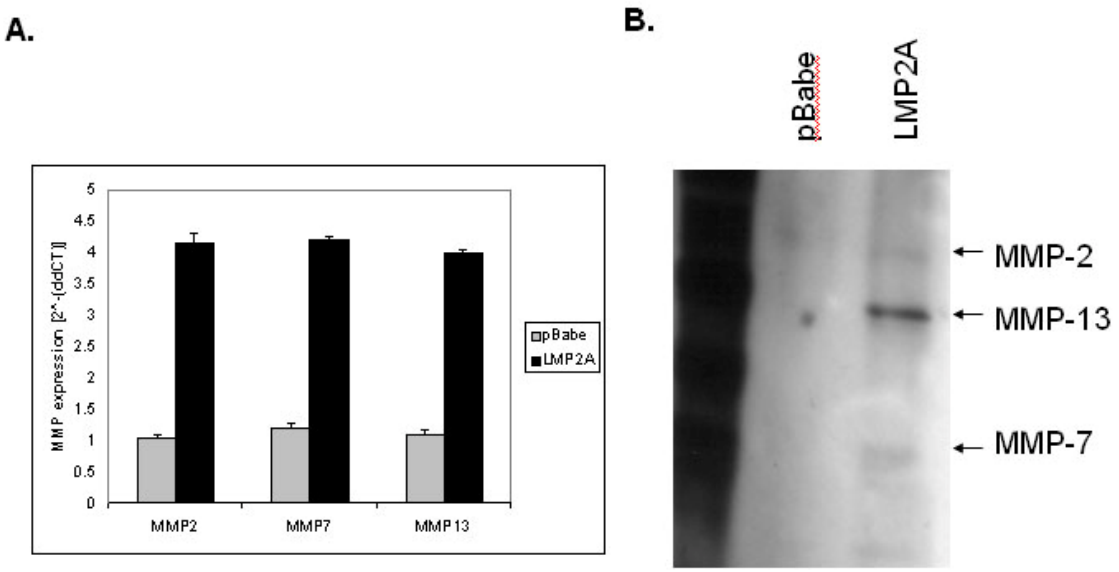

Figure 2: MMP RNA levels and protein secretion are increased in HFK-LMP2A cells. (A) Graph of qRT-PCR results showing increased MMP RNA in LMP2A expressing cells. Graph represents the results of four independent experiments performed in triplicate. Error bars represent standard deviation. (B) TCA precipitation of HFK supernatants. Supernatants from HFK-pBabe and LMP2A cells were treated with TCA and precipitate was subjected to Western blot analysis for the presence of MMP-2, -7, and -13.

LMP2A induces MMP activity in HFK cells.

MMPs are typically translated as an inactive enzyme (proMMP) and contain a signal sequence for targeting to the secretory vesicles. To determine the gelatinolytic activity of MMP-2, -7, and -13 in HFK cells expressing LMP2A, we performed gelatin zymography on supernatants from HFK cells expressing either LMP2A or vector alone. As seen in Figure 3, clear bands appear at the appropriate sizes for MMP-2, -7, and -13 (72kDa, 20kDa, and 48kDa respectively), in the lane contained the LMP2A supernatants indicating that each MMP is able to digest the gelatin in the gel. These results indicate that MMP-2, -7, and -13 are active in LMP2A expressing cellular supernatants.

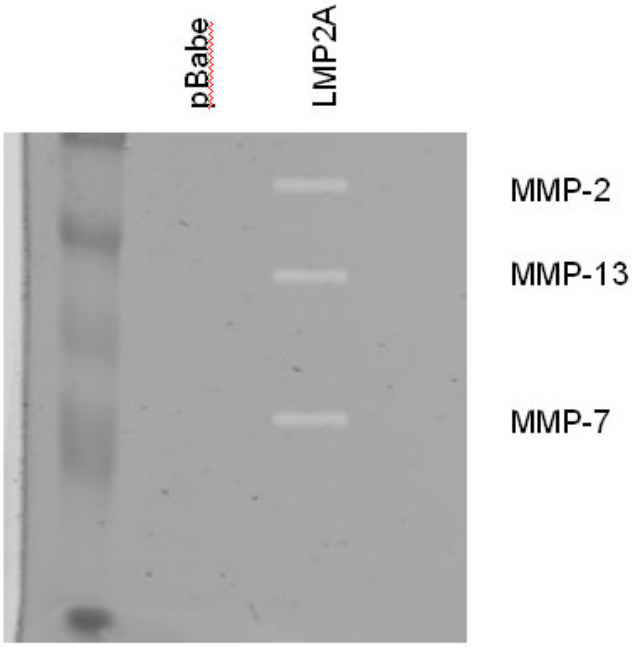

Figure 3: MMP activity is induced in HFK-LMP2A cells. Supernatants from HFK-pBabe and LMP2A cells were used to perform gelatin zymography. Clear band indicates the digestion of gelatin by an active MMP.

The PI3K/Akt pathway regulates MMP-7 and MMP-13 activity.

MMP-7 and MMP-13 are regulated by the binding of β-catenin to Tcf/Lef sites in their promoters (1, 2, 22). Since EBV LMP2A has critical effects on β-catenin nuclear localization in epithelial cells and this effect is regulated by PI3K/Akt (8), we investigated the role of the PI3K/Akt pathway in the activation of MMPs. To determine the role of PI3K/Akt in MMP expression in LMP2A expressing HFK cells, we treated cells with a PI3K inhibitor, LY294002 (LY), and an Akt inhibitor, triciribine (TCN) and then performed qRT-PCR to determine the effects of each inhibitor on MMP RNA levels. As depicted in Figure 4A, the LY and TCN inhibitors reduced the levels of MMP-7 and MMP-13 RNA in LMP2A expressing cells to nearly control cell levels. However, MMP-2 RNA levels were not affected by either the LY or TCN inhibitors. Next we performed gelatin zymography to determine the effects of the inhibitors on LMP2A mediated gelatinolytic activity. Both the LY and TCN inhibitors abolished MMP-7 and MMP-13 activity while not affecting MMP-2 activity (Figure 4B). To show the efficacy of the LY and TCN inhibitor treatments, we performed Western blot analysis to determine the phosphoylation status of Akt. As depicted in Figure 4C, phosphorylated Akt is detected in the untreated cells, but not seen in the inhibitor treated samples. These data indicate that the PI3K/Akt pathway is critical for MMP-7 and MMP-13 activity but not involved in the regulation of MMP-2 activity.

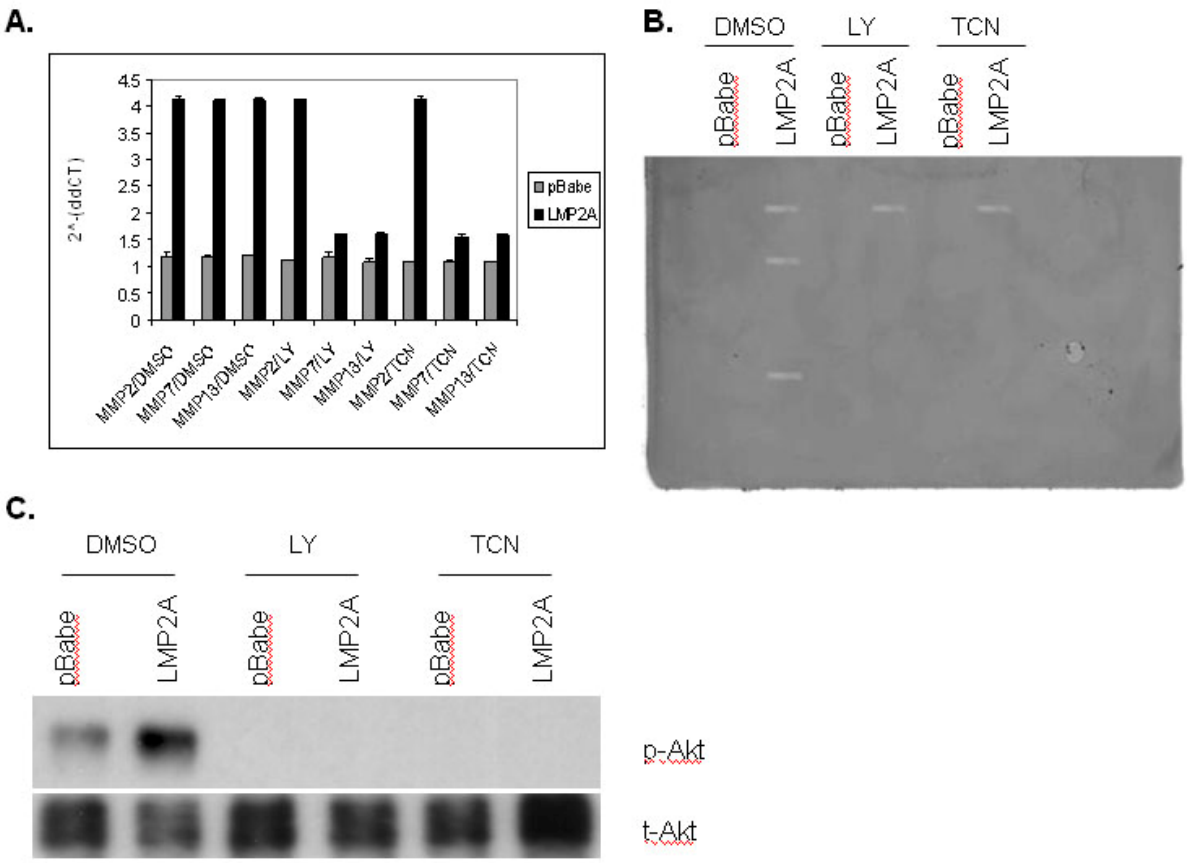

Figure 4: MMP-7 and MMP-13 RNA levels and activity are regulated by PI3K/Akt. (A) Graph of qRT-PCR results showing decreased MMP-7 and MMP-13 RNA levels in LMP2A expressing cells treated with PI3K/Akt inhibitors. Graph represents the results of three independent experiments performed in triplicate. Error bars represent standard deviation. (B) Supernatants from HFK-pBabe and LMP2A cells treated with PI3K/Akt inhibitors were used to perform gelatin zymography. Clear band indicates the digestion of gelatin by an active MMP. (C) Western blot of HFK-pBabe and LMP2A cells treated with PI3K/Akt inhibitors. Absence of p-Akt band in inhibitor lanes indicates the functionality of the inhibitor. Total Akt served as a loading control.

MMP-2 is regulated by MAPK in HFK-LMP2A cells.

Since several previous reports have implicated the MAPK target Erk in the regulation of MMP-2 (5, 7), we decided to pursue the role of the pathway in LMP2A mediated induction of MMP-2. First, we performed qRT-PCR on HFK cells expressing both LMP2A and vector alone treated with PD98059 (PD), a MAPK inhibitor or DMSO as a vehicle control to determine if inhibition of Erk would affect RNA levels of MMP-2. As seen in Figure 5A, treatment with PD reduced MMP-2 RNA levels in LMP2A cells to nearly vector alone levels. Of note, there was no effect on MMP-7 or MMP-13 RNA levels in cells treated with PD. Next we performed gelatin zymography on supernatants treated with PD or DMSO to determine changes in MMP-2 activity. As seen in Figure 5B, MMP-2 activity was ablated in PD treated LMP2A supernatants whereas no effect on MMP-7 or MMP-13 activity was observed. To show the efficacy of the PD inhibitor treatment, we performed Western blot analysis to determine the phosphoylation status of Erk. As depicted in Figure 5C, phosphorylated Erk is detected in the untreated cells, but not seen in the inhibitor treated samples. These data indicate that the MAPK pathway is important for MMP-2 regulation, but is not involved in the regulation of MMP-7 or MMP-13.

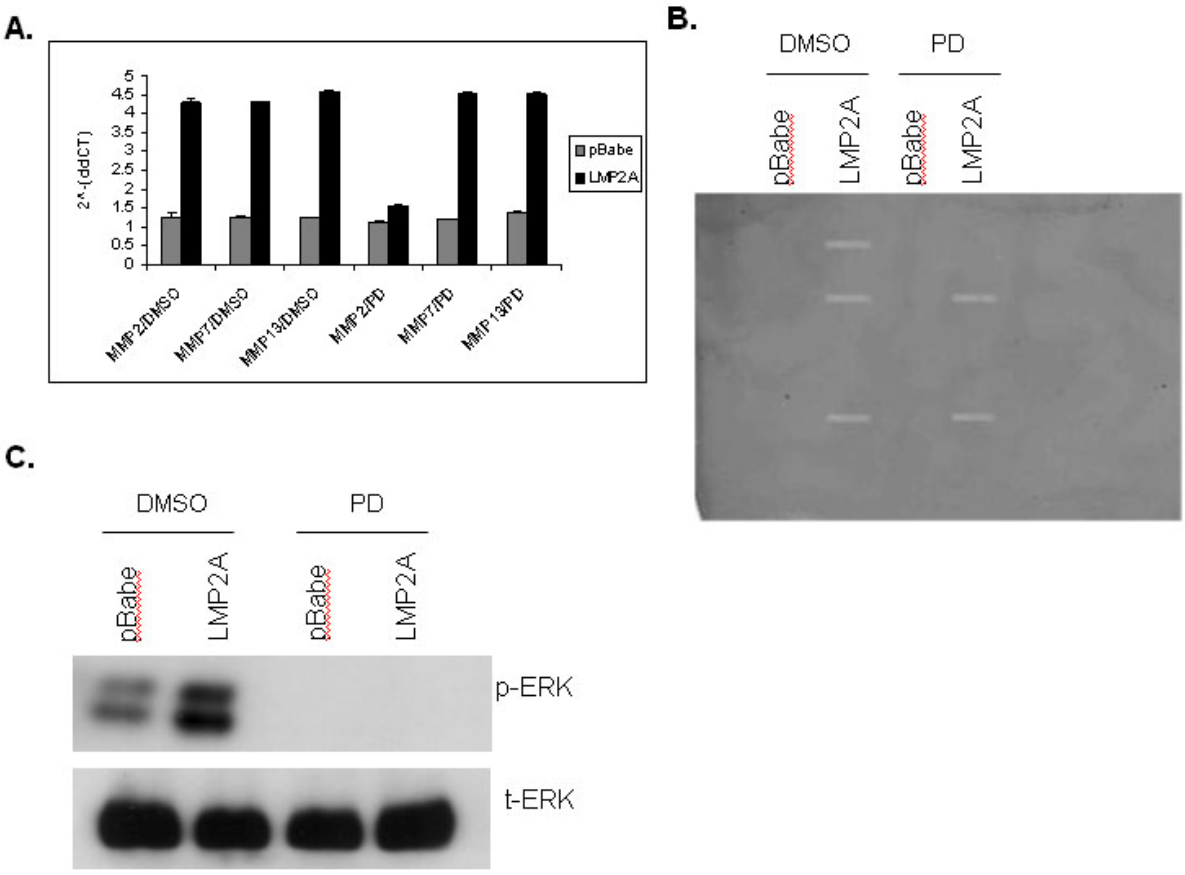

Figure 5: MMP-2 RNA levels and activity are regulated by MAPK. (A) Graph of qRT-PCR results showing decreased MMP-2 RNA levels in LMP2A expressing cells treated with a MAPK inhibitor. Graph represents the results of three independent experiments performed in triplicate. Error bars represent standard deviation. (B) Supernatants from HFK-pBabe and LMP2A cells treated with a MAPK inhibitor were used to perform gelatin zymography. Clear band indicates the digestion of gelatin by an active MMP. (C) Western blot of HFK-pBabe and LMP2A cells treated with MAPK inhibitor. Absence of p-Erk band in inhibitor lanes indicates the functionality of the inhibitor. Total Erk served as a loading control.

β-catenin immunoprecipitates on the MMP-7 and MMP-13 promoters. Previous studies indicated that β-catenin can regulate MMP-7 and MMP-13 at the transcriptional level by binding either a Tcf-4 or Lef-1 site in their respective promoters (1, 2, 22). Since LMP2A is known to induce β-catenin nuclear translocation in epithelial cells, we hypothesized β-catenin would strongly bind the MMP-7 and MMP-13 promoters in LMP2A expressing cells. To validate this hypothesis, HFK cells stably expressing LMP2A or pBabe vector alone were analyzed by ChIP using primers flanking the Tcf-4 site in the MMP-7 promoter and primers flanking the Lef-1 site in the MMP-13 promoter. The precipitation with β-catenin strongly detected the Tcf-4 and Lef-1 sites in the MMP-7 and MMP-13 promoters in both pBabe and LMP2A cells with the stronger detection of each site visible in LMP2A expressing cells (Figure 6A). In addition, β-catenin binding to the MMP-2 promoter was not detected. In order to evaluate the results of the ChIP, we performed qRT-PCR on the ChIP products. As depicted in Figure 6B, the binding of β-catenin to the MMP-7 and MMP-13 promoters was increased approximately two fold in LMP2A expressing cells, whereas MMP-2 promoter binding was not detected. Of note, previous work from our laboratory has shown nuclear β-catenin levels to be increased approximately two-fold in LMP2A expressing cells (8). These data indicate that LMP2A regulates MMP-7 and MMP-13 by increasing nuclear β-catenin which subsequently interacts with Tcf and Lef sites on the MMP promoters inducing transcription.

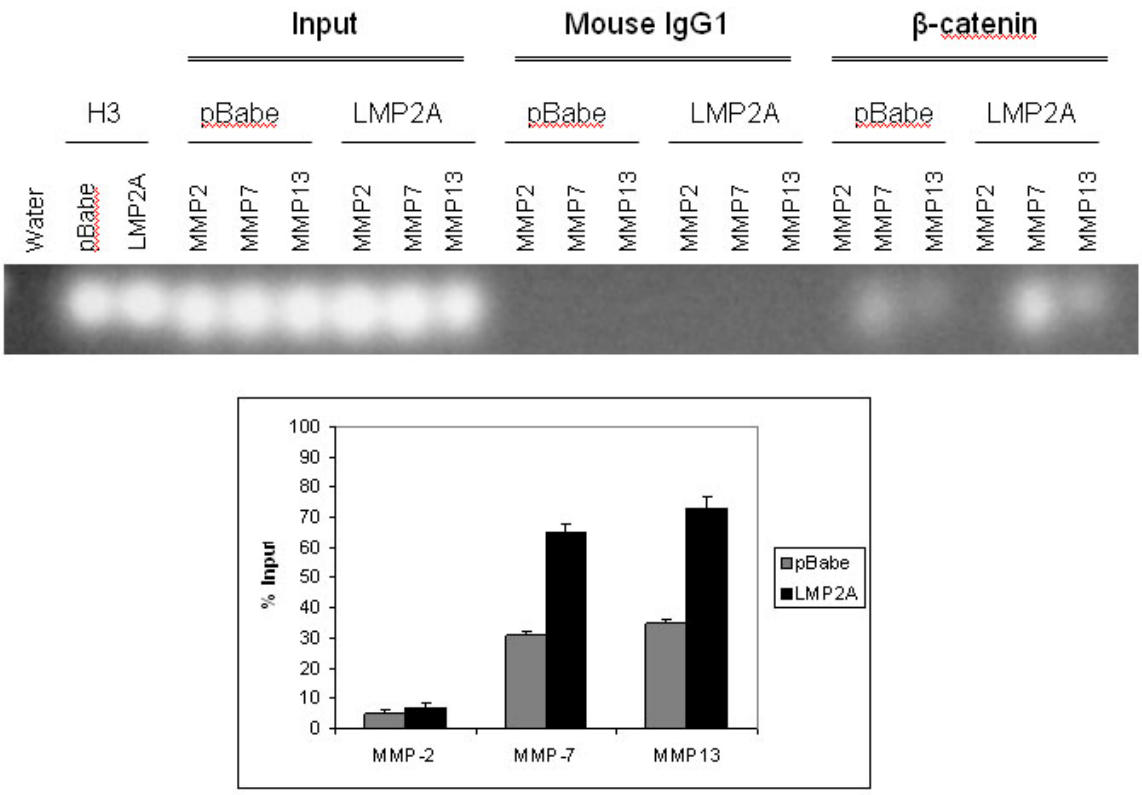

Figure 6: LMP2A increases binding of β-catenin to MMP-7 and MMP-13 promoters. (A) ChIP analysis using mouse IgG1 (isotype control), H3 histone (positive control) or β-catenin in HFK-pBabe and HFK-LMP2A expressing cells. Precipitated complexes were subjected to PCR with primer pairs specific to the Tcf4 and Lef1 sites in the MMP-7 and MMP-13 promoters, respectively. PCR was also performed on chromatin input (lanes 4-9). (B) Graphical depiction of qRT-PCR results on ChIP products. Real-time quantitative PCR was performed on precipitated DNA using primers specific for the TCF/LEF promoter regions of the MMP-7 and MMP-13 gene and for the promoter region of the MMP-2 gene. Values are normalized against the input DNA and are represented as the percentage of input for each given sample. Each value represents the mean of three independent measurements of the precipitated DNA.

DISCUSSION

This study reveals that LMP2A increases the expression and activity of MMP-2, -7, and -13 in epithelial cells. In LMP2A expressing HFK cells, the expression and activity of both MMP-7 and MMP-13, but not MMP-2, were directly related to the activation of the PI3K/Akt pathway. The expression and activity of MMP-2 was directly related to the activation of the MAPK pathway and the inhibition of this pathway had no effect on the levels or activity of MMP-7 or MMP-13.

Previous work from our laboratory has shown LMP2A to have profound effects on β-catenin localization in epithelial cells (8). In LMP2A expressing epithelial cells, β-catenin accumulates in the cytoplasm and subsequently translocates to the nucleus in an Akt dependent manner. Once in the nucleus, β-catenin can bind members of the T cell factor (TCF)/lymphoid enhancing factor (LEF) family of transcription factors to induce target gene expression. With regards to MMP-7, β-catenin has been shown to regulate MMP-7 expression by binding a TCF-4 site in the promoter (1, 2). Additionally, β-catenin can bind a LEF-1 site in the MMP-13 promoter resulting in activation (22). Therefore, it is reasonable that LMP2A would increase both the levels and activity of MMP-7 and MMP-13.

In the case of MMP-2, the activation of ERK is critical for MMP-2 function in brain cancer metastasis (7). Furthermore, the phosphorylation of ERK is necessary for MMP-2 activation and subsequent cell invasion in fibrosarcoma cells (5). LMP2A has been shown to interact with ERK and increase ERK phosphorylation in B cells (11). It is not surprising LMP2A modulates MMP-2 activity as MMP-2 is consistently associated with NPC (18, 19) and MMP-2 activity is not related to LMP1 expression (20).

Although previous studies have shown roles for other EBV proteins such as LMP1 and BZLF1 in the induction of MMPs in epithelial cells and NPC (14, 15, 20, 21), this is the first study to date that implicates LMP2A in the activation of MMPs in epithelial cells. Since NPC is a highly metastatic tumor that regularly expresses LMP2A, the activation of MMP-2, -7, and -13 may be a critical factor contributing to pathogenesis.

MATERIALS AND METHODS

Cell culture and retrovirus production. HFK cells (3) were maintained in keratinocyte serum free medium (K-SFM, Gibco) supplemented with epidermal growth factor (.2ng/ml), bovine pituitary extract (30μg/ml), and 1% antibiotic-antimycotic solution (Sigma) and grown in a humidified incubator at 37°C and 5% CO_2. Stable cell lines were generated using recombinant retrovirus expressing either the pBabe vector or pBabe vector subcloned with hemagglutinin (HA) tagged LMP2A as previously described (13) and used to transduce HFK cells. A stable pool of HFK cells expressing either pBabe or pBabe-LMP2A was selected using K-SFM media containing puromycin (Sigma) at .5μg/ml.

Western blot analysis and antibodies. Whole cell lysates were prepared using Nonidet P-40 (NP-40) lysis buffer containing 50mM Tris-HCl, 150mM NaCl, 2mM EDTA, 10% glycerol, 1% NP-40, 1mM sodium vanadate (Na_3VO_4), 0.4mM phenylmethylsulfonyl fluoride (PMSF), and protease and phosphatase cocktails (Sigma) at 1:100. Protein concentrations were determined using the Bio-Rad DC assay system according to manufacturer's instructions. Lysates were boiled in a protein loading solution containing SDS and β-mercaptoethanol for five minutes and subjected to SDS-10% polyacrylamide gel electrophoresis. Proteins were transferred to an Optitran nitrocellulose membrane (Schleicher & Schuell) and subjected to Western blot analysis. Antibodies used include anti-HA from Covance, anti-MMP-2 and MMP-13 from Calbiochem, and anti-MMP-7 from Santa Cruz. Horseradish peroxidase secondary antibodies (Amersham) and SuperSignal West Pico System (Pierce) were used to detect antibody bound proteins.

TCA protein precipitation. Supernatants from cells expressing pBabe or pBabe-LMP2A were harvested and 100% TCA was added at a ratio of 1 volume of TCA to 4 volumes of sample. Samples were then incubated for 10 minutes at 4°C and then spun at high speed to pellet the protein. Supernatant was then removed and pellet washed with cold acetone three times. Pellet was then dried to remove any excess acetone before pellet was resuspended in 3X protein loading solution for running on SDS-PAGE.

Gelatin zymography. Supernatants from cells expressing either pBabe or pBabe-LMP2A were fixed with SDS loading buffer [50mM Tris-HCl (pH 6.8), 10% glycerol, 1% SDS, and 0.01% bromophenol blue] followed by a 20 minute incubation at 37°C. Fixed supernatants were then electrophoresed on a 10% SDS-PAGE gel containing gelatin (Sigma) at a final concentration of 0.1%. Gel was then rinsed with PBS containing 2.5% Triton X-100 for two hours. Again, the gel was rinsed with water to remove the Triton and incubated overnight at 37°C in a solution containing 50mM Tris-HCl (pH 7.6), 150mM NaCl, 10mM $CaCl_2$, and 0.02% NaN_3. The gel was then stained in 0.1% Coomassie blue R250 (Sigma) dissolved in 40% methanol and 10% acetic acid. To destain the gel, a solution containing 40% methanol and 10% acetic acid was used. The MMPs are identified as clear bands against the dark background of stained gel. Gel was scanned using a Typhoon 9400 scanner.

Inhibitor experiments. For qRT-PCR and gelatin zymography experiments, HFK cells were plated in 100mm dishes containing keratinocyte media. Within 24h post-seed, triciribine (TCN), LY294002, and PD98059 (Calbiochem) were added to the media at a concentration of 5μM and 10μM respectively. Control plates were treated with an equivalent

volume of DMSO (vehicle control). After 24-36h of inhibitor treatment, cells or supernatants were harvested for RNA isolation, gelatin zymography, or Western blot analysis.

Microarray analysis. Total RNA was extracted from HFK cells expressing LMP2A or vector alone using a RNeasy kit (Qiagen) according to manufacturer's instructions. RNA concentration was determined by NanoDrop and the quality of RNA checked by using an Agilent LabChip Bioanalyzer to ensure high-quality RNA for successful hybridization. Human 1A (V2) chips containing oligonucleotides representing 22K human genes were obtained from Agilent Technologies. Total RNA samples were amplified using T7 primers to make cRNA. The cRNA samples were incorporated with two different Cy dyes (Cy3 and Cy5) and hybridized with the microarray. Slides were scanned using an Axon 4000 scanner and images processed with GenePix software to generate raw data files for uploading to the University of North Carolina Microarray Database for normalization. Clustering analysis was performed within the database and data sets were combined in Microsoft Excel for sorting.

Quantitative real-time PCR. RNA from HFK cells expressing pBabe or pBabe-LMP2A was extracted using a RNeasy kit (Qiagen) according to manufacturer's instructions. Quantitative PCR was performed using QuantiTect SYBR Green kit (Qiagen) with the following specific primers: human MMP-7 (forward, 5´-GACATCATGATTGGCTTTGC-3´; reverse, 5´-GTGAGCATCTCCTCCGAGAC-3´), human MMP-13 (forward, 5´-AGCACCCTTCTCATGACCTC-3´; reverse, 5´-TCTTTTGGAAGACCCAGTTCA-3´),

human MMP-2 (forward, 5´-ATGGCAAGTACGGCTTCTGT-3´; reverse, 5´-CATAGGATGTGCCCTGGAAG-3´), and as an internal control, actin (forward, 5´-CCCAGCACAATGAAGATCAA-3´; reverse, 5´-ACATCTGCTGGAAGGTGGAC-3´). Amplification of target sequences was detected with an ABI 7900HT detection system (Applied Biosystems) and analyzed with SDS 2.0 software (Applied Biosystems). PCR conditions were as follows: One cycle for 30 minutes at 50°C for reverse transcription, one cycle for 10 minutes at 95°C for initial activation, followed by 40 cycles of 95°C for 15 seconds, 55°C for 15 seconds, and 72°C for 30 seconds followed by a dissociation curve cycle. Raw data was normalized to actin according to the Applied Biosystems manual.

Chromatin Immunoprecipitations. ChIPs were performed using the EZ ChIP kit (Upstate) according to manufacturer's instructions. In brief, 1 x 10^7 cells in 10ml of media were cross-linked in 1% formaldehyde at room temperature for 15 minutes followed by quenching with glycine. Cells were then washed in PBS and then harvested in PBS containing protease inhibitor, spun, and cell pellet was then lysed in SDS lysis buffer containing protease inhibitor. Lysates were sonicated, clarified, and pre-cleared with Protein G agarose. Supernatants were incubated without antibody or with β-catenin antibody and nutated overnight at 4°C. Lysates were immunoprecipitated with Protein G agarose for one hour and beads subsequently washed with a series of buffers. DNA/protein was eluted from the beads using elution buffer. Cross-linking was reversed at 65°C overnight in the presence of NaCl. Samples were then treated with RNase A for 30 minutes at 37°C and Proteinase K for 2 hours at 45°C. Sample DNA was purified using a nucleotide removal kit (Qiagen) as directed by the manufacturer. The Tcf4 site on the MMP-7 promoter was PCR amplified

using the primer set forward, 5´-AGCCCAAAATGGACTTCCAA-3´; reverse, 5´-CATCAAATGGGTAGGAGTCC-3´. The Lef1 site on the MMP-13 promoter was amplified with the primers forward, 5´-CATGCCAACAAATTCCATATTG-3´; reverse, 5´-CCAGCCACGCATAGTCATATAG-3´. The primer set used to amplify the MMP-2 promoter was forward, 5´-ACTTCCTCAGGCGGTGGCTG-3´; reverse, 5´-GTTGGAGCCTGCTCCGCGGC-3´. PCRs were performed using Hot Start Taq (Qiagen) under standard conditions.

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CHAPTER FOUR

General Conclusions

GENERAL CONCLUSIONS

Although the vast majority of EBV infections are asymptomatic, the ubiquitous nature of the virus allows for a significant number of individuals to develop EBV-associated diseases and malignancies. The substantial morbidity and mortality associated with EBV infection necessitates further study of the virus and its pathogenesis to properly develop and design effective therapies to combat this insidious infectious agent.

The two principle cells types infected by EBV are B lymphocytes and epithelial cells. Both lytic and latent phases of infection occur in B lymphocytes. Notably, this latent reservoir in the B cell compartment enables viral survival and persistence in the host. Epithelial cell infection has largely been associated with the lytic phase and production of progeny enabling the horizontal spread to the next host via salivary shedding or for infection of trafficking B lymphocytes in the area. However, EBV-associated malignancies of epithelial origin are primarily associated with latent infection. EBV infected malignant cells are clonal with respect to the virus, the viral genome is episomal in form, and viral gene expression is normally restricted to the latent genes (24).

The purpose of the current study was to further evaluate the role of EBV LMP2A and the rhesus EBV LMP2A homolog in epithelial cells. In contrast to epithelial cells, the vast majority of studies investigating the consequences of LMP2A expression have focused on B lymphocytes. In B lymphocytes, LMP2A is responsible for maintaining latency (15-18). LMP2A blocks BCR activation and signaling by absorbing BCR-associated signaling molecules and manipulating their turnover in addition to blocking BCR translocation into lipid rafts which prevents the initiation of signaling (6, 12, 31). Additionally, studies with

LMP2A transgenic mice revealed that LMP2A transmits survival signals in B lymphocytes, alters global B lymphocyte transcription, and allows B lymphocytes to circumvent normal developmental checkpoints to colonize the periphery (2, 3, 23). Clearly, much effort has been invested in elucidating the function of LMP2A in B lymphocytes. However, the role of LMP2A expression in epithelial cells is only beginning to unfold.

Several previous studies from our lab focused on the role of LMP2A expression in epithelial cells. One study in the HaCaT human keratinocyte cell line showed that LMP2A expression activated the PI3K/Akt signaling pathway, conferred anchorage independent cell growth, and tumorigenicity in nude mice (25). Studies continued using more "normal" telomerase immortalized human foreskin keratinocytes, known as HFK cells. Studies in HFK cells revealed LMP2A activated PI3K/Akt, as observed in HaCaT cells. The same study showed two Akt targets, GSK3β and FKHR were phosphorylated and subsequently inactivated in LMP2A expressing HFK cells. LMP2A expression in HFK cells resulted in cytoplasmic accumulation of β-catenin, PI3K dependent nuclear translocation of β-catenin, and activation of a TCF responsive reporter (20). Additional study identified key domains in LMP2A that are important for its effects on PI3K/Akt and β-catenin signaling. The ITAM motif was critical for Akt activation in epithelial cells and both the ITAM and PY motifs contributed to the effects of LMP2A on β-catenin signaling (21). In regards to epithelial cell biology, LMP2A was shown to inhibit epithelial cell differentiation. We have continued these studies of EBV LMP2A as well as initiated study of the rhesus LMP2A homolog in HFK cells.

The work presented in chapter two revealed that rhesus LMP2A expression in HFK cells activates PI3K/Akt, phosphorylates and inactivates the Akt target GSK3β, and allows

for the accumulation and nuclear translocation of β-catenin in an Akt dependent manner. Additionally, rhesus LMP2A was able to inhibit differentiation in epithelial cells. Notably, a mutant form of rhesus LMP2A lacking the last six transmembrane domains of the protein functioned similarly to wild-type rhesus LMP2A. These results indicate that the rhesus LMP2A homolog functions in a similar fashion to EBV LMP2A and underscores the suitability of the rhesus macaque model for the study of EBV pathogenesis.

Data presented in chapter three also revealed an important effect of LMP2A on epithelial cell biology and potential role in NPC metastasis. Although other EBV proteins such as LMP1 and BZLF1 have been shown to increase MMP activity, no previously published report indicated a role for LMP2A in increased MMP activity (11, 27, 28, 33, 34). Experiments indicated that LMP2A induced MMP-2, -7, and -13 expression and activity in HFK cells as indicated by microarray, qRT-PCR, and gelatin zymography. Additionally, MMP-2 activity was dependent on MAPK activation whereas MMP-7 and MMP-13 activity was dependent on PI3K/Akt activation. Notably, MMP-7 and MMP-13 are known TCF/LEF targets. ChIP results indicated that β-catenin did bind the TCF/LEF sites in the MMP-7 and MMP-13 promoters. These findings support previous results showing LMP2A expression in HFK cells activated a TCF responsive promoter (20). These effects of LMP2A in epithelial cells may provide a mechanism by which EBV-associated NPC metastasizes to the lymph nodes.

Overall, these data contribute to the expanding knowledge of pathogenesis mechanisms employed by EBV in the development of disease and malignancy. In epithelial cells, both EBV and LCV LMP2A can activate the PI3K/Akt and β-catenin pathways. The PI3K/Akt pathway is aberrantly activated in many types of cancers including lymphomas,

melanomas, and breast and prostate carcinomas. β-catenin is at the crux of the Wnt signaling pathway which is abnormally activated in over 90% of colon carcinomas as well as in an array of other malignancies. Aberrant activation of these pathways by LCV and EBV LMP2A allows cells to continually proliferate and survive, primed for transformation. Additionally, inhibition of epithelial cell differentiation by LCV LMP2A may further enable these processes to occur by maintaining a metabolically active host cell population. Notably, EBV and LCV are not the only viruses known to target the PI3K/Akt and β-catenin pathways. Another herpesvirus, KSHV and the human polyomavirus, JC virus have been shown to activate these pathways (8, 9, 19, 29). More recently, multiple studies have found that influenza A, poliovirus, human rhinovirus, hepatitis B, and rotavirus can all activate the PI3K/Akt pathway (1, 10, 13, 22, 26).

The upregulation of MMPs by EBV LMP2A is another potential mechanism by which EBV can enable tumorigenesis and metastasis. MMP overexpression has been associated with various cancers including colon, breast, bladder, and lung (5). In many instances this increased MMP expression is associated with an invasive and metastatic phenotype. As both LMP1 and LMP2A are both consistently expressed in NPC and both can activate MMPs, it is likely that these two proteins act additively or synergistically to enable NPC metastasis to secondary sites such as the lymph nodes. Of note, other viruses have been shown to activate MMPs. KSHV, a related herpesvirus, has been shown to activate MMP-9 in endothelial cells (30). Viruses such as dengue, human papilloma virus (HPV), and coxsackievirus B3 have also recently been shown to activate MMPs during infection (4, 14, 32).

In conclusion, these studies have identified key pathways targeted by LCV and EBV LMP2A in epithelial cells. LCV LMP2A activates the PI3K/Akt pathway and the Akt target, GSK3β, is phosphorylated and inactivated in LCV LMP2A expressing keratinocytes. Furthermore, LCV LMP2A expression in HFK cells results in the cytoplasmic accumulation of β-catenin with Akt dependent nuclear translocation, indicating activation of the β-catenin signaling pathway. LCV LMP2A was also able to inhibit differentiation in epithelial cells. Importantly, a mutant LCV LMP2A lacking six transmembrane domains was able to function similarly to full-length LCV LMP2A. EBV LMP2A was found to increase expression and activity of MMP-2, -7, and -13. The EBV LMP2A effects on MMP-2 were dependent on the MAPK pathway, whereas MMP-7 and MMP-13 activity were dependent on PI3K/Akt activation. These studies have contributed to our understanding of LCV and EBV biology in epithelial cells by providing validation for the rhesus macaque model in the study of EBV pathogenesis and insight into the mechanisms of EBV-associated diseases and malignancies.

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