The Epstein-Barr Virus Latent Membrane Protein 1 Regulates Gene Expression by Engaging Multiple Signaling Pathways

by

Che-Pei Kung

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in the partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Microbiology and Immunology

Chapel Hill

2009

Approved by:

Advisor: Nancy Raab-Traub, Ph.D.
Reader: Albert S. Baldwin, Ph.D.
Reader: Eng-Shang Huang, Ph.D.
Reader: Blossom Damania, Ph.D.
Reader: Dirk Dittmer, Ph.D.
ABSTRACT

Che-Pei Kung: The Epstein-Barr Virus Latent Membrane Protein 1 Regulates Gene Expression by Engaging Multiple Signaling Pathways
(Under the direction of Nancy Raab-Traub)

Epstein-Barr virus (EBV) is a ubiquitous human gammaherpesvirus that is associated with a variety of human malignancies of lymphoid and epithelial origins, such as post-transplant lymphoproliferative disorder (PTLD), Burkitt’s lymphoma (BL), Hodgkin disease (HL), nasopharyngeal carcinoma, and others. Latent membrane protein 1 (LMP1), which is expressed in latency type II and III, is considered the EBV oncoprotein due to its ability to transform rodent fibroblasts and its critical roles in EBV-mediated B cell transformation. Two major signaling domains of LMP1, carboxyl-terminal activating region (CTAR) 1 and 2, are responsible for the majority of LMP1-mediated signaling pathways. However, CTAR1 and CTAR2 induce distinctive signal transductions and only CTAR1 is essential for LMP1-mediated cell transformation. CTAR1 recruits TNFR-associated factors (TRAFs) to induce signaling pathways including PI3K/Akt, MEK/ERK, and complex NF-κB signaling. CTAR1 is also responsible for LMP1-regulated expression of cellular genes, such as epidermal growth factor receptor (EGFR).

In this study, multiple signaling pathways engaged by LMP1 through CTAR1 to mediate EGFR upregulation were evaluated. LMP1 has been shown to induce EGFR expression by activating transcriptional active complex p50-homodimer/Bcl-3. This study shows CTAR1
also induces the expression of Bcl-3 through activating transcriptional regulator of Bcl-3, STAT3, and the STAT3 activation is required for CTAR1’s complete activity to induce EGFR expression. Using mouse embryonic fibroblasts (MEF) defective for different NF-κB effectors and TRAFs, we identified that CTAR1-mediated EGFR upregulation is dependent on NIK but not canonical or noncanonical NF-κB pathways. However NIK alone is not sufficient to induce EGFR expression, indicating that additional pathways are also needed. Consistent with previous studies, TRAF2 and TRAF3 but not TRAF6 are required for CTAR1-mediated EGFR induction. TRAF2 potentially contributes to EGFR induction by regulating p50 activation, and this activation is not mediated through the canonical or noncanonical NF-κB pathways.

Both EGFR and STAT3 are activated by CTAR1 in a serum-independent manner, however, the CTAR1-activated EGFR can also activate STAT3 in response to EGF treatment and induce the expression of STAT3 target genes. CTAR1 was found to activate STAT3 through PKCδ, as inhibition of PKCδ by a chemical inhibitor reduced CTAR1-induced serine phosphorylation of STAT3 and Bcl-3 expression. These findings indicate that CTAR1 manipulates STAT3 signaling through multiple mechanisms. PKCδ was also shown to mediate CTAR1-induced ERK activation, and inhibition of PKCδ block CTAR1-mediated transformation of rodent fibroblast cells. These results suggest that CTAR1 induce EGFR expression by engaging multiple downstream signaling pathways that contribute to LMP1-mediated gene regulation.
To my parents and to Miin-Feng.
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Nancy Raab-Traub, for her guidance over the past five years. I couldn’t imagine having a more supportive and encouraging supervisor. I am confident to say that I made a great decision to join her lab, and I look forward to apply what I’ve learned from her to my future scientific career.

I would also like to thank my committee members, Dr. Blossom Damania, Dr. Dirk Dittmer, Dr. Albert Baldwin, and Dr. Eng-Shang Huang, for their suggestions and criticisms regarding my studies, as well as their support and time.

The Raab-Traub lab is a great place to work not only because of the science, but also because of the people. Betsy Edwards is the perfect lab manager. I have been spoiled by the fact that if there is anything I need, Betsy is always there to make it happen. She is also a great listener who you always want to tell your jokes. To Dr. Natalie Thornburg for guiding me during my rotation. To George Ward, Kat Bendt, Dr. David Everly, Dr. Bernardo Mainou, and Dr. Cathy Siler for making me feel welcome when I first joined the lab. To Stephanie Mazzucca for reminding me that it is possible to be forever young in spirit.

We have an outstanding group of postdocs from whom I have learned so much. Dr. Julie Fotheringham brings an unmatchable level of enthusiasm to the lab every day. She is also a great resource for scientific discussion and news around the “entertainment world”. Dr. Aron Marquitz is the expert of critical thinking. He shows me how to set a high standard for scientific research, and that is what I plan to follow for the rest of my career. My life in
the lab would not have been the same without his sense of humor and those man’s talks we’ve shared over the years, including virtual basketball worlds and the Duke-UNC rivalry. Dr. Kathy Shair is a great scholar. I have no doubt that she will have a phenomenal scientific career ahead of her and that’s what I also aspire to do because of her influence. The knowledge I learned from her, both for science and for life, will always be invaluable to me. The new member of the lab, Dr. David Meckes, has brought a new layer of expertise to an already excellent place. Our conversations as colleagues and desk-mates have generated new excitements which I enjoy a lot and hope to carry to my next destination.

I would like to thank the student service manager, Dixie Flannery, for making my life in graduate school so much easier. To the former director of IBMS program, Dr. Sharon Milgram, for taking a chance on me as a graduate student. To Dr. Darrel Stafford in the Department of Biology for offering me a job as a technician at UNC. To my mentor back in Taiwan, Dr. Hwan-You Chang, for leading me into the world of microbiology and encouraging me to explore the opportunity of studying abroad.

My family back in Taiwan, including my brother and my parents, is my rock. Leaving home so far away was the most difficult decision I have ever made in my life. Without their unconditional support, none of these would have been possible. For that, I am always in debt to them.

To my wife Miin-Feng. She was my dearest friend and partner before our journey in America, and now she is my only family on this continent. I can’t wait to move on to the next chapter of my life with her, knowing that she will always be there for me. Rain or shine.
# TABLE OF CONTENTS

LIST OF TABLES ................................................................................................................... x

LIST OF FIGURES .............................................................................................................. xi

LIST OF ABBREVIATIONS ............................................................................................... xiii

CHAPTER ONE: Introduction

Epstein-Barr virus ........................................................................................................ 2
The Life Cycle of EBV ................................................................................................. 3
Reverse Genetics with EBV Study .............................................................................. 6
Lytic Replication ........................................................................................................... 6
  Immediate Early Genes ......................................................................................... 7
  Early Genes .......................................................................................................... 8
  Late Genes ............................................................................................................. 9
Establishment of Latency ............................................................................................. 9
  LMP1 .................................................................................................................. 10
  LMP2A and LMP2B ........................................................................................... 14
  EBNA1 ............................................................................................................... 16
  EBNA2 ............................................................................................................... 16
  EBNA-LP ........................................................................................................... 17
  EBNA3A, 3B, 3C ............................................................................................... 18
  EBERs ................................................................................................................. 18
  BARTs ................................................................................................................ 20
  EBV and microRNAs ......................................................................................... 21
REFERENCES .......................................................................................................................... 106

CHAPTER THREE: LMP1 modulates distinctive NF-κB pathways through CTAR1
to regulate EGFR expression
ABSTRACT ............................................................................................................................. 116
INTRODUCTION ................................................................................................................... 117
MATERIALS AND METHODS .............................................................................................. 120
RESULTS ............................................................................................................................... 124
DISCUSSION ......................................................................................................................... 135
ACKNOWLEDGEMENTS ...................................................................................................... 140
REFERENCES ....................................................................................................................... 141

CHAPTER FOUR: EBV Latent Membrane Protein 1 (LMP1) Activates STAT3 and ERK through effects on EGFR and PKCδ
ABSTRACT ............................................................................................................................. 148
INTRODUCTION ................................................................................................................... 149
MATERIALS AND METHODS .............................................................................................. 152
RESULTS ............................................................................................................................... 156
DISCUSSION ......................................................................................................................... 165
ACKNOWLEDGEMENTS ...................................................................................................... 169
REFERENCES ....................................................................................................................... 170

CHAPTER FIVE: General Conclusions .............................................................................. 176
REFERENCES ....................................................................................................................... 188
LIST OF TABLES

CHAPTER ONE

Table 1: Gene Expression patterns of different types of EBV latency and EBV-related malignancies associated with each latency type ................................................................. 10

CHAPTER FOUR

Table 1: Quantitative RT-PCR primers ............................................................................................................. 155
LIST OF FIGURES

CHAPTER ONE

Figure 1: EBV life cycle ........................................................................................................ 5
Figure 2: LMP1-associated signaling pathways ............................................................. 13

CHAPTER TWO

Figure 1: CTAR1 of LMP1 upregulates EGFR mRNA levels ........................................ 92
Figure 2: CTAR1 of LMP1 upregulates Bcl-3 and induces nuclear translocation of Bcl-3 and p50 ........................................................................................................ 93
Figure 3: CTAR1 of LMP1 upregulates Bcl-3 and EGFR by activating STAT3 ...... 95
Figure 4: IL-6 production in C33A cells ........................................................................ 97
Figure 5: LMP1 CTAR1 induces binding of STAT3 to Bcl-3 promoter and intronic Enhancers ........................................................................................................... 99
Figure 6: A working model for LMP1 CTAR1-mediated induction of Bcl-3 and, consequently, EGFR ................................................................................. 104

CHAPTER THREE

Figure 1. EBV LMP1-CTAR1 mediates mild induction of EGFR expression in mouse embryonic fibroblast (MEF) cells ......................................................... 124
Figure 2. LMP1-mediated EGFR upregulation is NIK-dependent ......................... 126
Figure 3. Blocking NIK reduces LMP1-induced EGFR expression, but overexpression of NIK is not sufficient to mimic LMP1-mediated EGFR upregulation ...................................................................................... 128
Figure 4. CTAR1-mediated p50 activation is not dependent on canonical or noncanonical pathway ........................................................................................... 130
Figure 5. CTAR1 mediates p50 activation through proteasome-dependent pathways .................................................................................................................. 132
Figure 6. LMP1-mediated EGFR upregulation is TRAF2- and TRAF3-, but not TRAF6-Dependent ...................................................................................................... 134
CHAPTER FOUR

Figure 1. LMP1-CTAR1 activates EGFR and STAT3 independent of serum, and CTAR1-induced EGFR further activates STAT3 in response to EGF treatment ................................................................. 158

Figure 2. mRNA expression of EGFR ligands in CTAR1 cells ........................................................................ 159

Figure 3. Expression level of STAT3 target genes in EGF-treated CTAR1-expressing C33A cells ................................................................. 160

Figure 4. LMP1-induced serine phosphorylation of STAT3 is PKCδ-dependent ... 162

Figure 5. PKCδ inhibitor, Rottlerin, inhibited CTAR1-induced focus formation ... 163

Figure 6. Tyrosine-311 phosphorylation of PKCδ is elevated in CTAR1 C33A cells ................................................................................................. 164

CHAPTER FIVE

Figure 1. CTAR1-mediated signaling pathways for EGFR upregulation.............. 187
# LISTS OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIDS</td>
<td>Acquired immune deficiency syndrome</td>
</tr>
<tr>
<td>ATF5</td>
<td>Activating transcription factor 5</td>
</tr>
<tr>
<td>BART</td>
<td>BamHIA rightward transcript</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BL</td>
<td>Burkitt’s lymphoma</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin-immunoprecipitation</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Csk</td>
<td>Carboxy-terminal Src kinase</td>
</tr>
<tr>
<td>CTAR1</td>
<td>Carboxy-terminal activating region 1</td>
</tr>
<tr>
<td>CTAR2</td>
<td>Carboxy-terminal activating region 2</td>
</tr>
<tr>
<td>CXCL</td>
<td>CXC chemokine ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>CXC chemokine Receptor</td>
</tr>
<tr>
<td>CYLD</td>
<td>Cylindromatosis</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant negative</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNMT</td>
<td>DNA methyltransferase</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EBER</td>
<td>Epstein-Barr virus encoded RNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>EBNA</td>
<td>Epstein-Barr virus nuclear antigen</td>
</tr>
<tr>
<td>EBNA-LP</td>
<td>EBV-encoded nuclear antigen leader protein</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>EREG</td>
<td>Epiregulin</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>GAC</td>
<td>Gastric carcinoma</td>
</tr>
<tr>
<td>GC</td>
<td>Germinal center</td>
</tr>
<tr>
<td>gp</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>GRP78</td>
<td>Glucose-regulated stress protein</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HBEGF</td>
<td>Heparin-bound epidermal growth factor</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HCMV</td>
<td>Human cytomegalovirus</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>Hdm2</td>
<td>Human double minute</td>
</tr>
<tr>
<td>HHV</td>
<td>Human herpesvirus</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immune deficiency virus</td>
</tr>
<tr>
<td>HL</td>
<td>Hodgkin lymphoma</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HOXA10</td>
<td>Homeobox A10</td>
</tr>
<tr>
<td>HPS</td>
<td>Haemophagocytic syndrome</td>
</tr>
<tr>
<td>HRS</td>
<td>Hodgkin and Reed-Sternberg</td>
</tr>
<tr>
<td>HS</td>
<td>Hypersensitive site</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of NF-κB</td>
</tr>
<tr>
<td>Id</td>
<td>Inhibitor of differentiation</td>
</tr>
<tr>
<td>IE</td>
<td>Immediate early</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin like growth factor 1</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Infectious mononucleosis</td>
</tr>
<tr>
<td>IR</td>
<td>Internal repeats</td>
</tr>
<tr>
<td>I-TAC</td>
<td>Interferon-inducible T cell alpha chemoattractant</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine based activation motif</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi’s sarcoma-associated herpesvirus</td>
</tr>
<tr>
<td>LCV</td>
<td>Lymphocryptovirus</td>
</tr>
<tr>
<td>LD</td>
<td>Lymphocyte-depleted</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LMP</td>
<td>Latent membrane protein</td>
</tr>
<tr>
<td>LCL</td>
<td>Lymphoblastoid cell line</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MC</td>
<td>Mixed cellularity</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine double minute</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated ERK kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MICB</td>
<td>MHC class I polypeptide-related sequence B</td>
</tr>
<tr>
<td>MIG</td>
<td>Monokine induced by the interferon-gamma</td>
</tr>
<tr>
<td>MIP-2</td>
<td>Macrophage inflammatory protein-2</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB inducing kinase</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NotchIC</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>NPC</td>
<td>Nasopharyngeal carcinoma</td>
</tr>
<tr>
<td>NS</td>
<td>Nodular sclerosing</td>
</tr>
<tr>
<td>OHL</td>
<td>Oral hairy leukoplakia</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP)-Ribose Polymerase</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase c</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase r</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>pSS</td>
<td>Primary Sjögren’s syndrome</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PTLD</td>
<td>Post-transplant lymphoproliferative disorder</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>QRT-PCR</td>
<td>Quantitative real time PCR</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RACK</td>
<td>Receptor for activated protein kinase C</td>
</tr>
<tr>
<td>RASSF</td>
<td>RAS association domain family protein</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>RBPJκ</td>
<td>Recombination signal-binding protein 1 for J-kappa</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor interacting protein</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation assay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>SAP</td>
<td>Signals for lymphocyte activation molecule-associated protein</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TCL1</td>
<td>T-cell leukemia-1</td>
</tr>
<tr>
<td>TFs</td>
<td>Transcription factors</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>Th1</td>
<td>Type 1 T helper cell</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TPA</td>
<td>Phorbol diester 12-O-tetradecanoylphorbol 13-acetate</td>
</tr>
<tr>
<td>TR</td>
<td>Terminal repeats</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNF receptor-associated death domain</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor-associated factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor-of-apoptosis</td>
</tr>
<tr>
<td>XLP</td>
<td>X-linked lymphoproliferative syndrome</td>
</tr>
</tbody>
</table>
CHAPTER ONE

Introduction
Epstein-Barr Virus

A novel extranodal lymphoma occurring in children was identified in the 1940s by Denis Burkitt in equatorial Africa where malaria was endemic (33-35, 54). Denis Burkitt referred to this disease as “African Lymphoma” (currently called Burkitt’s lymphoma) and postulated that there may be an infectious agent involved due to the atypical epidemiological and clinical features of the disease. Epstein-Barr virus was discovered in 1964 by Anthony Epstein, Yvonne Barr, and Bert Achong in electron micrographs of the cells cultured from the tumor specimens provided by Denis Burkitt. They revealed a herpesvirus-like particle that did not react to antibodies from other known herpesviruses and did not replicate in cultured cells (79).

EBV, originally known as human herpesvirus 4 (HHV4), is a prototype member of the gamma herpesvirus subfamily and belongs to the lymphocryptovirus (LCV; gamma 1) genus (190). EBV is an enveloped virus with a linear double-stranded DNA genome of more than 180 kilobase pairs in length (17, 59, 310, 313). EBV and most LCVs share four characteristic features to their genomes: Single overall format and gene rearrangement, Tandem and reiterated direct repeats at both termini (TR), six to twelve tandem and reiterated direct internal repeats (IR1), as well as short and long unique sequence domains (U_S and U_L) that contain the most of the viral coding capacity (46, 47, 107, 108).

Gamma herpesviruses are lymphotropic, generally replicate in B or T cells, and have a relatively limited range of hosts. Similarly, EBV can infect B lymphocytes to establish lifetime persistence while retain the ability to undergo productive replication in its only natural host, humans (190). EBV is a ubiquitous infectious agent that more than 90% of the world adult population is infected with EBV (190). EBV infection during the childhood is
normally asymptomatic and does not cause diseases. However, delayed infection in adolescence may cause infectious mononucleosis (IM) and in individuals like immunocompromised patients EBV infection is associated with pathological conditions, including the development of cancer (190). One defining biological property of EBV is its ability to transform resting B cells \textit{in vitro} into lymphoblastoid cell lines (LCLs) (190). EBV is also capable of infecting epithelial cells in vitro and in vivo and is associated with cancers of epithelial origins (190, 212, 358). It has also been reported that EBV can infect cell types other than B cells and epithelial cells, such as T cells, natural killer (NK) cells, monocytes, and neutrophils (116, 155, 337).

\textbf{The Life Cycle of EBV}

A schematic illustration of the EBV life cycle is shown in Figure 1. EBV is normally transmitted through saliva that contains both cell-free virus and EBV-infected cells that eventually lead to the infection of oral epithelial cells (321). The mechanism by which EBV infects the oral epithelium is still not well understood, although one early study suggested that EBV infection begins with the fusion of EBV-infected B cells to epithelial targets (21). This model is supported by the observation that infection of epithelial cells is more efficient when in close proximity with EBV-producing B cells than with cell-free virus (153, 391). Infection of the oral epithelium leads to production of progeny virions through lytic replication (204, 205, 357). Oral epithelium-released virions infect naïve B cells circulating near the oral epithelium or in lymphoid organs (255, 278). Interestingly, EBV infects naïve and memory B cells at similar efficiency \textit{in vitro} (429).
EBV infects B cells through the binding of gp350/220 to cell-surface receptor CD21 to initiate endocytosis into the host cells, as well as the binding of gp42 to class II human leukocyte antigen (HLA, also called major histocompatibility complex or MHC) as a co-receptor (87, 211, 274, 372). Attachment of EBV to B cells leads to viral fusion with the endosomal membrane at a low pH (249). However, whether CD21 plays a role in EBV infection of epithelial cells remains to be determined, indicating that different mechanisms may be required for EBV binding and entry of epithelial cells. It has been shown that EBV needs gH and gL to bind to several epithelial cell lines, but the participating receptors have not yet been identified (256, 284). Recently it has been reported that EBV glycoprotein BMRF2 interacts with integrin α5β1 and the binding can be blocked by antibodies against integrin or BMRF2 (391). Moreover, endocytosis is not required in EBV infection of epithelial cells, and fusion occurs at neutral pH (249). Interestingly, cell tropism of EBV infection can be modified depending on the cell types in which the virus is produced. Virions that are made in HLA-class-II-positive B cells are depleted of gp42, and therefore target HLA-class-II-negative epithelial cells more effectively than B cells. Conversely, virions produced in epithelial cells can infect B cells more effectively (391).

Once viral entry is complete, the EBV genome circularizes to form a viral episome in the B cells and initiates a limited latency program through transcription from the viral Wp promoter using cellular RNA polymerase II (150, 336). Activation of Wp promoter in resting naïve B cells leads to expression of EBNA2, which then turn on a “growth program” (similar to type III latency described below) which activates the Cp promoter to initiate B cell proliferation (377, 415). These B cells can undergo germinal center (GC) differentiation and become memory B cells (377). Lifetime viral persistency is established and most viral
EBV infects the oral epithelium through saliva contact. Lytic infection in the oral epithelium leads to amplification of progeny EBVs, which then infect naïve B cells in the surrounding lymphoid tissues and drive their proliferation and growth. The infected B cells then undergo germinal center differentiation to become memory B cells. Most viral protein expression is turned off at this point, except for dividing memory B cells, which express EBNA1. Persistently-infected memory B cells occasionally undergo differentiation to become plasma cells, where viral reactivation takes place. Released virus may re-infect other B cells or be shed into saliva for spread to other hosts. (Adapted from (379))
proteins are not expressed (375). Persistently infected memory B cells occasionally undergo plasma-cell differentiation which triggers viral reactivation (14, 24, 201, 365). The newly released virus can be shed into saliva for spread to other hosts or for infection of surrounding B cells and epithelial cells.

**Reverse Genetics with EBV study**

Studies with genetic manipulations of EBV have been hindered due to the limited host range of EBV and the lack of permissiveness for EBV replication in most cell lines. Several approaches have been tried to conduct reverse genetic studies of EBV. By transfecting latently infected lymphocytes with mutated EBV DNA fragments cloned in *Escherichia coli*, recombinant EBV can be made following lytic program induction and homologous recombination (244, 402). Alternatively, a latently infected cell line with replication-competent EBV strain, P3HR1, has been used as the parental strain for homologous recombination following transfection of mutated EBV DNA fragments (383). A system using bacterial artificial chromosome (BAC) to produce recombinant EBV has also been developed to aid the genetic studies of EBV (63, 171).

**Lytic Replication**

Due to the availability of cell lines latently infected with EBV and the lack of good genetic tools for EBV primary infection, EBV lytic replication is normally studied by inducing the reactivation of EBV in latently infected cells. Since infection of epithelial cells in vitro does not achieve full lytic replication, transformed B cells remains the dominant model of infection (429). In vitro studies have focused on using phorbol esters (TPA) to
induce lytic replication through activating protein kinase c (PKC) pathway, which induces transcription of EBV immediate early genes including BZLF1 (10, 91). The lytic program can also be induced by activating the B cell receptors using soluble immunoglobulin (371). Induction of lytic replication results in the production of infectious virus following cytopathic effects on infected cells, such as marginization of heterochromatin, inhibition of host macromolecule synthesis, assembly of nucleocapsids, and envelopment of virions through the inner nuclear membrane (104, 179, 190, 337). Like other herpesviruses, EBV lytic replication follows a temporal and sequential order and production of lytic genes can be divided into three categories, immediate early (IE), early, and late gene expressions (371).

**Immediate Early Genes**

BZLF1 (also called Zta or ZEBRA) and BRLF1 (also called Rta) are EBV immediate early genes because of their expression in the presence of protein synthesis inhibitors, such as cycloheximide. BZLF1 and BRLF1 are located in the cell nucleus and function as transcriptional regulators to initiate and promote lytic infection (113, 122, 315, 362, 371). BRLF1 and BZLF1 have both been shown to transactivate early gene promoters and also to be required for efficient viral DNA replication (31, 48, 89, 92, 122, 142, 185, 339). Moreover, expression of either BZLF1 or BRLF1 during latency will result in the induction of the other to drive the virus into the lytic cycle (92, 223, 315). Uniquely, BZLF1 preferentially binds to methylated-forms of promoters of BRLF1 and other viral genes (25, 67). During the transition from latent to lytic cycle, BZLF1 also ensures the successful switch by inhibiting the activity of Cp promoter which regulates the expression of latency-associated EBNA genes (186).
Interaction between BZLF1 and the NF-κB subunit, p65, has been identified and it’s believed that they mutually inhibit each other’s transcriptional activity depending on the stages of EBV infection (117, 262). Furthermore, BZLF1 prevents an EBV-stimulated immune response by downregulating CIITA, tumor necrosis factor receptor 1 (TNFR1), and interferon gamma receptor. BZLF1 also promotes B-cell proliferation by inducing IL-13 (209, 263, 264, 387). Interestingly, it has been recently reported that in AIDS-associated primary effusion lymphoma (PEL) co-infected with Kaposi’s sarcoma-associated herpesvirus (KSHV) and EBV, lytic replication initiators for both viruses, K-RTA (KSHV) and BZLF1 (EBV), inhibits each other’s transcriptional activity (165). This mutual inhibition may be important for maintaining viral latency in dually infected cells.

Recently it has been shown that BRLF1 induces the expression of decoy receptor 3 (DcR3), which is also upregulated by EBV LMP1 (140, 141). The upregulation of DcR3 may contribute to evade immune surveillance and promote cell migration and invasion.

**Early Genes**

The expression of EBV early genes is dependent on active protein synthesis but not on viral replication. EBV-encoded early genes are involved with several early events during EBV infection, including viral gene transcription and DNA replication (90). For example, EBV DNA polymerase BALF5 is responsible for EBV genome replication during the lytic cycle and its activity is dependent on another early gene, BMRF1, which encodes the DNA polymerase processivity factor (142, 218). Another EBV early gene, BHRF1, has been identified as a viral homolog of cellular Bcl-2 both sequence-wise and functionally (125). Cellular Bcl-2 protects cells from programmed cell death and activation of Bcl-2 by
chromosomal translocation is characteristic of human follicular lymphomas (389, 390). Therefore, it has been suggested that BHRF1 may function similarly to protect EBV-infected cells from apoptosis during the viral life cycle. Notably, BHRF1 has been recently shown to be also expressed in EBV-transformed B cells and BHRF1 may contribute to B cell transformation by providing resistance to apoptosis (182). Interestingly, another EBV early gene detected in lytic cycle, BARF1, is also expressed during type II EBV latency (NPC and GC) and has been shown to possess tumorigenic potentials (62, 350, 441).

**Late Genes**

The last subset of genes expressed during the lytic cycle after viral DNA synthesis, usually referred to as late genes, encode viral structural components including tegument proteins and glycoproteins (236, 376). EBV late genes BLLF1 and BZLF2 encoded EBV glycoproteins gp350/220 and gp42, respectively (87, 211). Both gp350/220 and gp42 are important for viral entry of EBV into B cells as described previously. Due to its critical role in EBV infection, gp350 has served as a prime candidate for the development of EBV vaccines (225, 360). BCRF1 encodes a viral homolog of IL-10 and it has been shown that BCRF1 negatively regulates the host immune response, similar to cellular IL-10, by inhibiting activities of T cells, NK cell, and macrophage function (146, 257, 334, 367).

**Establishment of Latency**

Types of EBV latency are defined by the expression of specific subsets of EBV latent genes, including six EBV-encoded nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C, and LP), three latent membrane proteins (LMPs 1, 2A, and 2B), small non-polyadenylated RNAs (EBER1
and EBER2), and a group of spliced 3’ transcripts known as BamHIA rightward transcripts or BARTs (190, 429). Genes expressed in certain types of latency, as well as associated diseases, are summarized in Table 1. In type I latency of EBV infection, with Burkitt’s lymphoma as the classical example, only EBNA1 is expressed at the protein level along with transcripts for the BARTs and EBERs. Type 0 latency is referred to the cell state where infected resting memory B cells do not express EBNA1 before cells undergo cell-division. During type II latency, major EBV oncoproteins like LMP1, LMP2A, and LMP2B are expressed in addition to EBNA1 as well as the transcripts of BARTs and EBERs. Type II latency is normally seen in EBV-associated Hodgkin disease and EBV-associated diseases of epithelial origins, such as nasopharyngeal carcinoma and gastric carcinoma. In type III latency, expression of most EBV latent genes can be detected, including all EBNAs, LMPs, EBERs, as well as of BARTs. Typical examples of type III latency include EBV-generated LCLs in vitro and post-transplant lymphoproliferative disorders (PTLDs) (30).

<table>
<thead>
<tr>
<th>Latency Type</th>
<th>EBV Genes Expressed</th>
<th>Associated Malignancies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>EBNA1, BARTs, EBERs</td>
<td>Burkitt’s Lymphoma</td>
</tr>
<tr>
<td>Type II</td>
<td>EBNA1, BARTs, EBERs, LMP1, LMP2A and 2B, +/- BARF1</td>
<td>Hodgkin Disease, Nasopharyngeal Carcinoma, Gastric Carcinoma</td>
</tr>
<tr>
<td>Type III</td>
<td>EBNA1, 2, 3A, 3B, 3C, and LP, BARTs, EBERs, LMP1, LMP2A and 2B.</td>
<td>Lymphoblastoid Cell Lines, Post-transplant Lymphoproliferative Disorder</td>
</tr>
</tbody>
</table>

Table 1. Gene Expression pattern of different types of EBV latency and EBV-related malignancies associated with each latency type.

**LMP1**

EBV latent membrane protein 1 (LMP1) is expressed in latency type II and III. LMP1 is the EBV major oncogene since it is essential for EBV-mediated B-cell transformation and LMP1 alone can transform rodent fibroblasts in vitro (180, 401). LMP1
expression in epithelial cells can significantly affect cell growth, morphological transformation, and inhibit cell differentiation (60, 82). Moreover, LMP1 expression in mouse models can cause hyperplasia and B cell lymphomas (197, 414). LMP1 is an integral membrane protein which has 386 amino acids and encodes a 63kDa protein product. LMP1 can be basically divided into three domains: first, the N-terminal cytoplasmic tail (aa 1-23), which is responsible for the orientation of LMP1; Second, six transmembrane domains (aa 24-186), which are involved in self aggregation and oligomerization; third, the long C-terminal cytoplasmic tail (aa 187-386), which is responsible to mediate most of LMP1’s signaling activities (429).

In latently infected B cells, LMP1 mRNA is more abundant than EBNA mRNAs despite being regulated by a weak promoter (85). Transcription of LMP1 is regulated through multiple TATA-less sites in the first terminal repeat (TR) to result in a transcript with a long 5’ untranslated region (105, 330). The half-life of LMP1 is less than two hours and post-translational modifications of LMP1 include phosphorylation of multiple serine and threonine, but not tyrosine, residues (18, 242, 258). It has been shown that LMP1 associates with cytoplasmic cytoskeleton and also incorporates in cholesterol-rich lipid rafts on the cell membrane (131, 215, 245). Although most studies of LMP1 focus on its function in the plasma membrane, only half of the cellular LMP1 is found in the plasma membrane, indicating that LMP1’s function may be regulated by its cellular localization (216, 241). Interestingly, a variety of LMP1 sequence variants have been identified and it seems they arise from the onset of primary infection. These sequence variants appear to associate with compartmentalization (e.g. oral cavity, peripheral blood, or NPC tumors) and could potentially provide cell growth advantages (72, 73, 252, 354, 355).
LMP1 functions as a constitutively active tumor necrosis factor receptor (TNFR) by associating with tumor necrosis factor receptors-associated factors (TRAFs) (160, 250, 265). LMP1 is functionally similar to another TNFR member, CD40, and can partially substitute for CD40 in B cells (392). A myriad of signaling pathways have been shown to be activated by LMP1, including mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), phosphatidylinositol 3-kinase (PI3K)-Akt, and NF-κB pathways (77, 239, 290, 320). In order to control cell cycle progression, cell proliferation, and migration, LMP1 regulates transcriptional expression of many cellular genes, including EGFR, Bcl-2, A20, inhibitor of differentiation (Id) 1 and 3, p27, and plakoglobin, (81, 93, 126, 251, 345). LMP1 has two major signaling domains in its c-terminal tail, carboxyl-terminal activating regions 1 and 2 (CTAR1 and CTAR2), which mediate LMP1-associated signaling pathways through recruiting different set of TRAFs. CTAR1 recruits TRAF1/2/3/5 through a TRAF-binding PxQxT motif at aa 204-208 (PQQAT), whereas CTAR2 recruits TRAF2 and TRAF6 through TNFR-associated death domain (TRADD), the receptor interacting protein (RIP), and BS69 (159, 361).

CTAR1 and CTAR2 have different effects on LMP1-mediated signaling and cellular transformation, possibly due to their distinctive TRAF binding patterns. CTAR1 induces MAPK, PI3K/Akt/GSK3β signaling, while CTAR2 activates JNK pathway (76, 239, 240). CTAR2 can strongly activate canonical NF-κB signals, while CTAR1 weakly induces NF-κB signalings including canonical, noncanonical, and atypical pathways (232, 250). Interestingly, it has been reported that expression of LMP1 can be regulated by NF-κB (65, 168). Furthermore, CTAR1 is essential for LMP1-mediated transformation of B-lymphocyte and rodent fibroblast, while CTAR2 is dispensable (160, 239). CTAR1 also has unique
Figure 2. LMP1-associated signaling pathways. CTAR1 associates with TRAF1, 2, 3, and 5 and activates a variety of signaling pathways, including PI3K/Akt/GSK3β, MAPKK/ERK, and NF-κB pathways. CTAR1 also regulates expression of proteins involved with proliferation and cell cycle regulation, including Id1/Id3, p27, Rb, and EGFR among others. CTAR2 recruits TRAF2 and 6 through TRADD and BS69, followed by activation of JNK and canonical NF-κB pathway. Full-length LMP1 is required for the inhibition of plakoglobin, as well as the initiation of the cadherin switch.
effects on regulating cellular gene expression in induction of EGFR, TRAF1, EBI3, Id1, and 
Id3, as well as the repression of p27 (66, 81, 250).

It has been reported that LMP1 downregulates E-cadherin gene expression via 
activation of DNA methyltransferases to induce cell migration (386). More recent studies 
revealed that in addition to downregulation of E-cadherin, LMP1 contributes to cell 
migration and NPC metastasis by downregulating junctional protein plakoglobin and 
initiating a “cadherin switch” in LMP1-expressing epithelial cells (345, 346). A schematic 
illustration of the LMP1-associated signaling pathways is shown in Figure 2.

LMP2A and LMP2B

LMP2A and LMP2B are two EBV integral membrane proteins. LMP2A and LMP2B 
share similar structural features, with 12 transmembrane domains and a 27-amino-acid 
cytoplasmic C-terminus (429). LMP2A has an extra 119-amino-acid cytoplasmic N-
terminus that contains an immunoreceptor-tyrosine activation motif (ITAM) and a 
phosphotyrosine (PY) motif. The LMP2s are expressed in type II and type III latency, 
however they are not required for EBV-mediated B-cell transformation (226, 227). LMP2-
mutant EBV-infected cells undergo normal lytic replication upon viral reactivation (192, 
228). B-cell-targeted expression of LMP2A in transgenic mice can abrogate normal B-cell 
development by mimicking the signaling mediated by B-cell receptor (BCR), resulting in 
colonization of Ig-negative cells in peripheral lymphoid organs (39). LMP2A can also 
transform epithelial cells and induce cell adhesion and migration, as well as inhibit cell 
differentiation (340). These results suggest that LMP2A potentially contribute to EBV-
associated tumorigenesis. The function of LMP2B is not well understood, but recent studies
have suggested that LMP2B negatively regulates the function of LMP2A, one of which is to prevent the switch from latent to lytic EBV replication (317, 326).

LMP2A blocks BCR signaling by sequestering the Src family of tyrosine kinases and the Syk tyrosine kinase through activated ITAM motif, in which Tyr74 and Tyr85 are phosphorylated (32, 94). Lyn tyrosine kinase can be recruited to a membrane-proximal tyrosine residue Tyr112 and mediate phosphorylation of other tyrosine residues of LMP2A (95). Interestingly, LMP2A can be phosphorylated by Csk in epithelial cells instead of Src kinase (341). LMP2A also recruits NEDD4-like ubiquitin protein ligases, such as AIP4, WWP2, and Nedd4, through the PY motif to promote the degradation of Lyn and LMP2A, providing another mechanism for regulation of BCR signaling (151). LMP2A also contributes to cell transformation by actively activating several signaling pathways, including PI3K/Akt/GSK3β, MAPKK/ERK/JUN, and Wnt/β-catenin pathways (45, 260, 261, 340).

LMP2A has been found to induce expression of a variety of genes that are involved with cell cycle progression, inhibition of apoptosis, and evasion of immune response, indicating LMP2A’s role in EBV-associated tumorigenesis through transcriptional regulation (429). Interestingly, the transcriptional profiles of LMP2A-expressing B cells or LMP2A transgenic mice have been reported to resemble those observed in malignant Hodgkin and Reed Sternberg (HRS) cells of Hodgkin disease or germinal center B cells, indicating the roles of LMP2A in vivo (307, 308). A recent study has shown that LMP2A can also cause epigenetic abnormality in gastric carcinoma cell line by inducing STAT3-dependent DNA methyltransferase 1 (DNMT1) upregulation to decrease transcription of PTEN through promoter methylation (135).
EBNA1

EBV-encoded nuclear antigen 1 (EBNA1) is expressed in all types of EBV latency. The two main functions of EBNA1 are to maintain the episomal EBV genome and to regulate transcription of viral genes. Through binding to the plasmid origin of viral replication, OriP, EBNA1 tethers the viral episome to chromosomes in dividing cells to ensure that every daughter cell retains the viral episome after mitosis (285, 424, 425). Recent studies have suggested that EBNA1 binds to OriP through RNA-dependent mechanisms (282, 283). EBNA1 contributes to transcriptional regulation by binding to the promoters controlling viral latent genes, including EBNAs and LMP1, in a zinc-dependent manner (6, 11, 97). An internal Gly-Ala repeat sequence between the amino- and carboxyl-terminus of EBNA1 stabilizes EBNA1 by preventing its proteasome-mediated degradation (206, 207). This block of proteasome-mediated degradation and processing of EBNA1 may also contribute to the lack of immune response elicited by EBNA1 expression in EBV-infected cells (385). The oncogenic potential of EBNA1 is still under debate. Although it has been shown that EBNA1 is not essential for EBV to establish LCLs, EBNA1 does seem to provide certain survival signals to EBV-associated Burkitt’s lymphomas (149, 183). Moreover, EBNA1’s ability to cause cancer in transgenic mouse models has been inconsistent, with various results observed among mice with different genetic backgrounds (174, 175, 413).

EBNA2

Expressed in type III latency, EBNA2 is considered essential for EBV-mediated B cell transformation initially from studying the lack of transforming ability of EBNA2-deficient EBV strain P3HR-1 (56). EBNA2 contributes to EBV-mediated transformation by
regulating transcription of both cellular and viral genes, such as CD21, CD23, BATF, LMP1, and LMP2A (2, 57, 114, 167). The transactivating function of EBNA2 is mediated through its ability to interact with downstream target of Notch, Jκ-recombination-binding protein (RBP-Jκ), and abolish RBP-Jκ-mediated transcriptional repression (114, 145, 332, 438). Other than Notch, EBNA2 has also been suggested to regulate signaling pathways mediated by other transcriptional factors, such as STAT3 (269).

EBNA-LP

EBV-encoded nuclear antigen leader protein (EBNA-LP) is not essential for EBV-mediated transformation but is important for the outgrowth of EBV-transformed B cells (56, 243, 353). The main function identified so far for EBNA-LP is that it interacts with the acid activation domain of EBNA2 and cooperate with EBNA2 in RBP-Jκ-mediated transcriptional activation of cellular and viral genes (120, 281). Moreover, it has been shown that EBNA-LP’s activity may be mediated through the interaction with Sp100 (222). Three evolutionarily conserved regions of EBNA-LP are important for its nuclear translocation and functions, and EBNA-LP interacts with the tumor suppressor genes p53 and retinoblastoma (Rb) (299, 368). EBNA-LP also binds the anti-apoptotic protein Bcl-2 and its EBV counterpart BHRF1, indicating that it regulates apoptosis in EBV-infected cells (247). Interestingly, a recent study showed that a truncated form of EBNA-LP is expressed in some BL cells infected with EBV strains defective for EBNA2. The truncated EBNA-LP induced resistance to caspase-mediated apoptosis through its interaction and sequestration of phosphatase 2A (PP2A) (101). These findings further suggest that in addition to cooperating
with EBNA2, EBNA-LP has independent functions to contribute to EBV-mediated transformation.

**EBNA3A, EBNA3B, EBNA3C**

Similar to EBNA2 and EBNA-LP, EBNA3s are only expressed in type III latency of EBV infections. *In vitro* infections using recombinant EBV have shown that EBNA3A and EBNA3C are essential for EBV-mediated B cell transformation but EBNA3B is dispensable (382, 383). The EBNA3s repress EBNA2/EBNA-LP-mediated RBP-Jκ transactivation through competing for binding with RBP-Jκ (166, 322, 400, 435). EBNA3A has been shown to repress the EBV Cp promoter in both epithelial and B cells (53). The roles of EBNA3B in EBV infection is unclear since it’s not required for EBV-mediated transformation, but it may be important for maintaining viral latency by regulating chemokine receptor CXCR4 (43). In cooperating with RAS, EBNA3C can disrupt cell cycle checkpoints and transform rodent embryonic fibroblast (292, 293). The transforming activity of EBNA3C may reflect its ability to interact with cellular proteins that modulate gene transcription and apoptosis, such as histone deacetylase 1 (HDAC1), p53, Rb, and cyclin A (195, 292, 314, 426). Despite the aforementioned negative effects on EBNA2, EBNA3C can also induce LMP1 transcription in conjunction with EBNA2 (436). A recent study reported that EBNA3A and EBNA3C cooperatively regulate BL pathogenesis through downregulation of the pro-apoptotic tumor suppressor gene Bim (9).

**EBERs**
EBER1 and EBER2 are two small nonpolyadenylated, non-coding, RNAs that are expressed in all types of EBV latency and thus serve as good targets for detection of EBV infection using in situ hybridization (118, 144). Although the EBERs are not essential for EBV-mediated B-cell transformation, they are shown to be able to enhance the tumorigenic potential of B cells in cell culture and in immunodeficient mice by preventing apoptosis (196, 329, 423). Similarly, overexpression of EBERs can also increase the resistance of NPC cell lines to apoptosis (273, 416). A recent study suggests that despite their structural similarities, EBER2 contributes to B-cell transformation more significantly than EBER1 (417).

The effects of the EBERs on apoptosis may be due to its ability to assemble into ribonucleoprotein complexes and bind with double-stranded RNA-activated protein kinase PKR (272, 370). PKR has been known to induce pro-apoptotic pathways (68). By binding and inhibiting PKR activity, EBERs may increase the tumor cell’s resistance to apoptosis, and promote viral persistence (272, 348). However, this hypothesis has been recently challenged by a study which showed that EBERs are not able to block PKR-induced protein phosphorylation mediated by interferon (328). This result argues that EBER-mediated inhibition of apoptosis is not due to direct interaction with PKR. EBERs may also provide anti-apoptotic signals by inducing the expression of anti-apoptotic factor Bcl-2, and contribute to tumorigenesis by inducing expression of pro-proliferative factors, such as IL-10 in B cells and insulin-like growth factor 1 (IGF-1) in EBV-associated NPC or gastric carcinoma (157, 158, 194, 196).

Due to their status as the most abundant transcripts in EBV-infected cells, a recent study has taken advantage of this fact to develop shRNA fusion transcripts controlled by the EBERs promoter (49). SiRNAs processed from these fusion transcripts exhibit stronger
efficiency of knocking down target genes without activating double-stranded RNA-dependent protein kinase activity. This strategy may be used for more effective gene therapy, especially in EBV-infected cells.

**BARTs**

BamHIA rightward transcripts (BARTs) are a group of abundantly expressed RNAs that are highly spliced and encoded by the BamHIA region of the EBV genome (331, 359). The BARTs were first identified and characterized in NPC and were shown to be highly abundant compared to transformed lymphocytes (44, 138, 177, 331). However, using partially-deleted EBV cosmid recombinants, the BARTs were shown to not required for EBV-mediated latent infection or B-cell transformation (323).

Three potential open reading frames (ORFs), *rk103* (RPMS1), *rb2* (A73), and *rkbarf0*, are located in BARTs. Transcripts containing RK-BARF0 and RB2 are prevalently expressed in NPC tumors (331). Although the protein products of these ORFs have not been conclusively identified in vivo, despite the detection of specific antibodies in NPC patients, recombinant technologies have been used to evaluate their potential function (106). RK-BARF0 has been found to interact with human I-mfa domain-containing protein (HIC), epithelin, scramblase, Notch3, and Notch4 by yeast-two-hybrid screenings (200, 378). RK-BARF0 interacts with both processed and unprocessed Notch4 but only induces the nuclear translocation of unprocessed Notch4. Moreover, interaction of RK-BARF0 with Notch induces proteasome-mediated degradation of Notch, which may explain the low level of endogenous Notch expression in EBV-positive cell lines (378). These results indicate that RK-BARF0 may contribute to EBV-mediated tumorigenesis by regulating Notch pathways.
Interestingly, RK103 may also contribute to Notch regulation by interacting with RBP-Jκ, a Notch target, to inhibit transactivating activities of NotchIC or EBNA2 (433). Furthermore, it has been reported that overexpression of RK103 exhibits oncogenic potential in 293 cells and in nude mice (208).

RB2 has been shown to be a cytoplasmic protein which can interact with receptor for activated protein kinase C (RACK1), a modulator of protein kinase C (PKC) or Src kinases (359). Interestingly, a recent study suggests that multiple polymorphisms in the rb2 gene may be correlated with NPC development (434). Another transcript product of BARTs, BARF1, is expressed both as an early gene in the lytic cycle or as a secreted latent protein in EBV-associated NPC (62). Overexpression of BARF1 displays oncogenic activity in B cells, rodent fibroblasts and monkey primary epithelial cells by stimulating the cell cycle (335, 350, 351).

**EBV and MicroRNAs**

EBV is the first virus to be identified encoding microRNAs (miRNAs) (301). Multiple approaches have been applied to identify EBV miRNAs, including computational prediction, microarray analysis, and small RNA cloning (38, 115, 300, 301, 439). To date, approximately 30 EBV miRNAs have been identified, mainly distributed within two areas, BARTs introns and BHRFs transcripts. Similarly to the BART transcripts, the expression of BART miRNAs is tissue-specific, BART miRNAs are expressed at high level in latently infected epithelial cells, such as NPC or GC, but at much lower level in B cells (38, 191). In contrast, the BHRF miRNAs are expressed in a latency type-dependent manner. BHRFs miRNAs are produced from an intron within the EBNA2 transcript and expressed at high
levels in B cells in type III latency but are undetectable in B cells or epithelial cells of type I or II latency (38). During transcription, miRNAs are processed prior to the splicing reaction and a specific form of transcript favors miRNA production. Therefore, the BART structure may account for the difference in EBV miRNA abundance (71). Interestingly, the induction of lytic replication enhances the expression of many EBV miRNAs (38, 422).

Several studies have suggested that EBV miRNAs may contribute to EBV tumorigenesis by targeting both viral and cellular genes. BART miRNAs have been suggested to promote cancer development and viral latency by regulating expression of LMP1 and viral DNA polymerase BALF5, respectively (20, 224). EBV miRNAs also provide cancer cells with immune-evasive and survival signals by targeting cellular genes involved with the immune response and apoptosis, such as CXCL-11, MICB, and PUMA (50, 271, 418).

EBV infection also has significant effects on cellular miRNA expression (110). EBV infection has been shown to induce expression of human oncogenic miRNAs, or oncomirs, like miR-155 and miR-146a to regulate cellular transcription machinery (230, 267, 427). It has been suggested that EBV regulates the expression of cellular miRNAs through LMP1-activated transcriptional factors, such as NF-κB (103, 267, 316). These quickly-accumulating data suggest that EBV can regulate expression of viral and cellular genes through novel miRNA pathways to promote tumor progression and maintain viral latency.

**EBV-Associated Diseases and Malignancies**

**Infectious Mononucleosis**
Primary infection of EBV, through oral transmission during childhood, is normally asymptomatic. However, if the primary infection is delayed until adolescence or early adulthood, half of the patients develop benign lymphoproliferative disease infectious mononucleosis (IM) (277). IM has been shown to be a significant risk factor for EBV-associated Hodgkin’s lymphoma with about 1 in 1,000 patients with IM developing HL later in life (139, 164). During IM, complex infection involving multiple strains of EBV occurs in the oral cavity, peripheral blood lymphocytes, and the cell-free plasma (356). Patients with IM shed high titers of infectious virus and could suffer clinical symptoms with various degrees of severity, including fever, pharyngitis, headache, malaise, lymphadenopathy, and splenomegaly (55, 80). These symptoms are mainly due to hyperexpansion of CD8$^+$ T cells and subsequent cytotoxic immune response, such as induction of IFN-$\gamma$ and TNF-$\alpha$, induced by EBV-infected B-lymphocytes (4, 40, 137). Polymorphisms in the interleukin-10 gene have been linked to the severity of lymphoproliferation of symptomatic primary EBV infection (124). Recent studies showed that class I human leukocyte antigen (HLA)-A1 fails to present LCL-expressed, highly immunogenic peptides of EBV latent antigens and thus represent one of the risk factors of acute IM and Hodgkin lymphoma (29, 279). Moreover, class I HLA polymorphisms within HLA-A1 and HLA-A3 was suggested to predispose patients to development of IM upon primary EBV infection (248).

Chronic Active EBV Infection

In most IM cases, the primary infection is controlled by immune system. However, in some young patients recurrent fever, lymphadenopathy, hepatosplenomegaly, chronic fatigue, and other symptoms occurs even several years after the primary infection (289).
Patients with chronic active EBV infection have very high EBV genome loads in the peripheral blood mononuclear cells (PBMC), high titers of antibodies against EBV-encoded antigens, and expression of EBV proteins and transcripts in the affected tissues (127, 237, 342). It has been suggested that defects in cytotoxic activity of T cells and natural killer (NK) cells contribute to the occurrence of chronic active EBV infection (96, 169, 388). A recent study showed that EBNA1-specific CD4 (+) T cells are capable of killing EBV-infected NK and T cells derived from patients with chronic active EBV infection, indicating the potential of this approach in vaccine design and immunotherapy of EBV infection (64).

**Oral Hairy Leukoplakia**

Characterized by productive infection of EBV in the squamous epithelial cells of the lateral tongue border, oral hairy leukoplakia (OHL) often develops in HIV-infected or otherwise immunocompromised patients and is the only known pathological manifestation of permissive EBV infection in epithelial cells (112). Without obvious symptoms, OHL develops as white or gray patch on the tongue or the inside of the cheek and is identified histologically by epithelial acanthosis, lack of inflammatory infiltrate, and hyperkeratosis (133). It has been shown that OHL is infected by multiple EBV strains characterized by sequence variation within the LMP1 gene (355, 397, 399). In addition to lytic gene products, the EBV latent genes EBNA1, EBNA2, and LMP1 are also detected in OHL lesions, as well as signaling pathways contributing to EBV transformation, such as LMP1-induced NF-κB and JNK activation (398, 406, 407). The EBV glycoprotein BMRF-2 is highly expressed in OHL epithelium and is thought to play an important role in cell-to-cell spread of EBV within the oral epithelium through association with various integrins (419, 420).
Virus Associated Hemophagocytic Syndrome

Haemophagocytic syndrome (HPS) is a rare but often fatal disease despite treatment. HPS is caused by dysregulations in T-cell and NK-cell functions, resulting in activation and proliferation of lymphocytes with out-of-control haemophagocytosis and cytokine overproduction (61, 363). Characterized by clinical symptoms such as fever, hepatosplenomegaly, liver dysfunction, cytopenias, and hyperferritinaemia, HPS is associated with viral infections, including EBV, human cytomegalovirus (HCMV), human herpesvirus 8 (HHV8), HIV, influenza virus, and hepatitis virus (88, 325). Among all of the viruses associated with this disease, EBV is the most common triggering agent and EBV-associated syndrome results in the worst prognosis. In EBV-associated HPS, EBV infects CD8⁺ T cells and results in the lack of production of EBV-specific cytotoxic T cells, followed by severe cytokine storm and organ dysfunctions (154, 178). Normally occurs during reactivation, EBV-associated HPS also develop with other EBV-caused diseases, such as IM, chronic active infection, and NK T-cell lymphoma (5, 51, 286). It has been recently suggested that LMP1-induced ATF5 may contribute to the pathogenesis of HPS by transcriptionally downregulating signals for lymphocyte activation molecule-associated protein (SAP) to enhance Th1 cytokine secretion in T cells (52).

X-linked Lymphoproliferative Syndrome

X-linked lymphoproliferative syndrome (XLP; also called Duncan’s disease) is a rare immunodeficiency disease identified by its inability to control EBV infection-induced immune response (121, 219). XLP patients exhibit polyclonal B-cell proliferation, defective
NK cell cytotoxicity and prolonged cytotoxic T-cell activity, resulting in often fatal infectious mononucleosis and malignant lymphoma in response to EBV infection (119, 276, 344). The phenotype of XLP is thought to result from defective-mutations within several genes on the human X chromosome, including SAP and X-linked inhibitor-of-apoptosis (XIAP), lead to defects in the activity of NK-cells to kill EBV-infected B cells (275, 294, 319). A recent study identified a homozygous missense mutation of IL-2-inducible T cell kinase (ITK) in two Turkish girls who suffered fatal immunoproliferative disorders following EBV infection, resembling the pathogenesis of XLP (148).

Post-Transplant Lymphoproliferative Disorder

Solid organ transplant patients who receive immunosuppressive therapy are vulnerable to the development of post-transplant lymphoproliferative disorders (PTLD), including a variety of B-cell diseases which are mostly EBV-positive (408). Although capable of developing much later after the transplantation, most PTLDs occur within the first year of allografting with the most severe immunosuppression and the weakest cytotoxic T-cell activity. The majority of PTLD tumors are in type III latency, with expression of all latent genes, although occasionally lytic gene of EBV or more intermediate patterns can also be detected (270, 381, 428). Data from studying gene sequencing and rearrangements of immunoglobulin (Ig) variable regions showed that in PTLDs EBV can infect B cells at any stage of differentiation, including naïve cells, memory cells, germinal center B cells, or even B cells possessing hypermutations of Ig (15, 28, 381). These novel EBV infections of non-naïve B cells in immunocompromised patients may create a pool of EBV-infected B cells with permanent transforming potential by trapping these infected cells into the “growth
program” of EBV gene expression (15, 297). An in vitro study showed that EBV infection results in transcriptional and posttranscriptional defects of surface immunoglobulin, indicating that EBV infection contributes to the development of PTLD by rendering B-cell receptor (BCR)-deficient and pre-apoptotic germinal center B cells independent from survival signals normally supplied by the BCR (22). Several genetic mutations are associated with PTLD, including c-myc and bcl-6 (41, 214, 374, 428).

Gastric Carcinoma

EBV is associated with approximately 10% of ordinary gastric carcinoma (GAC), while some rare subtypes are associated with EBV more frequently, such as lymphoepithelioma-like GAC (80%) and post-operative gastric stump carcinoma (35%) (152, 369). EBV-associated GAC normally localize to the upper stomach area, and EBV infection is considered a late event in GAC development due to the lack of detection of EBERs in the preneoplastic cells (442). EBV-associated GAC exhibits a unique latency expression pattern, with both latent and lytic genes being expressed (143). Moreover, only LMP2A, but not LMP1 and LMP2B, is expressed in EBV-associated GAC (364). A recent study showed that LMP2A induces the expression of survivin through NF-κB pathway in GAC cell lines, potentially providing survival advantage for EBV-associated GAC (134). The BARF1 gene is also expressed in GAC and can induce expression of cyclin D1 and increase ratio of Bcl2 to Bax to provide anti-apoptotic properties for GAC cells (403, 412, 441). Increasing amount of evidence have suggested that frequent aberrant methylations of CpG island occur in a global but also target-specific manner in EBV-associated GAC (173). Multiple genes are downregulated through methylation-mediated silencing mechanisms, including p14, p16,
p73, PTEN, HOXA10, and RASSF2A (135, 172, 246, 333, 393). Interestingly, despite these implications that EBV provide survival advantages to GAC, EBV infection has been linked to better patient survival (394).

Multiple Sclerosis

EBV has been associated with multiple sclerosis (MS) by epidemiological studies investigating the relationship between infectious mononucleosis and MS, a complex inflammatory CNS disease (235, 373). Elevated humoral and cellular immune response against EBNA1 has been found in MS patients (233, 235). A strong correlation between EBV infection and MS was recently demonstrated by the discovery of selectively-expanded EBNA1 specific CD4+ Th1 cells that also possess the ability to cross-recognize MS-associated myelin antigens to trigger immune response (234). It has also been suggested that potential genetic defects of CD8+ T cells result in decreased T cell reactivity to EBV-infected LCLs and predispose patients to MS by accumulating EBV-infected B cells in the CNS of MS patients (298). Characterized by high titers of anti-EBV antibodies, increased EBV viral load and expression of EBV DNA/RNA, EBV infection has also been linked to other autoimmune diseases, including systemic lupus erythematosus (SLE), primary Sjögren’s syndrome (pSS), and rheumatoid arthritis (RA) (304, 384).

Burkitt’s Lymphoma

EBV-associated Burkitt’s lymphoma (BL) can be basically divided into three categories: endemic, sporadic, and AIDS-associated. BL is 100% EBV-positive in New Guinea and areas of Africa where malaria is holoendemic, and is approximately 85% EBV-
positive in Brazil and North Africa where incidence of BL is lower (16, 34, 36, 238, 305). In these areas, EBV-associated BL is the leading cause of childhood cancers and normally develops at extranodal sites in the jaw during molar eruption or in the CNS (3, 190). Only 15% of sporadic BL in the developed countries are associated with EBV and these cases normally develop at a later age and frequently present with an abdominal mass and occasionally as leukemia (238). Approximately 30-40% of AIDS-associated BLs are associated with EBV. These tumors appear as small B cell lymphoma in adult HIV-carriers and also often occur as the first sign of AIDS development in relatively immunocompetent patients (98, 190).

All BLs appear to be monoclonal and may originate from the germinal center B cells due to expression of germinal centroblasts’ markers, such as CD10, CD77, and Bcl6 (75, 311). It has been suggested that HIV-infection and holoendemic malaria may contribute to BL development by inducing lymphadenopathy and germinal center activity, or recruiting EBV-infected B cells into germinal center reactions (12, 23). BL can have one of three characteristic chromosomal translocations that place the c-myc oncogene under the control of the Ig heavy chain promoter on chromosome 14 or one of the light chain loci on chromosome 2 or 22 (58). These translocations result in deregulation of c-MYC expression and are important for BL proliferation (123, 213, 303). This is especially critical for BL pathogenesis considering that EBV-associated BL tumors have type I latency pattern with expression of only EBNA1 but not major oncoproteins EBNA2 or LMPs (303, 327). However, EBNA1 does seem to provide survival signals for BLs in addition to its normal function of maintaining viral episomes (183). Interestingly, in a subset of BLs where type III latency expression pattern is mostly retained, EBNA2 is deleted from the viral genome (181).
Transcriptional silencing of tumor suppressor genes are often found in BLs in addition to mutations of p53, p14, Bax, and Rb, as well as methylation-mediated inhibition of p16, p73, PUMA, and cyclin D2 (102, 221, 352). Upregulations of several oncogenes, such as TCL1 and MDM2, have also been reported in BLs (193, 220).

**Hodgkin Lymphoma**

A common cancer in developed countries, incidence of Hodgkin lymphoma (HL) peaks at young adults aged 15-34 years and more prevalent in male (163). HL is an unusual tumor, where the neoplastic cells only account for 1% of the cells within the tumor (99). Neoplastic cells in HL, including mononucleate Hodgkin cells and multinucleate Reed-Sternberg cells (collectively called HRS cells), are surrounded by non-malignant inflammatory infiltrate. Different types of infiltrate define HL into three subtypes: nodular sclerosing (NS), mixed cellularity (MC), and lymphocyte-depleted (LD) subtypes. Approximately 40% and nearly 100% of HL cases are associated with EBV-infection in developed and developing countries, respectively (147). And individuals with a prior history of IM have higher risk of developing HL, further indicating the importance of EBV-infection in HL (109). Interestingly, in developed countries EBV-associated HL cases include most MC and LD subtypes but only a minority of the NS subtype, which makes up for the “peak” group of young adults. This phenomenon reflects the fact that EBV-associated HL cases are most frequent in the older population of developed countries. In developing countries, however, HL cases are most frequent in childhood without the peak in young adults (13, 147). Cytokines released from both HRS and inflammatory infiltrate cells, such as IL-1, IL-6, TGF-β, and TNF-α result in the clinical symptoms of HL (187).
In EBV-positive HLs, the virus is found only in malignant HRS cells. Genotyping of Ig V locus has identified that HRS are likely derived from nonfunctional postgerminal center B cells that atypically survived the GC reaction (26, 27, 176, 199). EBV-infected HRS cells exhibit a type II latency expression pattern and have no chromosomal abnormalities (8, 409, 410). EBV-positive HLs demonstrate constitutive activation of NF-κB, and PI3K pathways, which are potentially induced by LMP1 or LMP2A to ensure the proliferation and survival of HRS cells (19, 69, 307, 343). Interestingly, EBV-negative HLs also show constitutive NF-κB activities by inactivating inhibitor of κBα (IκBα) or amplifying the REL gene (198). Deregulation of cellular genes expression have also been shown in HL, including increased IL-10, STAT1, STAT3, and Bcl-3, as well as decreased p27,p53, CDK6, cyclin E, and BCL-XL (100, 306).

Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma (NPC) is a subset of gastric adenocarcinomas and certain salivary-gland carcinomas (309). Its anatomical location is in the upper throat and directly behind the nasal cavities, and the carcinoma arises from the epithelium lining the surface and subterranean chambers of the nasopharynx (162, 395). The World Health Organization (WHO) classifies NPC into three subtypes based on the degree of differentiation: keratinizing squamous cell carcinoma (type 1), non-keratinizing carcinoma (type 2), and undifferentiated carcinoma (type 3), which is the most frequent form of the disease and also shows the most consistent association with EBV infection (309, 347). EBV-associated NPC has specific geographical and ethnic distribution. In most part of the world, including Europe and North America, the incidence of NPC is lower than 1 case per 100,000 human population.
However, the frequency of NPC incidence increases dramatically in Arctic region, South China, and Southeast Asia, with 15-30 cases person 100,000 population per year (430). In Chinese Cantonese males in Hong Kong or Guangzhou, the incidence rates can even climb up to a jaw-dropping 150 cases per 100,000 population per year (129). Notably, the incidence rate of NPC remains high in populations moved from high-incidence areas, as well as in their descendants, regardless of where they live (132). This suggests a genetic predisposition to the disease in addition to environmental triggers such as salted fish consumption and exposure to chemical carcinogens (1, 430). EBV association with more differentiated forms of NPC has also been shown in these high-incidence areas (295).

NPC appears as a mixture of malignant epithelial cells and a prominent, non-malignant, EBV-negative lymphocytic infiltrate, which likely contributes to tumor propagation by producing cytokines (297, 429). NPC cells also express immune regulatory factors, indicating the functional interaction between NPC cells and surrounding lymphoid cells (4). EBV-associated NPC is in type II latency which includes the expression of EBNA1, EBERs, BARTs transcripts, LMP1 (in ~50% of cases), and LMP2A (in ~45% of cases) (130, 297, 309, 312, 429). It has been shown that EBV-associated NPC has monoclonal infection of EBV, indicating that EBV infection occurs before the clonal expansion of malignant cells (311). Some NPC cells may spontaneously undergo productive replication and linear EBV genome can be detected in NPC patients as well as viral lytic antigens (128).

NPC tumor cells express human leukocyte antigen (HLA) class I and II that process antigens efficiently (188, 203). HLA haplotype contributes to NPC pathogenesis as genes linked to HLA are associated with different degrees of risk for NPC development (231).
HLA haplotypes HLA-A2, BW46, A19, and B17 of class I, as well as HLA-DR β10803 of class II are associated with an increased risk for NPC, while HLA-A11 and HLA-B13 of class I are associated with a decreased risk for NPC (73, 190). A distinctive predominance of specific LMP1 strains in endemic NPC has been identified with sequence changes in HLA-restricted epitopes. This finding suggested that LMP1 evade immune recognition by having specific sequence variation in NPC (73). The association between HLA haplotypes and NPC prevalence may explain the high incidence rates of NPC in Chinese descendant who no longer live in high-risk areas.

Other than HLA haplotypes, other genetic factors could be important for EBV infection and NPC development. This is supported by the fact that EBV is monoclonal in malignant cells and also appears in pre-malignant lesions in the nasopharynx (296). Multiple genetic alterations, such as deletions or methylations, have been reported in NPC, locating in chromosomes 3p (RASSF1A), 9p (p16), 11q, 13q, and 14q (280, 429). Interestingly, mutations of p53 are detected in NPC tumors passaged in nude mice but much more infrequently in primary NPC samples, indicating that p53 mutations are not essential for NPC development (74).

Therapeutic Approaches

Traditional treatments of EBV-associated diseases include antiviral agents, immune modulators, and chemotherapeutic drugs. Two major directions of therapeutic approach are being developed to treat EBV-associated tumors, including pharmacological and Immunological-based therapies. In pharmacological methods, one approach is to induce EBV lytic cycle in order to enhance cytotoxic immune response. For example,
demethylating agent 5-azacytidine can de-repress lytic cycle of EBV to induce EBV-encoded kinases that subsequently activate nucleoside analogue gancyclovir for cytotoxic activities (42). Delivery of EBV immediate early genes may elicit similar effects (7, 84, 156). More selective approach was experimented using EBNA1-responsive gene-therapy construct to express cytotoxic proteins or p53 in tumor cells (210). Approaches directly targeting individual EBV proteins have also been tested using single-chain antibodies, antisense RNA, and small chemical compounds or EBNA2-TAT peptide inhibiting NF-κB and RBP-Jκ pathways (37, 83, 184, 302).

Immunotherapy approach has been proven successful firstly in preventing bone-marrow-transplant patients from developing PTLD. Effector T cells were prepared from the bone marrow donor by autologous LCL stimulation and expansion ex vivo before being infused to the recipient to provide EBV-specific, mostly EBNA3s-specific, cytotoxicity (324). Similar adoptive immunotherapy has also been developed in solid organ transplant cases and showed promising results (338). Similar methods are also being developed to target other EBV proteins that are less immunogenic, such as LMPs (396). Dendritic cells pulsed with LMP2 epitopes have been used to treat EBV-positive nasopharyngeal carcinoma patients with noticeable CD8+ T-cell responses (217). Alternative treatments using monoclonal antibodies to target EBV receptor CD21 or B-cell antigen CD24 and CD20 have also been used to try to eliminate EBV-infected B cells (287). Moreover, anti TNF-α agents have been used to treat X-linked lymphoproliferative syndrome (288).

**EBV Vaccines**
The development of EBV vaccines has been hampered by some limitations and concerns. The nature of EBV’s life cycle and tumorigenesis make it difficult to design vaccines delivered to people at risk of developing malignancies such as NPC or lymphoproliferative diseases before primary infection. Moreover, potential antigens expressed by EBV during latency normally have oncogenic potentials and will be evaluated as vaccine candidates with great skepticism (189, 266). However, with increasing understandings of EBV biology as well as immunological controls of EBV-associated diseases, multiple vaccines have been under development for prophylactic protection (IM) and therapeutic purposes (Malignancies) (229). Recombinant gp350 subunit vaccine has been shown to induce neutralizing antibody response to prevent the development of IM caused by EBV-infection after phase I and II clinical study (161, 268, 360). Single peptide-based vaccine using CD8+ T-cell epitope from EBNA3 conjugated with tetanus toxoid as a source of CD4+ T-cell help has shown positive results, after phase I trial, in preventing IM without predisposing recipients to diseases (78). However, the HLA diversity across human population will limit the efficacy of vaccines with single peptide. This problem can potentially be solved by using recombinant virus technique to express multiple epitopes at once (111). A recent mice study showed that a tetrameric vaccinia virus-based vaccine expressing epitopes from gp110, gp350, EBNA2, and EBNA3C elicited the production of neutralizing antibodies and EBV-specific T-cell responses (225). This “cocktail” strategy may prove to be efficient against both EBV primary infection and EBV-associated malignancies.

**Epidermal Growth Factor Receptor in NPC**
Upregulation of the epidermal growth factor receptor (EGFR) has been detected in over 70% of EBV-associated NPC samples and correlated with LMP1 expression level (291, 349, 437). Moreover, the level of EGFR expression is positively associated with the stage of the disease (291). Treatment with EGFR tyrosine kinase inhibitors induces cell cycle arrest, inhibits cell proliferation of NPC cell lines, and exhibits potent antitumor effects on NPC xenografts using in vivo mouse model (366, 404, 421, 440).

EGFR is a member of the ErbB receptor tyrosine kinase family which includes EGFR (ErbB-1), HER2/c-neu (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4). Upon ligand binding, ErbBs undergo dimerization, autophosphorylation, activation, and subsequently degradation/turnover from an originally inactive conformation (86, 432). Multiple signaling pathways are activated downstream of EGFR, including Ras/MAPK, Src kinases, JAKs/STATs, and PI3K-AKT pathways (170). Deregulated EGFR signaling has been associated with tumor progression, invasion, and metastasis (411). EGFR signaling has been shown to be targeted by oncogenic viral proteins to regulate tumorigenesis, including v-ErbB, E5, and HBVx (254). EGFR also serves as cellular receptor for HCMV entry and signaling (405).

One important mechanisms by which EGFR becomes active and potentially oncogenic is through activating mutations (431). Numerous activating mutations have been identified in EGFR that can affect ligand binding, receptor dimerization and phosphorylation, as well as its enzymatic activity and substrate specificity (318). However, somatic mutations were not detected in a comprehensive sequencing analysis in NPC (202). This indicates that alternative mechanisms, such as amplification of EGFR found in NPC, play major roles in NPC development. EGFR was previously shown to be induced by LMP1 in epithelial cell
line C33A at transcriptional level (250, 251, 253). This transcriptional upregulation was found to be mediated by a novel NF-κB complex comprised with p50 homodimer and Bcl-3 (379, 380). LMP1-induced EGFR may play a very important role in EBV tumorigenesis and is a prime target for development of therapeutic strategies. Interestingly, in current EGFR-targeted treatments using chemical inhibitors and monoclonal antibodies, patients with elevated EGFR expression level generally exhibit better response (70, 136, 259). This further suggests NPC patients could more likely benefit from EGFR-specific treatments.
OBJECTIVES

EBV LMP1 is the main oncoprotein of EBV during latent infection. LMP1 is required for EBV-mediated B cell transformation and overexpression of LMP1 results in deregulated proliferation and transformation of rodent fibroblast cells. The oncogenic potential of LMP1 is due to multiple signaling pathways mediated through two major signaling domains of LMP1, CTAR1 and CTAR2. CTAR1 is capable of inducing cell transformation while CTAR2 is dispensable, indicating that signaling transduction downstream of CTAR1 are critical for LMP1-mediated transformation and ultimately, EBV-associated tumorigenesis. The goal of the studies presented in this dissertation is to evaluate several signaling pathways engaged by LMP1-CTAR1 to regulate cellular gene expression and cell transformation focused on CTAR1-specific induction of EGFR.

Aim 1. LMP1 induces EGFR upregulation in epithelial cells through STAT3 activation and Bcl-3 induction.

In C33A cells and NPC, LMP1-CTAR1 induces EGFR transcriptional expression through a transactivating complex comprised of p50 and Bcl-3. The effects of LMP1 on Bcl-3 expression and translocation in C33A cells were analyzed in this study. The connection between CTAR1-mediated STAT3 activation and EGFR upregulation was established using chemical inhibitors and chromatin-immunoprecipitation analysis.

Aim 2. LMP1 modulates distinctive NF-κB pathways through CTAR1 to regulate EGFR expression.

In addition to EGFR induction, CTAR1 also activates multiple forms of NF-κB. Mouse embryonic fibroblasts (MEFs) defective for different NF-κB effectors were used in
this study to evaluate the effects of NF-κB pathways on CTAR1-mediated EGFR induction. MEFs defective for NF-κB effectors or different TRAFs were also used to identify the factors involved with CTAR1-induced p50 activation.

**Aim 3. Effects of CTAR1 on signaling transductions involved with EGFR and STAT3.**

In addition to increased EGFR expression, we show that overexpression of CTAR1 also induced EGFR activation intrinsically and in response to an EGFR ligand. EGFR-mediated STAT3 activation and potential downstream targets were evaluated. Importantly, PKCδ was identified as the serine kinase of STAT3 in CTAR1-expressing C33A cells and was required for activation of ERK. The effects of PKCδ on CTAR1-mediated transformation were tested using focus formation assay.
REFERENCES


CHAPTER TWO

Epstein-Barr Virus Latent Membrane Protein 1 Induces Expression of the Epidermal Growth Factor Receptor Through Effects on Bcl-3 and STAT3

Che-Pei Kung and Nancy Raab-Traub

This work was originally published in Journal of Virology (2008), Vol. 82 No. 11:

5486-5493
ABSTRACT

Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) activates multiple signaling pathways. Two regions, CTAR1 and CTAR2, have been identified within the cytoplasmic carboxy terminal domain that activates NF-κB. C-terminal activating region 2 (CTAR2) activates the canonical NF-κB pathway which includes p50/p65 complexes. C-terminal activating region 1 (CTAR1) can activate both the canonical and noncanonical pathways to produce multiple distinct NF-κB dimers, including p52/p50, p52/p65, and p50/p50. CTAR1 also uniquely upregulates the epidermal growth factor receptor (EGFR) in epithelial cells. Increased p50-Bcl-3 complexes have been detected by chromatin precipitation on the NF-κB consensus motifs within the egfr promoter in CTAR1-expressing epithelial cells and NPC cells. In this study, the mechanism responsible for the increased Bcl-3 has been further investigated. The data indicate that LMP1-CTAR1 induces Bcl-3 mRNA and increases the nuclear translocation of both Bcl-3 and p50. LMP1-CTAR1 constitutively activates STAT3 and this activation was not due to induction of IL-6. In LMP1-CTAR1-expressing cells, increased levels of activated STAT3 were detected by chromatin immunoprecipitation on STAT-binding sites located within both the promoter and second intron of Bcl-3. A STAT3 inhibitor significantly reduced the activation of STAT3, as well as the CTAR1-mediated upregulation of Bcl-3 and EGFR. These data suggest that LMP1 activates distinct forms of NF-κB through
multiple pathways. In addition to the canonical and noncanonical pathways, LMP1-CTAR1 constitutively activates STAT3 and increases Bcl-3. The increased nuclear Bcl-3 and p50 homodimer complexes positively regulate EGFR expression. These results indicate that LMP1 likely regulates distinct cellular genes by activating specific NF-κB pathways.

INTRODUCTION

Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) is an essential factor in EBV-induced transformation and is expressed in many of the malignancies associated with EBV, including post-transplant lymphoma, Hodgkin's disease, and nasopharyngeal carcinoma (NPC) (12, 28, 51, 66). LMP1 is considered a constitutively activated member of the tumor necrosis factor receptor (TNFR) family and binds TNF associated factors (TRAFs) (27, 41, 44). Two major signaling domains have been identified within the cytoplasmic C-terminal domain of LMP1, CTAR1 and CTAR2, that can activate NF-κB (25). However, CTAR1 has several unique properties and is essential for transformation while CTAR2 is dispensable (34, 35). LMP1-CTAR1 uniquely induces expression of the epidermal growth factor receptor (EGFR) at the mRNA level and this induction requires NF-κB and is mediated through the TRAF signaling pathway (41-43). Subsequent studies have identified other genes that are uniquely activated by
LMP1-CTAR1, including TRAF1 and EBI3 (14).

The NF-κB transcription factors dimerize and bind NF-κB consensus sequences in cellular and viral promoters to regulate the expression of genes controlling inflammation, cell cycle regulation, apoptosis, and oncogenesis (21, 38). There are five mammalian NF-κB family members including p50, p52, p65 (RelA), c-Rel, and RelB. The activation of NF-κB family members is regulated through interactions with inhibitors of NF-κB (IκB), which sequester NF-κB members in the cytosol. Activation of a kinase cascade that includes IKKα, IKKβ, and IKKγ results in phosphorylation, ubiquitination, and degradation of an IκB, leading to the release and nuclear translocation of NF-κB. The p50 and p52 precursor proteins, p105 and p100 respectively, can also function as IκB.

Early studies initially showed that LMP1-CTAR1 activated multiple forms of NF-κB including p50/p65, p50/p52, and p50 homodimers and also greatly increased the processing of p100 to p52 in epithelial cells and the nuclear translocation of p50 (41, 48). It has subsequently been shown that the induction of processing of p100 represents another mechanism for activation of NF-κB. This is considered the noncanonical NF-κB pathway and the activation of this pathway is specific for LMP1-CTAR1 (1, 15, 33, 53). Noncanonical activation of NF-κB requires IKKα and is mediated through the NIK kinase to induce processing of p100 and activate p52/relB. Canonical activation requires IKKβ and IKKγ to activate p50/p65. The activation of specific genes by LMP1
has been linked to the canonical and noncanonical pathways using engineered mouse
fibroblasts (33). MIP-2 was activated by canonical pathway, which is
IKKβ/IKKγ-dependent. Induction of the cellular chemokine, CXCR4, required IKKα
and was considered activated by the noncanonical pathway. An atypical pathway was
also identified that was IKKβ dependent but independent of IKKγ and regulated
expression of MIG and I-TAC.

The link between LMP1-CTAR1 activation of unique genes and distinct forms of
NF-κB was demonstrated in studies that showed that LMP1-CTAR1 induced the binding
of NF-κB p50 and Bcl-3 to the NF-κB sites in the egfr promoter in C33A cells (59).
LMP1 effectively induces the nuclear translocation of p50 and p50/p50 homodimers are
the major NF-κB complex activated in LMP1-expressing cells and EBV-positive
xenografted NPC tumors (48, 58, 59). In addition, elevated levels of p50/p50
homodimers and Bcl-3 are found in classical Hodgkin lymphoma and anaplastic
large-cell lymphomas that are associated with EBV infection (37).

In this study, the effects of LMP1-CTAR1 on Bcl-3 expression and EGFR
induction were further evaluated. LMP1-CTAR1 induced Bcl-3 transcription resulting
in increased levels of nuclear Bcl-3. The transcriptional activation of Bcl-3 required
STAT3, which bound to sites within the Bcl-3 promoter and intron 2. LMP1-CTAR1
expression increased both the serine and tyrosine phosphorylations of STAT3 that are
indicative of activation. These data indicate that LMP1 activates distinct forms of NF-κB through different pathways. In addition to activation of the canonical and noncanonical pathways, LMP1-CTAR1 also activates p50/p50 homodimers by increasing expression of Bcl-3 through its effects on STAT3.

MATERIALS AND METHODS

Retrovirus production and transduction

Recombinant retrovirus production and transduction were performed as previously described to establish C33A stable cell lines expressing full-length LMP1, CTAR1 (1-231), CTAR2 (d187-351), or vector control pBabe (34). Briefly, ~80% confluent 293T cells were triple transfected using FuGEGE 6 transfection reagent (Roche) according to the manufacturer’s instruction with 5µg pBabe (vector), pBabe-HA-LMP1, pBabe-HA-1-231, or pBabe-HA-d187-351, and 5µg pVSV-G and 5µg pGag/Pol expressing plasmids. After 24 hours incubation at 37°C, media were replaced with fresh media and cells were incubated at 33°C for another 24 hours. Cell supernatant then was centrifuged at 1000g for 5 min to remove cell debris and virus-containing supernatant was collected. C33A cells with ~70-80% of confluence were then transduced with clarified supernatant with 4µg/ml polybrene for 24 hours at 37°C.
**Cell Culture and stable cell lines**

C33A cervical carcinoma cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (Sigma) and antibiotic/antimycotic (Gibco) at 37°C with 5% CO₂. C33A stable cell lines expressing full-length LMP1, CTAR1 (1-231), CTAR2 (d187-351), or vector control pBabe were established by retroviral transduction followed by selection and passage in the presence of 1µg/ml puromycin (Sigma).

**Fractionation of Cells**

After cultured cells reached ~80-90% confluence, cells were scrape harvested, washed once with cold phosphate-buffered saline (PBS, Gibco), centrifuged at 1000g, and lysed with RIPA buffer (20mM Tris-HCl [pH 7.5], 150mM NaCl, 1mM EDTA, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS], 0.1% deoxycholic acid) supplemented with phenylmethysulfonyl fluoride (PMSF), sodium orthovanadate (Na₃VO₄), protease and phosphatase inhibitor cocktail (Sigma). Lysates were then clarified by centrifugation at 13,000 rpm, 4°C for 15 min and supernatants containing whole cell lysates were removed to new tubes. Nuclear extracts were made as previously described with slight modification (59). Briefly, cells were scrape harvested, washed once with cold PBS, and lysed by incubation in a hypotonic buffer (20 mM HEPES, 10 mM KCl, 0.1 mM EDTA,
0.1 mM EGTA) supplemented with PMSF, Na$_3$VO$_4$, protease and phosphatase inhibitor cocktail (Sigma) for 15 min on ice. Nonidet P-40 was then added to a final concentration of 1%, followed by 1 min of vortex. Nuclei were pelleted by low-speed centrifugation at 1,200 rpm for 10 min at 4°C and the supernatant were collected as cytoplasmic fraction. The nuclei fraction were purified using the Optiprep reagent (Sigma) as directed by the manufacturer, as previously described (58). Nuclei were lysed with nuclear extraction buffer (20 mM Tris-HCl [pH 8.0], 420 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 25% glycerol, PMSF, Na$_3$VO$_4$, protease and phosphatase inhibitor cocktail [Sigma]) with the salt concentration adjusted to 400 mM with 5 M NaCl. All lysates were stored at -80°C.

*Western Blot Analysis*

Protein concentration of cell lysates was determined using Bio-Rad DC protein assay system according to manufacturer’s instruction. Equal amounts of protein were used for SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Optitran® (Schleicher and Schuell) for Western blot analysis. Primary antibodies used include anti-p50, anti-β-actin, anti-GRP78, anti-STAT3, anti-PARP (Santa Cruz), anti-Bcl-3 (Upstate Biotechnology), anti-phospho-STAT3 (Ser 727 and Tyr 705) (Cell Signaling), anti-phospho-EGFR (Tyr 1068) (BD Biosciences), and anti-HA tag (Covance). A rabbit
antiserum raised against the carboxyl-terminal 100 amino acids of the EGFR fused to glutathione S-transferase (kindly provided by H. Shelton Earp) was used to detect total EGFR. Secondary antibodies used to detect bound proteins include horseradish peroxidase-conjugated antimouse, antirabbit (Amersham Pharmacia), and antigoat (DAKO). Blots were developed using Pierce Supersignal West Pico chemiluminescence system followed by exposure to film.

**Chromatin Immunoprecipitation (ChIP) Analysis**

ChIP analysis was performed using a ChIP kit (Upstate Biotechnology) according to manufacturer's protocol. Briefly, cells were cultivated in 100 mm plates to 90% confluency and scrape harvested. Cells were then fixed for 5 min in 1% freshly-made formaldehyde, washed with PBS, and lysed for 10 min in lysis buffer provided in the kit. Chromatin was sheared by sonication to an average size of ~200-500 bps, clarified, and precleared for 1 h at 4°C with salmon sperm DNA-saturated protein G-Sepharose beads. The supernatant was incubated with normal rabbit IgG, with anti-STAT3 (Santa Cruz), or with anti-phospho-STAT3 (Ser 727, Cell Signaling) and nutated overnight at 4°C. Lysates were immunoprecipitated with salmon sperm DNA-saturated protein G-Sepharose beads for 1 h at 4°C and washed extensively according to the manufacturer's instruction. Input and immunoprecipitated protein/DNA complexes were eluted at room
temperature and the cross-linking was reversed overnight at 65°C in the presence of 200 mM NaCl. After RNase A (37°C for 30 min) and proteinase K (45°C for 2 h) treatment, sample DNAs were purified as directed by the manufacturer for further analysis. PCR of ChIP products were performed with HotStar Taq polymerase (Qiagen) and Primer pairs used for different ChIP target sequences include: Bel-3-Pro: 5’ TGACCCGGACTCAACCCCAG 3’ and 5’ TCTCCTCCCCTCCTCCCTC 3’: HS3: 5’ CGCTTCTCCCAACCTTAACC 3’ and 5’ TGCCCAGTCCCTAACCTCTT 3’: HS4: 5’ CATTGAGGATGGGAAGTTGG 3’ and 5’ CAGGGTTAAGTGAGGCGAGA 3’.

Quantitative Real Time Polymerase Chain Reaction (QRT-PCR)

Total cell RNA was isolated using RNAeasy kit as directed by the manufacturer (Qiagen). Primer pairs used in this paper include actin: 5’ TCACCACACTGTGCCCATCAGA 3’ and 5’ CAGCGGAACCGCTCATTCGCAATGG 3’: EGFR: 5’ CTGCAGTCTTCTGTCCGAGATG 3’ and 5’ TTGCTCACCCCTCCAGAGG 3’: Bel-3: 5’ ACAACAGCCCTTAGCATGGTG 3’ and 5’ GCTGAGTGCAGGGCGGAGCT 3’: IL-6: 5’ AGCCACTCACCTTCAGAAG 3’ and 5’ GCTGCTTTCACACATGTTACTCTT 3’. Quantitative real-time-PCR (QRT-PCR) was performed using Quantitect SYBR Green RT-PCR kit (Qiagen) according to the manufacturer’s instruction. Amplification of PCR
products was detected using ABI 7900HT sequence detection system (Applied Biosystems) and analyzed using SDS 2.0 software (Applied Biosystems). The cycle threshold ($C_T$) was determined as the number of PCR cycles required for a given reaction to reach an arbitrary fluorescence value within the linear amplification range. The change in $C_T$ ($\Delta C_T$) was determined between the same target gene primer sets and different samples, and the change in $\Delta C_T$ ($\Delta\Delta C_T$) was determined by adjusting for the difference in the number of cycles required for actin to reach the $C_T$. The fold change was determined as $2^{\Delta\Delta C_T}$ since each PCR cycle results in a twofold amplification of each PCR product. Quantitative real time-PCR was also performed to amplify ChIP products and primer pairs used for different ChIP target sequences include: Bcl-3-Pro: $5'$ TGACCCGGACTCAACCCCAG 3' and 5' TCTCCTCCCCTCCTCTCCCT 3'; HS3: 5' CGCTTCCCTCCAACCTTAACC 3' and 5' AAGAGGAGCCGGTGCGGCGAG 3'; HS4: 5' TTACTGGAAGTCCGAGGGCT 3' and 5' TTCAGAGAAACCGTCCAGGC 3'.

RESULTS

**CTAR1 of LMP1 induces EGFR mRNA and protein.** The CTAR1 domain of LMP1 has previously been shown to induce expression of the EGFR. The full length LMP1, 1-231 (contains only CTAR1 but not CTAR2), and d187-351 (contains only CTAR2 but not CTAR1) previously cloned into the pBabe retroviral expression vector
were stably transduced into C33A epithelial cells (Fig. 1B) (17). Expression of LMP1 and the CTAR deletion mutants and EGFR expression was evaluated by immunoblotting (Fig. 1A). As previously shown, LMP1 and CTAR1 but not CTAR2 of LMP1 induced EGFR expression with highly elevated levels induced by CTAR1 (43). In addition, high levels of phosphorylated, activated EGFR were detected using a phospho-specific antibody, indicating that the EGFR induced by CTAR1 is functionally active. Real-time quantitative reverse transcription PCR (QT-PCR) confirmed previous studies that indicated that LMP1 upregulates EGFR at the mRNA level (42) (Fig. 1C).

Quantification of the immunoblot indicated that in C33A cells LMP1 and CTAR1 induced EGFR mRNA expression 7-fold and 17-fold, respectively. LMP1-CTAR2 did not affect EGFR mRNA level or the levels of phosphorylated EGFR protein. Although the levels of LMP1 expression were very similar, the total and activated EGFR induced by LMP1-CTAR1 was significantly higher than full-length LMP1. This suggests that CTAR2 or sequences between CTAR1 and CTAR2 may inhibit the ability of CTAR1 to induce specific targets, such as EGFR.

LMP1-CTAR1 upregulates Bcl-3 expression and induces nuclear translocation of Bcl-3 and p50. In studies of EBV-positive NPC xenografts, p50 and Bcl-3 were detected by chromatin immunoprecipitation (ChIP) to be bound to the EGFR promoter while other forms of NF-kB were not detected (58). In addition, in C33A cells,
transient overexpression of Bcl-3 and/or p50 slightly increased EGFR expression and p50/Bcl-3 complexes could be detected by CHIP on the EGFR promoter in C33A cells expressing LMP1-CTAR1 (59). To determine the effect of LMP1 and LMP1-CTAR1 on the localization and expression levels of Bcl-3 and p50, lysates of whole cell and nuclear fractions were analyzed by western blotting. Both LMP1 and LMP1-CTAR1 increased levels of Bcl-3 in the whole cell lysates, approximately 2.1-fold and 4.6-fold respectively (Fig. 2A). Elevated levels of p50 and Bcl-3 were also detected in the nucleus of the LMP1-expressing C33A cells (Fig. 2B). Equal loading was confirmed by
immunoblotting for the cytosolic and nuclear proteins, GRP78 and PARP.

Quantification using the ImageJ software and normalization to the intensity of PARP bands indicated that LMP1-expressing C33A cells had a 1.4–fold increase of nuclear Bcl-3 and LMP1-CTAR1 expressing cells had an approximately 1.9–fold increase.

Fig. 2. CTAR1 of LMP1 upregulates Bcl-3 and induces nuclear translocation of Bcl-3 and p50. A) Bcl-3 expression in stable C33A cells was examined by western blotting and quantitated using ImageJ software. Data shown are the mean values of three independent experiments. B) Nuclear p50 and Bcl-3 in C33A stable cells were shown by western blotting and quantitated using ImageJ software. Data shown are the mean values of four and three independent experiments for Bcl-3 and p50, respectively. C) mRNA of Bcl-3 was examined by quantitative RT-PCR. Fold change was normalized to actin. Data shown are the mean values of three independent experiments, each being performed in triplicate.
LMP1-CTAR2 did not affect the levels of whole cell or nuclear Bcl-3 compared to vector control cells. Expression of LMP1 or LMP1-CTAR1 also significantly induced the nuclear translocation of NF-κB p50, approximately 13- and 16-fold, respectively (Fig. 2B) (48). Although LMP1-CTAR2 induces greater levels of NF-κB activity in reporter assays, in C33A cells expressing LMP1-CTAR2 nuclear p50 was only increased 5-fold (25, 57). QT-PCR using Bcl-3 specific primers indicated that Bcl-3 mRNA was increased approximately 1.9-fold and 3.3-fold inductions in LMP1- and LMP1-CTAR1 expressing cells (Fig. 2C). LMP1-CTAR2 did not affect the Bcl-3 mRNA level. These results indicate that LMP1-CTAR1 not only induces the nuclear translocation of NF-κB Bcl-3 and p50, it also transcriptionally activates Bcl-3.

**STAT3 is constitutively activated by LMP1-CTAR1.** Previous studies have shown that LMP1 can activate signal transducer and activator of transcription 3 (STAT3) and that the activated STAT3 may regulate LMP1 expression through effects on the novel LMP1 promoter within the terminal repeats that is active in NPC (9, 10, 32, 52). STAT3 has also been shown to transcriptionally activate Bcl-3 through enhancer sequences detected within the Bcl-3 introns (4). The transcriptional activity of STAT3 is regulated by phosphorylation. Phosphorylation at tyrosine 705 induces STAT3 dimerization while phosphorylation at serine 727 affects DNA binding and transcriptional activity (3, 13). To determine the effects of LMP1 and LMP-1 on STAT3 activation, serine and tyrosine
phosphorylated STAT3 was identified using phospho-specific STAT3 antibodies, quantified by ImageJ, and normalized to loading control, GRP78 (Fig. 3A). The fold induction is indicated beneath the corresponding bands of a representative experiment out of three independent attempts. Cells expressing LMP1 had an approximately 2.4-fold

**Fig. 3.** CTAR1 of LMP1 upregulates Bcl-3 and EGFR by activating STAT3.  A) Total and phosphorylation levels of STAT3 were examined by western blotting. Fold inductions are listed beneath their corresponding bands. The blot is representative of three independent experiments.  B) Phosphorylation of STAT3, as well as expression of Bcl-3 and EGFR, was examined in 1-231-expressing C33A cells treated with the STAT3-specific inhibitor, Cucurbitacin.  C) mRNA of EGFR was examined by quantitative RT-PCR in pBabe control, 1-231 cells treated with DMSO, and 1-231 cells treated with Cucurbitacin. Fold change was normalized to actin. Data shown are the mean values of four independent experiments, each being performed in triplicate.
increase in tyrosine-phosphorylated STAT3 and 2.5-fold increase in serine-phosphorylated STAT3 compared to vector control. Cells expressing LMP1-CTAR1 had an approximately 3-fold increase in tyrosine-phosphorylated STAT3 and 4-fold increase in serine-phosphorylated STAT3. LMP1-CTAR2 had an approximately 1.6-fold increase in tyrosine-phosphorylated STAT3 and 1.7-fold increase in serine-phosphorylated STAT3. Although the levels of phosphorylated STAT3 were increased by LMP1, the total level of STAT3 was not affected. Cucurbitacin is a specific inhibitor of STAT3 activation through effects on the Janus kinases (2, 31). Treatment of the LMP1-CTAR1 expressing C33A cells with Cucurbitacin reduced both the tyrosine and serine phosphorylation of STAT3 induced by LMP1-CTAR1 (Fig. 3B). The effects of Cucurbitacin were dose-dependent in that 1μM of Cucurbitacin reduced LMP1-CTAR1-mediated induction to the level detected in control cells while 10μM eliminated phosphorylated STAT3. Importantly, treatment with Cucurbitacin significantly reduced the effects of LMP1-CTAR1 on Bcl-3 and EGFR and at 10μM expression of Bcl-3 was eliminated. QT-PCR of EGFR mRNA indicated that EGFR mRNA was reduced 63% and 86% by 1μM and 10μM of Cucurbitacin (Fig. 3C). These results suggest that CTAR1-mediated STAT3 activation is required for Bcl-3 induction and at least partially responsible for the induction of EGFR. Previous studies have shown that LMP1 can induce expression of IL-6 resulting in activation of STAT3 (4, 9, 10,
To determine whether LMP1-mediated STAT3 activation resulted from IL-6 induction in C33A cells, QRT-PCR was performed to detect IL-6 mRNA (Fig. 4). In C33A cells, IL-6 mRNA level was induced by LMP1, LMP1-CTAR1, or LMP1-CTAR2 to 1.6-, 2.7-, and 4.2-fold, respectively. The highest induction was detected in LMP1-CTAR2 cells that didn’t activate STAT3. These data indicate that STAT3 is constitutively activated by LMP1-CTAR1 in C33A cells through mechanisms that are not dependent on IL-6 induction.

![IL-6 QRT-PCR](image)

**Fig. 4. IL-6 production in C33A cells.** Expression level of IL-6 mRNA in stable C33A cells was analyzed by quantitative RT-PCR. Fold change was normalized to actin. Data shown are the mean values of four independent experiments, each being performed in triplicate.

LMP1 CTAR1 induces STAT3 binding to sites in the Bcl-3 promoter and introns. Two enhancers, HS3 and HS4, have been identified within introns of Bcl-3 that mediate STAT3 induction of Bcl-3 (4). HS3 contains one and HS4 has three putative
STAT3 binding sites, respectively (Fig. 5A). The online program, ALGGEN-PROMO, was also used to predict STAT3 binding sites in the Bcl-3 promoter. One potential site (Bcl-3-Pro) was identified at approximately 1200 bps upstream of the Bcl-3 transcriptional start site (Fig. 5A) (18, 39). To determine if LMP1-CTAR1 induces binding of STAT3 to these putative binding sites, ChIP analysis was performed in C33A cells stably expressing pBabe vector control and LMP1-CTAR1 using primers specific for each of the three predicted sites (Fig. 5B). In the pBabe control cells, precipitation with STAT3 or serine-phosphorylated-STAT3 antibodies did not increase the amplification compared to precipitation with normal rabbit immunoglobulin. In LMP1-CTAR1 cells, precipitation with STAT3 antibody slightly increased amplification of all three putative STAT3 binding sites. However, precipitation with the serine-phosphorylated specific STAT3 antibody detected increased binding between STAT3 and all three putative STAT3 binding sites in the presence of LMP1-CTAR1. LMP1-CTAR1 did not increase interaction between STAT3 and a nonspecific target within EGFR Exon2, indicating the specificity of the effect (data not shown). This result was confirmed using quantitative PCR to amplify ChIP products and the data were normalized to determine the enrichment fold in LMP1-CTAR1 cells compared to pBabe control cells (Fig. 5C). In LMP1-CTAR1 cells, the binding of serine phosphorylated STAT3 to Bcl-3-Pro, H3, and
H4 was increased 4.2-, 3-, and 4.6-fold, respectively. These data indicate that LMP1 CTAR1 likely regulates Bcl-3 by increasing the interaction of serine phosphorylated STAT3 to multiple binding sites that regulate Bcl-3 expression.

Fig. 5. LMP1 CTAR1 induces binding of STAT3 to Bcl-3 promoter and intronic enhancers. A) Schematic representation of putative STAT binding sites. HS3 and HS4 were suggested in Broeke-Heidrich K et al. paper. Bcl-3-Pro was predicted using ALGGEN-PROMO online program. B) Chromatin immunoprecipitation (ChIP) analysis using normal IgG (lane 2), STAT3 (lane 3), and Serine-phosphorylation-STAT3 (lane 4) antibodies in pBabe control and CTAR1-expressing C33A cells. Precipitated complexes were subjected to PCR with primer pairs specific to Bcl-3-Pro, HS3, and HS4 regions. PCR was also performed with chromatin input (lane 1). C) Quantitative real time-PCR was performed to amplify ChIP products from pBabe control and CTAR1-expressing cells. Enrichment fold was calculated by normalizing results from CTAR1 cells to pBabe cells. Data shown are the mean values of two independent experiments, each being performed in triplicate.
DISCUSSION

The induction of EGFR expression by LMP1 is likely a contributing factor to the development of cancer. The EGFR is frequently expressed at high levels in a variety of human cancers, including NPC (40, 54, 69). Stimulation of the tyrosine kinase activity of EGFR affects multiple signaling pathways leading to deregulation of cellular growth control and tumorigenesis (5, 65). Thus the effect of LMP1 on EGFR expression is likely an important factor in carcinogenesis. The unique activation of EGFR expression by LMP1-CTAR1 provides a novel system to assess the contribution of NF-kB activation.

It was initially thought that p50/p50 or p52/p52 homodimers were transcriptionally inactive as these forms of NF-kB lack transactivation domains. However, subsequent studies determined that Bcl-3, which contains a transcriptional transactivation domain, can convert these complexes into active forms (19, 46, 47). It is thought that one of the major functions of Bcl-3 is to bring p50 into the nucleus and the increased nuclear presence of p50 correlates with elevated Bcl-3 in LMP1 CTAR1-expressing cells (61, 68).

It has also been suggested that Bcl-3 might contribute to p50 activation by inhibiting the ubiquitination and subsequent degradation of DNA-bound p50 homodimers (7). The data presented here indicate that Bcl-3 is also regulated by LMP1-CTAR1 resulting in increased Bcl-3 mRNA, protein, and nuclear translocation of Bcl-3. The data also indicate that the effects of LMP1 on Bcl-3 are linked to its effects on STAT3. STAT3 is
known to be activated in EBV-infected and LMP1-expressing epithelial cells and B-cell lymphomas (6, 9, 10, 32, 55). The induction of IL-6 by LMP1 can result in activation of STAT3 and increased IL-6 production has been detected in EBV-infected and LMP1-expressing epithelial cells (9, 10). In multiple myeloma cells, Bcl-3 transcription has been shown to be induced by IL-6 via STAT3 binding to intronic enhancers (4). However as shown here, IL-6 transcription was slightly induced in LMP1- and CTAR1-expressing C33A cells but was induced the highest in CTAR2-expressing cells, where no EGFR or Bcl-3 induction was detected. In addition, treatment with recombinant IL-6 did not increase serine phosphorylation of STAT3 or EGFR expression in pBabe control or CTAR1-expressing cells (data not shown). These data indicate that LMP1 CTAR1 mediates constitutive activation of STAT3 independently of effects on IL-6. IL-6-independent STAT3 activation by EBV has been suggested in studies where retinoic acid treatment of EBV-immortalized B lymphocytes inhibited IL-6-dependent but not constitutive STAT3 activation (67).

It is not yet clear what exact signaling pathways activate STAT3 in LMP1-expressing cells. It was previously suggested that a novel activating region between CTAR1 and CTAR2 of LMP1 interacts with Janus kinase 3 (JAK3) and activates JAK/STAT signaling pathway (22). However, in EBV-transformed lymphoblastoid cell lines, the putative activating region did not mediate JAK3 association or JAK/STAT3
activation (23). In addition, in data presented here LMP1-CTAR1 is a deletion mutant, containing aa 1-231, that lacks this domain yet activates STAT3 considerably more effectively than full-length LMP1 (Fig. 3). These data indicate that the putative JAK-binding domain is not important for LMP1-mediated STAT3 activation in C33A cells. However, the inhibition of this activation by Cucurbitacin which is thought to inactivate STAT3 through inhibiting JAK2 and JAK3 activity may indicate that regulation of JAK activity contributes to LMP1-mediated STAT3 activation (2, 56). It will be of interest to determine how the CTAR1/TRAF complexes possibly affect STAT3 activity or the Janus kinases.

Several signaling pathways affected by LMP1 have been associated with STAT3 activation. Serine phosphorylation of STAT3 has been shown to be mediated by PI3K/AKT and ERK signaling pathways which are both activated by LMP1-CTAR1 (11, 20, 34, 35, 50, 62). Interestingly, EGFR also activates STAT3 which suggests a positive signaling loop of STAT3→Bcl-3→EGFR→STAT3 may be established in LMP1-expressing cells and during EBV-mediated transformation (8, 49).

A recent study suggested that LMP1 induces Bcl-3 expression through CTAR2 and NF-κB pathway (45). This study showed that deletion of CTAR2 or the NF-κB binding sites in the Bcl-3 promoter abolished activity of a Bcl-3 reporter construct in LMP1-expressing Jurkat cells. The difference between this study and the data presented
here may reflect that the construct used in that study contained amino acid 1-331 of LMP1 protein, which is 100 amino acids longer than the CTAR1 construct (1-231) tested in our study. This region of aa 231-331 of LMP1 may have an inhibitory effect on CTAR1-mediated Bcl-3 induction. Possible inhibition by this domain is also suggested by the fact that CTAR1 alone has higher activity of inducing Bcl-3 and EGFR than full-length LMP1 (Fig. 1 and Fig. 2). Importantly, the LMP1-CTAR2 deletion mutant was only tested using a Bcl-3 reporter construct and CTAR1 was not tested for its effects on Bcl-3 expression in cell lines. In addition, the reporter construct did not include the STAT3-binding site located approximately 1200 bps upstream of Bcl-3 transcriptional start site which had the strongest interaction with STAT3 and serine phosphorylated STAT3 in CTAR1-expressing C33A cells. This STAT3 binding site is likely important for CTAR1-mediated Bcl-3 induction.

It is intriguing that CTAR1 had a considerably stronger effect than full length LMP1 although both were expressed at very similar levels. It is possible that the presence of CTAR2 downregulates the ability of CTAR1 to induce Bcl-3 and EGFR. LMP1 CTAR2 mediates downstream signaling pathways through TRAF2 or TRAF6 which both are potential E3 ubiquitin ligases (26, 63). Since it has been shown that both EGFR and Bcl-3 can be regulated by ubiquitination, it is possible that either CTAR2-recruited TRAF2 or TRAF6 affects LMP1-mediated induction of EGFR and
Bcl-3 (24, 29, 30, 36, 60).

In summary, the data here suggest a model of LMP1-mediated induction of EGFR with constitutive activation of STAT3 by CTAR1 through induction of tyrosine and serine phosphorylation of STAT3 (Fig. 6). The serine phosphorylated STAT3 would increase Bcl-3 expression through interaction with multiple STAT-binding sites in both the promoter and introns of Bcl-3. The increased levels of Bcl-3 and the elevated levels of p50 form a transcriptional active complex to transactivate EGFR. The link between

Fig. 6. A working model for LMP1 CTAR1-mediated induction of Bcl-3 and, consequently, EGFR.
STAT3 and EGFR has also been shown by microarray data of epithelial cells overexpressing STAT3 (64). It has been suggested that the unique activation of distinct genes by LMP1-CTAR1 reflects its activation of the noncanonical pathway (33). However, the data presented here reveal that LMP1-CTAR1 has an additional mechanism to activate NF-κB through its effects on STAT3 and Bcl-3. As multiple genes have been shown to be regulated by the Bcl-3/p50-homodimer complex, it is likely that this pathway is responsible for other genes induced by LMP1 in EBV-associated tumorigenesis.

ACKNOWLEDGMENTS

We thank H. Shelton Earp for the anti-EGFR rabbit antiserum and David Everly for pBabe-LMP1, pBabe-CTAR1, and pBabe-CTAR2 constructs. We also thank Aron Marquitz and Kathy Shair for critical reviews of the manuscript.

This work was supported by NIH grant CA32979 to N.R.-T.
REFERENCES


27. **Izumi, K. M., K. M. Kaye, and E. D. Kieff.** 1997. The Epstein-Barr virus LMP1
amino acid sequence that engages tumor necrosis factor receptor associated factors is critical for primary B lymphocyte growth transformation. Proc Natl Acad Sci U S A \textbf{94}:1447-52.


CHAPTER THREE

LMP1 modulates distinctive NF-κB pathways through CTAR1 to regulate EGFR expression

Che-Pei Kung and Nancy Raab-Traub
ABSTRACT

Epstein-Barr Virus (EBV) latent membrane protein 1 (LMP1) is required for EBV B-lymphocyte transformation, transforms rodent fibroblasts, and can induce lymphoma and epithelial hyperplasia in transgenic mice. Two domains have been identified within the intracellular carboxy-terminus that can activate NF-κB, CTAR1 and CTAR2, through interactions with tumor necrosis receptor associated factors (TRAFs). CTAR1 can activate both the canonical and noncanonical NF-κB pathways and has unique effects on cellular gene expression. The epidermal growth factor receptor is highly induced by LMP1-CTAR1 in epithelial cells through activation of a novel NF-κB form containing p50 homodimers and Bcl-3. To further understand the regulation of NF-κB in CTAR1-induced EGFR expression, we evaluated the ability of CTAR1 to induce EGFR in mouse embryonic fibroblasts (MEFs) defective for different NF-κB effectors. CTAR1-mediated EGFR induction required the NF-κB Inducing Kinase (NIK) but not the IKK complex components that regulate canonical or noncanonical NF-κB pathways. CTAR1-induction of nuclear p50 occurred in IKKβ-, IKKγ-, and NIK-defective MEFs, indicating that this form is not dependent on the canonical or noncanonical NF-κB pathways. The CTAR1-induction of p50 was proteasome-dependent. EGFR and nuclear p50 were expressed at high levels in TRAF2- fibroblasts and were not induced by LMP1. EGFR was low in TRAF3- fibroblasts and was not induced by LMP1 while in TRAF6- MEFs, EGFR was not expressed but was induced by LMP1-CTAR1. These findings suggest that endogenous EGFR regulation is independent of TRAF2 and that the effects of LMP1-CTAR1 require TRAF3 but are independent of TRAF6. Importantly, this novel NFkB pathway is differentially regulated by TRAF2 and TRAF3.
INTRODUCTION

The Epstein-Barr virus is a human gammaherpesvirus that infects more than 95% of the world population and is associated with multiple malignancies, including Hodgkin’s disease (HD), NK or T-cell lymphoma, Burkitt’s lymphoma (BL), post-transplant lymphoproliferative disease (PTLD), gastric carcinoma (GAC), and nasopharyngeal carcinoma (NPC) (44). Latent membrane protein 1 (LMP1) is considered the EBV oncogene and is essential for EBV-mediated B-cell transformation (21). Moreover, LMP1 can transform rodent fibroblast cells and LMP1-transgenic mice develop B-cell lymphoma and epithelial hyperplasia (24, 33, 55, 56). LMP1 is expressed in multiple EBV-associated malignancies and acts as a constitutively active tumor necrosis factor receptor (TNFR) by recruiting TNFR-associated factors (TRAFs) to the cell membrane (22, 51). Numerous cellular genes have been shown to be induced by LMP1, including ICAM-1, TRAF1, A20, Id1, Id3, Bcl-2, Bcl-3, and EGFR (25, 26, 37, 46, 50). LMP1 has two major signaling domains, c-terminal activating region (CTAR) 1 and 2, that bind different TRAFs and activate distinct signaling pathways. CTAR1 recruits TRAF1, 2, 3, and 5, and uniquely activates noncanonical NFkB, phosphatidylinositol 3-kinase (PI3K)-Akt, and the MAPK pathways. CTAR2 recruits TRAF2 and TRAF6 through adaptors TRADD and BS69 to activate canonical NFkB and c-Jun N-terminal kinase (JNK) signaling pathway (8, 33, 34, 51). (19, 43). CTAR1 is required LMP1-mediated fibroblast transformation and for B-lymphocyte transformation while CTAR2 is dispensable (19, 33, 34, 43).

The nuclear factor-κB (NF-κB) is a transcription-factor family that dimerize and bind to κB sites within the promoter/enhancers to regulate transcription of genes that impact a variety of biological processes, including cell cycle progression, apoptosis, differentiation,
inflammation, angiogenesis, and cell proliferation (16, 35). The NF-κB family consists of five members, p50, p52, p65 (RelA), RelB, and c-Rel, all of which share a Rel homology domain responsible for dimerization and DNA binding. The transcription activation domain required for gene regulation only exists in p65, RelB, and c-Rel. The activation of NF-κB is tightly regulated through interactions with inhibitors of NF-κB (IκBs), which include p105 (precursor of p50), p100 (precursor of p52), IκBα, IκBβ, IκBγ, IκBε, IκBζ, and Bcl-3. IκBs mainly function by sequestering inactive NF-κB dimers in the cytoplasm to prevent their activity. Upon receiving an extracellular stimulus, such as binding of TNFα to its receptor, activation of a kinase cascade that includes IκB kinase alpha (IKKα), IKKβ, and IKKγ (NEMO) results in the phosphorylation of IκBs and leads to their ubiquitination and degradation. NF-κB members are then released into the nucleus for transcriptional regulation. In the canonical NF-κB pathway, an IKK complex consisting of IKKα/IKKβ/IKKγ is activated and results in IKKα/IKKβ-mediated phosphorylation and degradation of IκBα. Degradation of IκBα releases multiple NF-κB dimers, primarily p50/p65 into the nucleus. In the noncanonical NF-κB pathway, NF-κB inducing kinase (NIK) phosphorylates and activates IKKα in a IKKβ/γ-independent manner. Activated IKKα phosphorylates p100 and triggers proteasome-mediated processing of p100 to p52, liberating p52-containing NF-κB dimers, such as p52:RelB into the nucleus. Due to the very different mechanisms of activation, canonical and noncanonical NF-κB pathways regulate distinctive transcriptional regulation of target genes. In addition to participating in both NF-κB pathways, IKKs/NIK have also been reported to phosphorylate many other proteins besides the IκBs. For example, IKKα and IKKβ can both directly phosphorylate p65 (42). IKKα has also been implicated in the phosphorylation of several nuclear substrates, including SMRT,
histone H3, and CBP (17, 18, 58). NIK was recently suggested to function as serine kinase of STAT3 (40). Aberrant regulation of NF-κB pathways has been implicated in development of many human cancers (35, 45).

Initial studies characterizing LMP1 activation of NFκB showed that although CTAR2 had greater NF-κB activation as determined using reporter assays, CTAR1 induced more complex NF-κB detected by EMSA (20, 36, 52). Subsequent studies revealed that CTAR2 only activates the canonical NF-κB pathway, whereas CTAR1 can activate both the canonical and noncanonical pathways (1, 9, 31, 47). LMP-CTAR1 can transcriptionally activate EGFR expression and this ability was shown to be mediated through a unique NF-κB complex containing p50 homodimers and Bcl-3 (25, 37, 53). The induction of this transcription complex requires activation of STAT3 by CTAR-1 to increase Bcl-3 expression. Although considered a member of the IkB family, Bcl-3 contains a transactivating domain and can bind to p50 and p52 homodimers to induce their transcriptional activating potential (4, 14).

To determine if NFκB-regulated EGFR expression was a result of canonical or noncanonical NF-κB, the effects of LMP1-CTAR1 were analyzed in genetically engineered mouse embryonic fibroblasts (MEFs). LMP1-CTAR1 upregulated EGFR was independent of canonical or noncanonical regulation as it did not require IKKα, IKKβ, or IKKγ, but was dependent on NIK. In addition, TRAF2 and TRAF3, but not TRAF6, were required for LMP1-CTAR1-mediated induction of EGFR in MEF cells. These data indicate that in addition to canonical and noncanonical NF-κB pathways, LMP1 manipulates unique NF-κB complexes through CTAR1 to regulate target gene expressions.
MATERIALS AND METHODS

Reagents and Cell Culture

C33A cervical carcinoma cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (Sigma) and antibiotic/antimycotic (Gibco) at 37°C with 5% CO₂. Wild-type, IKKα−/−, IKKβ−/−, IKKγ−/−, NIK<sup>aly/aly</sup>, TRAF2−/−, and TRAF6−/− mouse embryonic fibroblast (MEF) cells were kindly provided by Elliot Kieff (Harvard university, Boston) and were immortalized by infecting with a human papilloma virus 16 E6/E7 retrovirus to make MEF cells susceptible for transfection or transduction (7, 27, 30-32, 48). TRAF3−/− MEF cells were obtained from Michael Karin (University of California at San Diego) and were spontaneously immortalized by continuous passaging (15). Immortalized MEF cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (Sigma) and antibiotic/antimycotic (Gibco) at 37°C with 5% CO₂. To inhibit proteasome activity, 1 to 10μM of MG132 (26S inhibitor) or clasto-Lactacystin β-Lactone (20S inhibitor) (Calbiochem) was added to ~90% confluent cells for 5 hours before preparing cell lysates.

Plasmids

Generation of plasmid constructs expressing both myc-tagged (pCDNA3) and HA-tagged (pBabe) full-length LMP1, LMP1-CTAR1 (which contains aa 1-231 of LMP1), and LMP1-CTAR2 (which has aa 187-351 of LMP1 deleted) was described previously (11). Myc-tagged and HA-tagged LMP1 constructs contain neomycin- and puromycin-resistant cassette, respectively. HA-tagged LMP1, LMP1-CTAR1, and LMP1-CTAR2 were also
subcloned into pCDNA3 vector with zeocin-resistant cassette by digesting pBabe-LMP1 / CTAR1 / CTAR2 with BamHI / EcoRI and ligated to BamHI / EcoRI-digested pCDNA3.1-zeocin vector (Invitrogen). Wild-type and dominant-negative NIK constructs were kindly provided by Christian Jobin (University of North Carolina at Chapel Hill) and Elliot Kieff (Harvard University).

Retrovirus production and transduction

Recombinant retrovirus production and transduction were performed as previously described to transduce full-length LMP1, CTAR1 (1-231), CTAR2 (d187-351), or pBabe vector control (25). Briefly, ~60-80% confluent 293T cells in 100mm plates were triply transfected using FuGEGE6 transfection reagent (Roche) according to the manufacturer’s instruction with 5µg pBabe (vector), pBabe-HA-LMP1, pBabe-HA-CTAR1, or pBabe-HA-CTAR2, and 5µg pVSV-G and 5µg pGag/Pol expressing plasmids. After 24 hours of incubation at 37°C, the culture media were replaced with fresh media and the cells were transferred to 33°C for another 24 hours of incubation. Cell supernatants were centrifuged at 1000g for 5 min to remove cell debris and virus-containing supernatant was collected and stored in -80°C if not immediately used. Cells to be transduced were grown to ~70-80% of confluence and then transduced with virus-containing supernatant with 4µg/ml polybrene for 24-48 hours at 37°C.

Generation of stable cell lines

C33A stable cell lines expressing CTAR1 (1-231) or vector control pBabe were established by retroviral transduction followed by selection and passages in the presence of
1μg/ml puromycin (Sigma). IKKβ-/- and NIK<sup>aly/aly</sup> MEF cells stably expressing CTAR1 were generated by transducing cells with pBabe- and CTAR1-containing retrovirus solution with 4μg/ml polybrene for 24-48 hours at 37°C, followed by selection and passages in the presence of 1μg/ml puromycin. IKKα-/- and IKKγ-/- MEF cells stably expressing CTAR1 were made by transfecting cells with myc-tagged pCDNA3 vector or pCDNA3-CTAR1 construct using FuGEGE6 transfection reagent for 48 hours at 37°C, followed by selection and passages in the presence of 0.8mg/ml G418 (Mediatech). Stable LMP1-, CTAR1-, and CTAR2-expressing TRAF2-/- and TRAF6 MEF cells were generated by transfecting cells with HA-tagged pCDNA3.1 vector, pCDNA3.1-LMP1, pCDNA3.1-CTAR1, or pCDNA3.1-CTAR2 construct using FuGEGE6 transfection reagent for 48 hours at 37°C, followed by selection and passages in the presence of 400μg/ml Zeocin (Invitrogen).

**Fractionation of Cells**

Cells were fractionated as previously described (25). Briefly, after cultured cells reached ~90% confluence, cells were scrape harvested, washed once with cold phosphate-buffered saline (PBS, Gibco), centrifuged at 1000g for 5-10 min to get cell pellets. Whole cell lysates were made by lysing cells with RIPA buffer (20mM Tris-HCl [pH 7.5], 150mM NaCl, 1mM EDTA, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS], 0.1% deoxycholic acid) supplemented with phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate (Na<sub>3</sub>VO<sub>4</sub>), protease and phosphatase inhibitor cocktail (Sigma). After incubation at 4°C for 15min, lysates were then clarified by centrifugation at 13,000 rpm, 4°C for 15 min and supernatants containing whole cell lysates were transferred to new tubes. Nuclear extracts were made by lysing initial cell pellets in a hypotonic buffer (20 mM
HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) supplemented with PMSF, Na$_3$VO$_4$, protease and phosphatase inhibitor cocktail (Sigma) for 15 min on ice. Nonidet P-40 was then added to a final concentration of 1%, followed by 1 min of vortex. Nuclei were pelleted by low-speed centrifugation at 2,000 rpm for 10 min at 4°C and the supernatant were collected as cytoplasmic fraction. The nuclei fraction was purified using the Optiprep reagent (Sigma) as directed by the manufacturer. Nuclei were lysed with nuclear extraction buffer (20 mM Tris-HCl [pH 8.0], 420 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 25% glycerol, PMSF, Na$_3$VO$_4$, protease and phosphatase inhibitor cocktail [Sigma]) with the salt concentration adjusted to 400 mM with 5 M NaCl. All lysates were stored at -80°C.

**Western Blot Analysis**

Protein concentration of cell lysates was determined using Bio-Rad DC protein assay system according to manufacturer’s instruction. Equal amounts of protein were used for SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Optitran® (Schleicher and Schuell) for Western blot analysis. Primary antibodies used include anti-p50 (Abcam), anti-p65 (RelA), anti-NIK, anti-RelB, anti-GAPDH, anti-GRP78, anti-PARP (Santa Cruz), anti-phospho-STAT3 (Ser 727 and Tyr 705) (Cell Signaling), anti-phospho-EGFR (Tyr 1068), anti-β-catenin (BD Biosciences), and anti-HA tag (Covance). A rabbit antiserum raised against the carboxyl-terminal 100 amino acids of the EGFR fused to glutathione S-transferase was kindly provided by H. Shelton Earp (University of North Carolina at Chapel Hill) and used to detect total EGFR expression. Secondary antibodies used to detect bound proteins include horseradish peroxidase-conjugated antimouse, antirabbit (Amersham Pharmacia), and antigoat (DAKO). After treatment with secondary antibodies, blots were
developed using Pierce Supersignal West Pico chemiluminescence system followed by exposure to film (ISCBioexpress).

RESULTS

**LMP1 Effects on EGFR expression in mouse embryonic fibroblast cells.** To assess the induction of EGFR expression by LMP1 in mouse embryonic fibroblast (MEF) cells, full length LMP1, LMP1-CTAR1, and LMP1-CTAR2 were expressed using the pBabe retroviral vector and transduced into wild-type MEF cells (11). The expression of LMP1 and deletion mutants and EGFR was determined by immunoblotting (Figure 1). EGFR was

![Image of immunoblot](image)

**Fig 1.** EBV LMP1-CTAR1 mediates mild induction of EGFR expression in mouse embryonic fibroblast (MEF) cells. Control vector pBabe, full length LMP1, truncated LMP1 containing only CTAR1, or truncated LMP1 containing only CTAR2 were transduced into wild-type MEF cells. Expression of EGFR and LMP1 derivatives were analyzed by immunoblotting with EGFR and HA-tag antibodies. Expression of GRP78 was measured by immunoblotting as loading control.
readily detected in the wt MEFs, and both LMP1 and LMP1-CTAR1 increased EGFR expression while LMP1-CTAR2 alone did not affect it (25, 53). The induction of EGFR by LMP1 was less in the MEF cells than was previously shown in the C33A cells, a difference that likely reflects the higher basal levels of expression in the MEFs.

**CTAR1-mediated EGFR upregulation is NIK-dependent, but NIK is not sufficient to mimic LMP1-mediated EGFR induction.** CTAR1 of LMP1 activates both the canonical and noncanonical NF-κB pathways to activate distinct NF-κB complexes, including p50/65, p52/p50, p52/RelB, and p50/50 (36, 43). The activation of canonical or noncanonical NFκB is linked to complexes that contain different members of the inhibitor of NF-κB kinase kinases (IKK). The canonical pathway requires IKKα, IKKβ and in some instances, IKKγ, while the noncanonical pathway requires the activation of IKKα resulting from phosphorylation by the NF-κB inducing kinase, NIK. The effects of expression of LMP1 or CTAR1 on EGFR and serine and tyrosine phosphorylation of STAT3 were evaluated in MEF cells lacking these regulators of NF-κB pathway (Figure 2). The MEFS had differing levels of endogenous EGFR, however, LMP1 or LMP1-CTAR1 induced EGFR protein in IKKα-, IKKβ-, and IKKγ-defective MEF cells. As these IKKs are required for activation of the canonical NF-κB pathway, these data indicate that LMP1-mediated EGFR upregulation is not dependent on the canonical NF-κB pathway. Moreover, since IKKα is the critical regulator in the noncanonical NF-κB pathway, the result also reveals that the noncanonical NF-κB pathway is not required for LMP1-mediated EGFR induction. Interestingly, EGFR expression was not induced by CTAR1 in NIK-defective MEF cells. These results suggest that LMP1 induces EGFR through an IKKα-independent but NIK-specific pathway.
Expression of LMP1 increased serine phosphorylation of STAT3 in wild-type MEF cells, IKKα, and IKKγ-defective MEF cells, but not in IKKβ or NIK null mice both of which had high basal levels. Elevated tyrosine phosphorylation was detected in wild-type, IKKγ- and NIK-defective cells, but not in IKKα- or IKKβ-defective MEF cells. Importantly, the effects of LMP1 on EGFR and STAT3 were not linked to the canonical or noncanonical NF-κB pathways but were linked to the presence of NIK.

A previous study showed that NIK could function as a serine kinase of STAT3 although the high basal levels of serine phosphorylated STAT3 in NIK null cells indicate that

---

**Fig 2.** LMP1-mediated EGFR upregulation is NIK-dependent. Full-length LMP1 or CTAR1 were transduced into wild-type MEF cells or MEF cells defective for IKKα, IKKβ, IKKγ, or NIK. Expressions of LMP1 or CTAR1 were confirmed by immunoblotting with specific antibody against HA-tag or Myc-tag. Expression levels of EGFR, serine-phosphorylated STAT3, or tyrosine-phosphorylated STAT3 were analyzed by immunoblotting. Expression of GRP78 was measured by immunoblotting as loading control. V: vector control; L1: LMP1; C1: CTAR1.

<table>
<thead>
<tr>
<th></th>
<th>WTMEF</th>
<th>IKKα-/-</th>
<th>IKKβ-/-</th>
<th>IKKγ-/-</th>
<th>NIK-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V</td>
<td>L1</td>
<td>V</td>
<td>C1</td>
<td>V</td>
</tr>
<tr>
<td>LMP1/CTAR1</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
</tr>
<tr>
<td>EGFR</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
<td><img src="image11" alt="Image" /></td>
</tr>
<tr>
<td>GRP78</td>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
<td><img src="image15" alt="Image" /></td>
<td><img src="image16" alt="Image" /></td>
<td><img src="image17" alt="Image" /></td>
</tr>
<tr>
<td>Ser-P-STAT3</td>
<td><img src="image19" alt="Image" /></td>
<td><img src="image20" alt="Image" /></td>
<td><img src="image21" alt="Image" /></td>
<td><img src="image22" alt="Image" /></td>
<td><img src="image23" alt="Image" /></td>
</tr>
<tr>
<td>Tyr-P-STAT3</td>
<td><img src="image25" alt="Image" /></td>
<td><img src="image26" alt="Image" /></td>
<td><img src="image27" alt="Image" /></td>
<td><img src="image28" alt="Image" /></td>
<td><img src="image29" alt="Image" /></td>
</tr>
</tbody>
</table>
other kinases can clearly phosphorylate STAT3 (40). To confirm the requirement for NIK in LMP1-induced EGFR expression, CTAR1-expressing C33A cells were transiently transfected with dominant-negative NIK construct (DNNIK), DNNIK with aly-mutation (G860R; DNNIK\(^{aly}\)), or kinase-dead NIK (NIK(K-A)) for 48 hours and the expression levels of EGFR and serine-phosphorylated STAT3 were analyzed by immunoblotting (Figure 3A). DNNIK and DNNIK\(^{aly}\) express the c-terminus of NIK without the kinase domain. NIK (K-A) expresses the full-length NIK with a mutation in the kinase domain that abrogates its kinase activity. Transfection of NIK (K-A), but not DNNIK or DNNIK\(^{aly}\), reduced CTAR1-induced EGFR expression significantly by 35%. Similarly, NIK (K-A) decreased CTAR1-induced serine phosphorylation of STAT3 by 34%, while DNNIK and DNNIK\(^{aly}\) did not have significant effects. These findings indicate that NIK contributes to the EGFR upregulation by LMP1 through mediating induction of STAT3 serine phosphorylation.

To determine whether NIK itself is sufficient to induce ser-pSTAT3 and EGFR upregulation, wild-type NIK was transiently transfected into both C33A and 293T cells and EGFR expression and ser-pSTAT3 were assessed by western blot analysis (Figure 3B). NIK expression was clearly detected in 293T cells, however, overexpression of NIK did not induce ser-pSTAT3 or EGFR expression. In C33A cells, transfection of 4μg of NIK very slightly increased ser-pSTAT3, however failed to induce expression of EGFR. To determine whether NIK perhaps requires activation of additional pathways affected by CTAR1 to induce STAT3 activation and EGFR expression, CTAR1-expressing NIK-defective MEF cells were also transfected with increasing amounts of the WT NIK construct. Expression of CTAR1 was confirmed by western blot analysis using antibody to the HA tag and the effects on EGFR and ser-pSTAT3 were determined (Figure 3C). Similarly to the results observed in
Fig 3. Blocking NIK reduces LMP1-induced EGFR expression, but overexpression of NIK is not sufficient to mimic LMP1-mediated EGFR upregulation. **A)** C33A cells stably expressing CTAR1 was transfected with pCDNA3 control vector or dominant-negative NIK construct (DNNIK), DNNIK construct with aly-mutation (G860R; DNNIKaly), or kinase-dead NIK (NIK (K-A)). Expression level of serine-phosphorylated STAT3 and EGFR were analyzed by immunoblotting. Expressions of NIK mutants were detected by immunoblotting using antibody against C-terminus of NIK. Expression of GAPDH was measured by immunoblotting as loading control. Intensity of ser-P-STAT3 and EGFR bands were quantitated by Image J software and normalized to the expression level of GAPDH from three independent experiments. *P<0.05. **B)** C33A and 293T cells were transfected with increasing amount of wild-type NIK expression construct. **C)** NIK-defective MEF cells stably expressing CTAR1 were transfected with increasing amount of wild-type NIK expression construct. CTAR1 expression was confirmed by immunoblotting with antibody against HA-tag. Expression of NIK, EGFR, and serine-phosphorylated STAT3 were analyzed with immunoblotting. Expression of GAPDH was measured by immunoblotting as loading control.
293T and C33A cells, overexpression of NIK in NIK-defective MEF cells was not sufficient to induce either ser-pSTAT3 or EGFR induction, even in the presence of CTAR1. These results suggest that although NIK is required for CTAR1-mediated EGFR upregulation, NIK overexpression alone is not sufficient to induce these pathways and additional factors or processes are required for ser-pSTAT3 or EGFR expression.

CTAR1-mediated p50 activation is not dependent on the canonical or noncanonical NF-κB pathways. LMP1 induction of EGFR transcription is mediated through effects on Bcl-3 and induction of p50-homodimers (25, 52, 53). To determine the requirement for specific IKK complexes on CTAR1-mediated p50 activation, CTAR1-expressing wild-type, IKKβ-, IKKγ-, and NIK-defective MEF cells were fractionated and the nuclear lysates analyzed to detect nuclear expression of p65, p50, and RelB (Figure 4). The expression of PARP was analyzed as a loading control for the nuclear lysates. Nuclear translocation of p65 is characteristic of the canonical pathway and p65 was detected in the nuclear lysates from WT, IKKγ-, and NIK-defective MEFs but not in IKKβ-defective cells. Nuclear RelB is the major form of NF-κB that represents the noncanonical pathway and CTAR1 induced nuclear translocation of RelB in wild-type and IKKβ-defective MEF cells, but not in NIK-defective cells. These findings confirm that CTAR1-mediated translocation of p65 reflects activation of the canonical pathway and that the effects of LMP1 on RelB require the noncanonical pathway. In contrast, the nuclear expression of p50 was significantly induced by CTAR1 in IKKβ-, IKKγ- and NIK-defective MEF cells. This indicates that CTAR1-mediated p50 activation and nuclear translocation are distinct from both the canonical and noncanonical NF-κB pathways. A high basal level of nuclear p50 was
detected in wild-type MEF cells and may be linked to the higher level of EGFR in wt-MEFs and the slight effects of LMP1 on EGFR expression compared to that in C33A cells.

**Nuclear lysates**

![Image of nuclear lysates](image)

**Fig 4.** CTAR1-mediated p50 activation is not dependent on canonical or noncanonical pathway. Wild-type, IKKβ−/−, IKKγ−/−, and NIKaly/aly MEF cells stably transduced with vector or CTAR1 were fractionated and nuclei lysates were subjected to immunoblot analysis with antibodies specifically against p65 (RelA), p50, and RelB. PARP expression was analyzed by immunoblotting as loading control.

**LMP1-mediated p50 activation is proteasome-dependent.** The mechanisms responsible for p50 activation have not been clearly defined. Several studies have indicated that p50 can be activated by a proteasome-dependent mechanism that either modulates p105 processing or impairs ribosomal progression (23). To determine whether p50 is activated by CTAR1 through proteasome-mediated mechanisms, C33A cells stably transduced with vector or CTAR1 were treated with DMSO or proteasome inhibitors, MG132 (26S inhibitor) or clasto-Lactacystin β-Lactone (20S inhibitor) (Figure 5). The effects of these inhibitors on p105/50 were assessed by immunoblotting anti-p105/50 antibodies. β-catenin is a known
target of proteasomal degradation and was analyzed as a positive control for inhibition of proteasome activity. GAPDH and Emerin were analyzed as loading control for whole cell lysates and nuclear lysates, respectively. In CTAR1-expressing C33A cells where nuclear expression of p50 is significantly induced, treatment with 1μM or 10μM of MG132 reduced p50 translocation level by 72% and 64%, respectively (Figure 5A). Similarly, treatment with 1μM or 10μM of *clasto*-Lactacystin β-Lactone reduced CTAR1-induced p50 nuclear translocation by 35% and 69%, respectively. Nuclear p50 was not detected in the C33A cells transduced with the pBabe control plasmid, and treatment with proteasome inhibitors did not have a significant effect (data not shown). This result indicates that LMP1-CTAR1 induces p50 activation and nuclear translocation are mediated through effects on proteasome activity.

CTAR1 did induced the expression of β-catenin in C33A cells which is consistent with findings that LMP1 affects the expression of junctional proteins (49). In cells treated with proteasome inhibitors, β-catenin levels were increased and a higher molecular weight forms of β-catenin was detected, reflecting its ubiquitination and the successful inhibition of proteasomal activity. The level of p105-to-p50 processing was determined by calculating the ratio of p50 to the total p105/50 complex in whole cell lysates of C33A cells transduced with pBabe control or CTAR1 (Figure 5B). The processing of p105 to p50 was enhanced by approximately 15% in CTAR1-expressing C33A cells compared to pBabe control cells. After MG132 treatment, the percentage of processed p50 was not significantly affected in either vector control or CTAR1-expressing cells. Treatment with 20S proteasomal inhibitor *clasto*-Lactacystin β-Lactone did not affect p50 processing in pBabe control cells. However, treatment of 10μM *clasto*-Lactacystin β-Lactone reduced p50 processing in CTAR1-expressing cells to approximately 40%, which is similar to the level observed in pBabe
**Fig 5.** CTAR1 mediates p50 activation through proteasome-dependent pathways. C33A cells stably transduced with vector control or CTAR1 were treated with DMSO or proteasome inhibitor MG132 or β-lactone (1 or 10μM) for 5 hours and subjected to fractionation. **A)** Nuclear lysates and **B)** Whole cell lysates of these cells were analyzed by immunoblotting. Antibody against p105/50 was used to detect the processing and activation of p50. Expression of GAPDH and Emerin were detected as loading controls for whole cell lysates and nuclear lysates, respectively. Intensity of p105/50 bands were quantitated by Image J software and percentage of processed p50 in whole cell lysates was calculated by \([p50 / (p50+p105)] \times 100\%\). The level of p50 nuclear expression was quantitated by normalizing p50 to the expression level of Emerin.
control cells. These findings indicate that LMP1-CTAR1 mediates proteasome-dependent mechanisms to induce p50 activation.

**LMP1-mediated EGFR induction requires TRAF2 and TRAF3, but not TRAF6.** Initial studies showed that the TRAF-binding motif of LMP1 was required for CTAR1-induced EGFR expression and NF-κB activation and these effects were decreased by dominant negative TRAF2 and TRAF3 (36, 38). To further evaluate this requirement, LMP1 and LMP1-CTAR1 were expressed in MEF cells deficient for TRAF2, TRAF3, or TRAF6 (Figure 6). The expression levels of EGFR, and LMP1 and deletion mutants were determined using immunoblotting of whole cell lysates (Figure 6A). LMP1 and CTAR1 were detected using antibodies against the HA- or myc-epitope tags. EGFR was expressed at high levels in TRAF2-/− MEFs, intermediate levels in TRAF3-/− MEFs, and was barely detected in TRAF6-/− MEFs. This confirmed that the presence and absence of TRAFs modulate expression of the EGFR. Expression of LMP1 or CTAR1 changed these patterns of expression. In the TRAF2-null and TRAF3-null MEFs, LMP1 or CTAR1 expression did not increase EGFR expression. In contrast, in the TRAF6-null MEFs, LMP1 and CTAR1 clearly induced EGFR expression. The ability of LMP1-CTAR1 to induce EGFR in TRAF6-null MEFs but not in TRAF2- or TRAF3-null MEFS is consistent with previously identified interactions between CTAR1 with TRAF2 and TRAF3 but not with TRAF6.

The nuclear level of p50 was also analyzed in TRAF2-/−, TRAF3-/−, and TRAF6-/− MEFs by immunoblotting (Figure 6B). Compared to the vector control cells, CTAR1 induced nuclear expression of p50 in TRAF3-/− and TRAF6-/− cells but did not affect nuclear p50 levels in TRAF2-/− MEF cells that had elevated basal nuclear p50. These results indicate that TRAF3 and TRAF6 are not required for LMP1-CTAR1 effects on p50 activation and
that in the absence of TRAF2, nuclear levels of p50 are elevated and LMP1-CTAR1 did not further increase nuclear p50. TRAF6 was not required for CTAR1-mediated effects on p50 translocation and EGFR upregulation.
DISCUSSION

LMP1 is a member of the TNFR family and potently activates NF-κB transcriptional activity. Early studies identified that two distinct NF-κB activating domains in the carboxy terminus, CTAR1 and CTAR2 and determined that CTAR2 had greater activity in NF-κB reporter assays (19, 43). However, identification of the specific complexes using electrophoretic mobility shifts assay (EMSA) revealed that CTAR1 activated multiple forms of NF-κB while CTAR2 primarily activated a complex containing p65. In addition, it was shown that CTAR1 significantly enhanced processing of p100 to p52. Subsequent studies have further characterized activation of NF-κB and defined the canonical pathway that involves the IKK complex containing IKKα, IKKβ, and IKKγ to phosphorylate and negatively regulate the inhibitor of NFkB, IκBα. It is now known that CTAR2 activates IKKγ (NEMO) / IKKβ-dependent canonical NF-κB pathway through TRAF6 / IRAK1 / TAK1 (30, 47, 57). CTAR1, on the other hand, activates IKKγ (NEMO)-independent but NIK / IKKα-dependent noncanonical NF-κB pathway, in addition to some activation of the canonical pathway (1, 9, 31). An atypical IKKβ-dependent / IKKγ-independent NF-κB activation has also been previously described in LMP1-expressing cell culture models (31). Our previous studies have identified an additional LMP1-mediated activation of NF-κB, in which CTAR1 of LMP1 activates two NF-κB members, p50 and Bel-3, that are induced to bind to the EGFR promoter by LMP1 and increase EGFR transcription (25, 52, 53). The data in this study indicate that this novel pathway is independent of factors required for activation of the canonical or noncanonical NF-κB pathways. IKKα, IKKβ, and IKKγ, all important components in canonical NF-κB pathway, were not required for CTAR1-induced EGFR expression (Figure 2). This result is consistent with previous data indicating that
CTAR2 alone was not able to induce EGFR expression despite its ability to activate strong canonical NF-κB signaling (Figure 1) (25, 36). Although the CTAR1-mediated EGFR induction was inhibited in NIK<sup>aly/aly</sup> cells, perhaps suggesting a requirement for the noncanonical NF-κB pathway, CTAR1-mediated induction of EGFR did not require IKKα, the functional kinase in the noncanonical NF-κB pathway. The decreased EGFR induction by the dominant-negative NIK constructs, kinase-dead NIK (K-A), in CTAR1-expressing C33A cells suggests that NIK does contribute to CTAR1-mediated EGFR induction (Figure 3A). The lack of effect by DNNIK<sup>aly</sup> on CTAR1-induced EGFR expression suggests that specific protein interactions and functions mediated through the aly residue (G860R) is important for EGFR upregulation. This result is consistent with the observation that CTAR1 could not induce EGFR expression in NIK<sup>aly/aly</sup> MEF cells. In contrast to NIK (K-A), DNNIK did not significantly reduce CTAR1-induce STAT3 serine phosphorylation and EGFR expression, despite both constructs contain the TRAF/IKK binding domain. This result indicates that N-terminus of NIK also contributes to EGFR upregulation. NIK expression was not sufficient for CTAR1-mediated STAT3 activation and EGFR upregulation in epithelial cells, and overexpression of wild-type NIK in the presence of CTAR1 did not induce ser-pSTAT3 or EGFR induction in NIK deficient cells (Figure 3B and 3C). These findings suggest that additional potentially NIK-associated factors or NIK-mediated effects on other kinases contribute to this pathway and that a balance of expression level between NIK and NIK-associated factors may be required. These findings are similar to the requirement for TRAF3 for LMP1 signaling. Although TRAF3 is required for LMP1-mediated signaling pathways and transformation, overexpression of TRAF3 in LMP1-expression cells impairs CTAR1-mediated NF-κB activation (2, 34, 36, 38). TRAF3 binds
strongly to CTAR1 and its overexpression likely alters the components in the LMP1/TRAF complexes that activate NF-κB.

It is known that both TRAF3 and TRAF2 regulate NIK and it is thought that TRAF3 is required to form a complex containing TRAF2 and cIAP that induces NIK turnover (54). This suggests a negative regulatory mechanism between TRAF2/TRAF3 and NIK. However, CTAR1 failed to induce EGFR expression in MEF cells defective for TRAF2, TRAF3, or NIK. It is possible that LMP1-CTAR1 may mediate EGFR upregulation through NIK by sequestering NIK regulators TRAF2 and TRAF3. This hypothesis would be consistent with the ability of LMP1 to modulate formation of TRAF-containing complexes and their effects on various targets.

The other important member of EGFR-inducing complex, p50 is activated and translocated into the nucleus in LMP1/CTAR1-expressing C33A cells (25, 43, 53). Several mechanisms have been suggested to mediate p50 activation. This study reveals that CTAR1-induced p50 activation is not dependent on either canonical or noncanonical NF-κB pathway since this occurred in IKKβ-, IKKγ- and NIK-defective MEF cells (Figure 4). Interestingly, CTAR1 did not induce significant p50 activation in wt-MEF cells. This may be due to the relatively high level of nuclear p50 in wt-MEF cells, and may partially explain why LMP1 only slightly induced EGFR expression in wt-MEF cells compared to C33A or other mutant MEF cells.

Multiple studies have suggested that p50 can be activated through processing of its precursor, p105. This processing could occur in ubiquitination-dependent or ubiquitination-independent fashions (3, 5, 23, 39). It was also suggested that p50 could be generated through a mechanism involved cotranslational ribosome halting (28, 29). One consensus
among these suggested mechanisms is that they are all proteasome-dependent. The data presented here indicate that CTAR1 indeed induces processing of p50, which is consistent with previous studies (43). Proteasome inhibition with both 26S and 20S inhibitors reduced p50 activation in CTAR1 expressing cells (Figure 5). These results indicate that CTAR1 activates p50 in part through proteasome-dependent processing of p105 to p50. Interestingly, both 26S inhibitor MG132 and 20S inhibitor clasto-Lactacystin β-Lactone inhibit CTAR1-induced nuclear translocation of p50, but only clasto-Lactacystin β-Lactone reduced processing of p105 to p50 in CTAR1 cells to a level similar to that in pBabe control cells. This suggests that while 20S proteasome is responsible for CTAR1-mediated processing of p50, MG132 blocks separate pathways that contribute to CTAR1-induced p50 activation (39). It is possible that both MG132 and clasto-Lactacystin β-Lactone block CTAR1-mediated nuclear translocation of p50, which only accounts for a small subset of CTAR1-induced p50 processing and the differences may not be detectable in the total cell lysates. It is likely that LMP1 regulates multiple pathways to mediate p50 activation. This is supported by the observation that CTAR1 induced p50 activation in MEF cells defective for IKKβ, which has been suggested as the kinase responsible for p105 processing (5). Moreover, LMP1-CTAR1 could also affect regulatory pathways to induce p50 translocation. Several proteins have been suggested previously to regulate p50 translocation, including Bcl-3 and Importin-3α (12, 59). The fact that LMP1-CTAR1 induces Bcl-3 expression and nuclear localization makes it an attractive candidate (25, 41). It will be of interest to determine if Bcl-3 and Importins contribute to CTAR1-mediated p50 activation and induction of EGFR.

LMP1-associated TRAFs are important for LMP1-mediated signaling pathways, including JNK, p38, and NF-κB activation (51). Our initial studies indicated that the TRAF-
binding motif of CTAR1 was required for LMP1 induction of EGFR expression (36). Expression of CTAR1 in MEF cells defective for different TRAFs revealed that TRAF2 and TRAF3, but not TRAF6, are required for CTAR1-mediated EGFR induction (Figure 6). This result is consistent with the findings that TRAF2 and TRAF3, but not TRAF6, interact with CTAR1 TRAF-binding motif. TRAF2 may contribute to EGFR induction by regulating p50 activation, while TRAF3 may contribute to EGFR induction through independent pathways.

It has been suggested that kinase Tpl-2/Cot functions downstream of TRAF2 to modulate p105 degradation (10). It will be interesting to see if Tpl-2/Cot plays a role in LMP1-induced EGFR expression. Although TRAF6 does not interact with CTAR1, it has been shown that TRAF6 can interact with NIK (6). It is possible that the interaction of CTAR2 with TRAF6 modulates the TRAF6 interaction with NIK and NIK-regulated activation of ser-pSTAT3 or downstream kinases. LMP1 containing CTAR1 but deleted for CTAR2 usually has elevated effects in comparison to full-length LMP1 in C33A cells (25). The effects of the two domains on various TRAF containing complexes are likely the basis for the enhanced activity of CTAR1 in the absence of CTAR2. It is also interesting to note that another potential STAT3 kinase, ERK, has been shown to be regulated by NIK and CTAR1-mediated ERK activation and transformation was also inhibited by dominant-negative TRAF2 or TRAF3, but not TRAF6 (13, 34). The interaction of LMP1 with TRAF2 and TRAF3 likely activates multiple potentially redundant kinases that affect transcription factors that regulate EGFR expression.

In conclusion, this study reveals that LMP1-CTAR1 induces EGFR expression through NIK, TRAF2, and TRAF3 independently of traditionally-defined canonical and noncanonical NF-kB pathways. Two important EGFR-inducing factors, STAT3 and p50, are
regulated distinctively in the presence or absence of various IKK and TRAF molecules. The effects of LMP1 on the ser-pSTAT3 potentially require NIK, whereas the effects on p50 require TRAF2 but are independent of NIK, TRAF3, and TRAF6. Additionally, LMP1-CTAR1 activates p50 through proteasome-dependent processing of p105 to p50. The data suggest that distinct combinations of LMP1-activated effectors regulate the effects of LMP1 expression and its ability to induce transformation. The further study of LMP1 and its activation of NF-κB and additional pathways will likely clarify the link between specific signal transducing complexes and the downstream effectors, as well as its contribution to EBV-associated cancer development.

ACKNOWLEDGEMENTS

We thank H. Shelton Earp for providing anti-EGFR rabbit antiserum. We thank Dr. Elliot Kieff and Dr. Michael Karin for providing MEF cells. We thank Dr. Elliot Kieff and Dr. Christian Jobin for providing NIK and DNIKK constructs. This work was supported by NIH grants CA32979, CA19014 to N.R.-T.
REFERENCES


CHAPTER FOUR

EBV Latent Membrane Protein 1 (LMP1) Activates STAT3 and ERK through effects on EGFR and PKCδ

Che-Pei Kung and Nancy Raab-Traub
ABSTRACT

Epstein-Barr virus is a ubiquitous herpesvirus that infects more than 90% of the world’s adult population and is linked to multiple malignancies, including Burkitt lymphoma, Hodgkin disease, and nasopharyngeal carcinoma (NPC). The EBV oncoprotein LMP1 induces transcription of the epidermal growth factor receptor (EGFR), which is expressed at high levels in NPC. EGFR transcription is induced by LMP1 through a p50 homodimer-Bcl3 complex, and Bcl3 is induced by LMP1 activation of STAT3. This study reveals that LMP1, through its carboxy terminal activation domain 1 (CTAR1), activated both STAT3 and EGFR in a serum-independent manner. LMP1-CTAR1 increased transcription of the EGFR ligand, epiregulin, which could potentially activate the EGFR through an autocrine mechanism. Upon treatment with EGF in serum-free medium, the CTAR1-induced EGFR was additionally phosphorylated and STAT3 became phosphorylated on tyrosine, concomitant with upregulation of selected STAT3 target genes, such as Fos and cyclin D1. The kinase responsible for CTAR1-mediated serine phosphorylation of STAT3 was identified to be PKCδ using the PKCδ specific inhibitor, Rottlerin. Interestingly, Rottlerin also inhibited CTAR1-induced ERK activation while the ERK inhibitor, U0126, inhibited activation of ERK but not STAT3. Inhibition of PKCδ blocked CTAR1-mediated transformation of Rat-1 cells, likely through the inhibition of ERK activation. These data suggest that PKCδ functions as a master regulator of STAT3 and ERK activation by LMP1-CTAR1. These data also reveal that LMP1 can modulate the STAT3 pathway through EGFR activation and PKCδ-mediated signaling to alter cellular gene expression.

INTRODUCTION
Infecting more than 90% of the world’s population, the Epstein-Barr virus (EBV) is a ubiquitous human gammaherpesvirus. EBV is transmitted through saliva to infect oral epithelium and B-lymphocytes resulting in a life-long infection (21). Persistent, latent EBV infection is present in several lymphoid and epithelial malignancies, including Burkitt lymphoma, Hodgkin disease, and nasopharyngeal carcinoma (NPC). Viral genes of latent infection are expressed in the malignant cells, which contain a homogeneous clonal form of the EBV episome (41).

EBV latent membrane protein 1 (LMP1) is considered the major oncogene as it is essential for B-lymphocyte transformation and can also transform rodent fibroblast cells (20, 56). LMP1 functions as a constitutively active tumor necrosis factor receptor (TNFR) due to aggregation through its six transmembrane domains and interactions with tumor necrosis factor-associated factors (TRAFs) (33, 37). Multiple signaling pathways are activated by LMP1, including mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), phosphatidylinositol 3-kinase (PI3K)/Akt, and NF-κB (8, 31, 38, 42). LMP1 induces transcription of multiple genes to affect apoptosis, cell cycle progression, cell proliferation, and migration (10, 11, 46). Two major signaling domains in the C-terminal tail of LMP1, carboxyl-terminal activating regions 1 and 2 (CTAR1 and CTAR2), mediate LMP1-associated signaling pathways by recruiting different TRAFs. CTAR1 recruits TRAF1/2/3/5 through a TRAF-binding motif residing from amino acid (aa) 204-208 (PQQAT), whereas CTAR2 recruits TRAF2 and TRAF6 through adaptors TRADD and BS69 (50).

In addition to distinctive TRAF binding, CTAR1 and CTAR2 also have different effects on signaling activation and cellular transformation. CTAR1 specifically induces MAPK, PI3K/Akt/GSK3β signaling, while CTAR2 activates the JNK pathway (7, 31, 32).
CTAR2 activates strong canonical NF-κB signaling, whereas CTAR1 induces more complex NF-κB signaling including canonical, noncanonical, and atypical pathways (29, 33). Moreover, CTAR1 has been shown to be required for LMP1-mediated transformation of B-lymphocyte and rodent fibroblast, while CTAR2 is dispensable (17, 31). This information suggests that CTAR1 may have more significant effects in the development of malignancy. The unique properties of CTAR1 were first identified in its ability to induce transcription of the EGFR and subsequently TRAF1, EBI3, Id1, and Id3, repression of p27, and activation of Bcl-3 have been identified (6, 10, 25, 33).

EGFR is a member of the ErbB receptor tyrosine kinase family, including EGFR (ErbB-1), HER2/c-neu (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4). In the absence of ligand, these receptors are in an inactive tethered conformation (57). Upon ligand binding, the receptors undergo dimerization, autophosphorylation, activation, and subsequently degradation/turnover. Multiple signaling pathways are activated by the EGFR, including Ras/MAPK, Src kinases, JAKs/STATs, and PI3K-AKT (19). EGFR signaling is targeted by proteins of several oncogenic viruses to mediate transformation, including v-ErbB, E5, HBVx, and LMP1 (35). NPC has elevated EGFR expression which correlated with the levels of LMP1 and treatment with EGFR tyrosine kinase inhibitors induces cell cycle arrest and inhibits cell proliferation of NPC cell lines (58, 59). These findings suggest that the effect of LMP1 on EGFR is an important factor in EBV carcinogenesis and is a prime target for development of therapeutic strategies.

Signal transducers and activators of transcription (STATs) consist of a family of cytoplasmic proteins that, upon stimulation of cytokines or growth factors, translocate into the nucleus and transactivate cellular target genes that are involved with cell proliferation,
cell cycle regulation, apoptosis, angiogenesis, and differentiation (22). The transcriptional activity of STAT3 is regulated by phosphorylation. Phosphorylation of tyrosine 705 and serine 727 affect dimerization/translocation and the DNA-binding/transcriptional activity of STAT3, respectively (1). In NPC and B-cell lymphomas that develop in LMP1-transgenic mice, STAT3 is activated (2, 45). A positive autoregulatory loop, in which LMP1-activated STAT3 regulates LMP1 expression through effects on the novel LMP1 promoter residing in the terminal repeats has been described (4). LMP1-mediated STAT3 activation is also responsible for several LMP1-induced cellular genes, including MUC1 and Bcl-3 (23, 25). LMP1-mediated tyrosine phosphorylation of STAT3 has been linked to JAK1 or JAK3, although whether LMP1 interacts directly with JAKs is controversial (13, 15). The mechanism through which LMP1 induces serine phosphorylation of STAT3 is unknown.

This study clarifies the mechanisms through which EGFR and STAT3 are activated by EBV LMP1-CTAR1. CTAR1 was found to induce activation of EGFR, STAT3, and Bcl-3 expression in a serum-independent manner. EGF treatment increased phosphorylation of EGFR, tyrosine phosphorylation of STAT3, and upregulation of STAT3 targets. Transcription of the EGFR ligand, epiregulin, was induced in CTAR1-expressing cells, suggesting that this may promote the continuous activation of the EGFR by LMP1. The data also indicate that the CTAR1-induced serine phosphorylation of STAT3 is dependent on PKCδ, whose inhibition also impaired LMP1-induced ERK activation and CTAR1-mediated transformation of Rat-1 cells. These data reveal that multiple LMP1-mediated signaling pathways, including effects on EGFR, STAT3, and PKCδ contribute to the transforming properties of LMP1.
MATERIALS AND METHODS

Cell Culture, plasmids, and reagents

Cervical carcinoma cell line C33A and 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (Sigma) and antibiotic/antimycotic (Gibco) at 37°C with 5% CO₂. Constructs expressing HA-tagged (pBabe) LMP1-CTAR1 (which contains aa 1-231 of LMP1) and LMP1(1-220) were generated as described previously (10, 32). Small chemical inhibitors, including Rottlerin, Gö6976, U0126, LY294002, and AG1478 were purchased from Calbiochem. To test the effects of inhibitors on CTAR1-mediated pathways, growth medium was replaced with serum-free DMEM supplemented with antibiotic/antimycotic for 24 hours. Inhibitors were added for 5 hr before preparing lysates. Dimethyl sulfoxide (DMSO; Sigma) was added at 1:1000 as the vehicle control. To test the activity of EGFR, serum-starved cells were added with 500ng/ml EGF (Austral Biologicals) and 100μM AG1478 for 10 or 30 min before harvesting cell lysates.

Retrovirus production and transduction

Recombinant retrovirus production and retroviral transduction were performed as previously described (32). Subconfluent 293T cells in 100mm plates were transfected by FuGENE 6 transfection reagent (Roche) with 5μg pBabe (vector), pBabe-HA-1-231, or pBabe-HA-1-220, and 5μg pVSV-G and 5μg pGag/Pol expressing plasmids. After incubating at 37°C for 24 hr, the medium was replaced with fresh medium (DMEM supplemented with 10% FBS and antibiotic/antimycotic) and cells were incubated at 33°C for 24 hr. The cell supernatant was clarified and the virus-containing supernatant was collected. Stable cell lines were produced by transduction with clarified virus supernatant.
with 4µg/ml polybrene for 24 hr at 37°C, followed by selection with 1µg/ml of puromycin (Sigma).

**Cell lysates harvesting and western blot analysis**

Cells were harvested at 90% confluence, washed with cold phosphate-buffered saline (PBS, Gibco), scrape harvested in cold PBS, centrifuged at 1000 × g for 5 min, and lysed with radioimmunoprecipitation assay (RIPA) buffer (20mM Tris-HCl [pH 7.5], 150mM NaCl, 1mM EDTA, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS], 0.1% deoxycholic acid) supplemented with 0.5mM phenylmethylsulfonyl fluoride (PMSF), 1mM sodium orthovanadate (Na3VO4), protease and phosphatase inhibitor cocktail (Sigma). Cell lysates were clarified and the protein concentration was determined using Bio-Rad DC protein assay system. Equal amounts of protein were used for SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Optitran® (Schleicher and Schuell) for Western blot analysis. Membranes were blocked for 1 hr in room temperature in Tris-buffered saline containing 0.1% Tween-20 and 5% nonfat dried milk. Primary antibodies include anti-GAPDH, anti-ERK, anti-phospho-ERK (Tyr 473) (Santa Cruz), anti-phospho-Akt (Ser 473), anti-phospho-STAT3 (Ser 727 and Tyr 705), anti-PKCδ (Cell Signaling), anti-phospho-EGFR (Tyr 1068) (BD Biosciences), anti-Bcl3 (Millipore), and anti-HA tag (Covance). A rabbit antiserum generated against the carboxyl-terminal 100 amino acids of the EGFR fused to glutathione S-transferase was kindly provided by H. Shelton Earp (University of North Carolina at Chapel Hill) and used to detect expression of total EGFR. Secondary antibodies used to detect Abs-bound proteins include HRP (horseradish peroxidase)-conjugated anti-mouse, anti-rabbit (Amersham Pharmacia), and anti-goat (DAKO). After incubation with secondary antibodies,
blots were developed using Pierce Supersignal West Pico chemiluminescence system according to the manufacturer’s instructions, followed by exposure to films (ISCBioexpress).

**Quantitative Real Time Polymerase Chain Reaction (QRT-PCR)**

Total RNA of cells was isolated using RNAeasy kit (Qiagen). Quantitative real time-PCR (QRT-PCR) was performed using Quantifast SYBR Green RT-PCR kit (Qiagen). PCR products were detected using ABI 7900HT sequence detection system (Applied Biosystems) and analyzed using SDS 2.0 software (Applied Biosystems). The cycle threshold ($C_T$) was determined as the number of PCR cycles required for reactions to reach an arbitrary fluorescence value within the linear amplification range. The change in $C_T$ ($\Delta C_T$) was determined between the same target gene primer sets and different samples, and the change in $\Delta C_T$ ($\Delta \Delta C_T$) was determined by adjusting for the difference in the number of cycles required for GAPDH to reach the $C_T$. The fold change was determined as $2^{\Delta \Delta C_T}$ since each PCR cycle results in a two-fold amplification of PCR products. Primers used in this study are listed in Table S1.

**Focus formation assay**

Focus formation assays were performed as described previously (31). Subconfluent Rat-1 fibroblast cells plated in 6-well plates were transduced with recombinant retrovirus for 24 hr. Fresh medium (DMEM supplemented with 10% fetal bovine serum and antibiotic/antimycotic) were then changed every other day for 10 days. The effect on focus formation by inhibition of PKCδ using Rottlerin was determined using 1μM Rottlerin or vehicle control DMSO added daily with freshly-changed medium. After foci can be clearly identified, cells were stained with 1% crystal violet (dissolved in 50% methanol) and images were taken under a stereomicroscope.
Data Analysis

The quantitative data were presented as the average value of at least triplicate of each experiment ± SE. Statistical significance was evaluated using a computerized, paired 2-tailed Student t test. Differences were considered significant at $P < 0.05$.

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primer sequence (from 5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Forward</td>
</tr>
<tr>
<td>GAPDH</td>
<td>TGCACCACCAACTGCTTAGC</td>
</tr>
<tr>
<td>EGFR</td>
<td>CTGCGTCTCTTTGCGGGAATG</td>
</tr>
<tr>
<td>TGFα</td>
<td>GCCAGCATGTGTCTGCCCCATT</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>TGGAGAATGCAAAATATGTAAGGA</td>
</tr>
<tr>
<td>Epiregulin</td>
<td>TTCCATCATGTATCCCCAGGAG</td>
</tr>
<tr>
<td>SOCS3</td>
<td>CCTCAAGACCTTCAGCTCCA</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>CTTGGAGTGGCCACTTACCTG</td>
</tr>
<tr>
<td>Fos</td>
<td>AAGGAGAATCGGGAAGGGAAA</td>
</tr>
<tr>
<td>CEBPD</td>
<td>ATCGACTTCAGCGCCTACAT</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>GATCAAGTGGAAGCCCGGACT</td>
</tr>
</tbody>
</table>

Table 1. Quantitative RT-PCR primers
RESULTS

LMP1 induces intrinsic activation of STAT3 and EGFR, as well as EGFR-dependent STAT3 phosphorylation. We have shown previously that LMP1, through CTAR1, induces EGFR expression through activation of NF-kB p50 and transcriptional upregulation of Bcl-3 by activated STAT3 (25, 34, 53). To investigate the effects of LMP1 on activation of EGFR and STAT3, C33A cells stably transduced with CTAR1 were cultured in 10% serum or serum-starved for 24 hr and whole cell lysates were analyzed by immunoblotting (Figure 1). In serum-supplemented medium CTAR1 induced expression and activation of EGFR as assessed by EGFR phosphorylation at Tyr1068 (lane 2). CTAR1 also induced expression of Bcl-3 and both tyrosine and serine phosphorylation of the transcriptional activator STAT3 but did not affect the total level of STAT3. Interestingly, EGFR and STAT3 were still phosphorylated and activated in the absence of serum, and expression of Bcl-3 was induced to similar levels as cells grown in 10% FBS (lane 3). These data indicate that CTAR1-mediated EGFR and STAT3 activations are independent of the presence of growth factors in the medium.

It has been suggested that EGFR interacts with STAT3 and can function as tyrosine kinase to activate STAT3 (40). To determine whether CTAR1-induced EGFR can be further stimulated to activate STAT3, serum-starved CTAR1-expressing C33A cells were treated with 500ng/ml of EGF with or without EGFR kinase inhibitor, AG1478, and analyzed by Western blotting. Treatment with EGF for 10 minutes greatly increased the tyrosine phosphorylation of STAT3 (lane 4) but did not affect the serine phosphorylation of STAT3 or expression of Bcl-3. The EGF-induced tyrosine phosphorylation of STAT3 was inhibited by the EGFR kinase inhibitor AG1478, indicating that EGF-induced STAT3 tyrosine phosphorylation...
phosphorylation is EGFR-dependent (lane 5). A similar result was observed with a 30 min EGF treatment with slightly lower tyrosine phosphorylation level of STAT3 compared to 10 min EGF treatment, suggesting that EGF-mediated EGFR activation decreases after 30 minutes (lane 6 and 7). Interestingly, the antibody against activated EGFR detected a subtly shifted band in both 10 min and 30 min EGF-treated samples (lane 4 and 6, pointed by arrows) that was not detected with simultaneous AG1478 treatment (lane 5 and 7). Although CTAR1 activated EGFR in the absence of serum, EGF treatment stimulated additional phosphorylation of EGFR to initiate signaling pathways downstream of EGFR, including the tyrosine phosphorylation of STAT3. Overall, the data indicate that LMP1-CTAR1 can induce activation of EGFR, STAT3, and Bcl-3 expression independently of external growth factors. Additionally, EGF treatment further stimulates the CTAR1-induced EGFR to induce tyrosine phosphorylation of STAT3.

CTAR1 induces expression of EGFR ligand epiregulin. To determine if CTAR1-mediated serum-independent activation of EGFR reflects effects on EGFR ligands, real-time quantitative RT-PCR was performed to assess expression of TGFα, heparin-bound EGF (HB-EGF), and epiregulin in serum-starved vector control and CTAR1-expressing C33A cells (Figure 2). EGFR expression was analyzed as a positive control and expression levels were normalized to GAPDH. The messenger RNA levels for TGFα and HB-EGF were not affected in CTAR1-expressing cells compared to vector control cells, while the mRNA expression of epiregulin was induced by 2.4-fold in CTAR1-expressing cells in the absence of serum compared to vector control cells. This result suggests that epiregulin transcription is induced by LMP1-CTAR1, and this may contribute to CTAR1-mediated EGFR activation.
EGF treatment induces expression of STAT3 target genes in CTAR1 cells. To determine if the STAT3 phosphorylation induced by EGF treatment increased expression of known STAT3 target genes, the RNA of EGF-treated CTAR1 cells was isolated and analyzed for expression of several known STAT3 targets, including SOCS3, Bcl-xL, Fos,
Figure 2. mRNA expression of EGFR ligands in CTAR1 cells. Total RNA of pBabe control and CTAR1-expressing cells were extracted and subject to real-time quantitative RT-PCR using primers for EGFR ligands. Results from RT-PCR were normalized to GAPDH and levels of EGFR ligands in pBabe control cells were set to 1-fold. RT-PCR of each ligand was performed four times, with each one done in triplicate.

CEBPD, and Cyclin D1, using real-time quantitative RT-PCR (Figure 3) (48). In the absence of serum CTAR1 induced EGFR expression 5.4-fold and treatment with EGF for 10 min further increased EGFR mRNA expression to 7-fold. However, after 30-min treatment of EGF this decreased to 6-fold. The RNA levels of SOCS3 and Bcl-xL were not significantly changed after 10 or 30 min treatment of EGF. Expression of cebpd went up by 2.2-fold with 10 min EGF treatment but decreased down to 1.4-fold after 30 min treatment with EGF. The expression of cyclin D1 increased by 2.7-fold after 10 min EGF treatment and stayed at 3-fold after 30 min treatment of EGF. The mRNA of fos changed most dramatically with a 2-fold induction after 10 min EGF treatment and an increase of approximately 15.8-fold following 30 min treatment of EGF. Statistical analysis revealed
that the changes of Cyclin D1 and Fos were significant with \(p<0.05\). These data indicate that CTAR1-induced EGFR in response to EGF activates STAT3 to increase expression of specific targets of STAT3.

**PKC\(\delta\) is responsible for CTAR1-induced serine phosphorylation of STAT3.** To identify the potential serine kinase responsible for CTAR1-mediated serine phosphorylation of STAT3, CTAR1-expressing C33A cells were treated with either DMSO or inhibitors of previously identified serine kinases of STAT3, including Rottlerin (PKC\(\delta\) inhibitor), Gö6976

**Figure 3. Expression level of STAT3 target genes in EGF-treated CTAR1-expressing C33A cells.** Total RNA was extracted from serum-starved CTAR1 cells treated with DMSO, EGF for 10min, or EGF for 30min and real-time quantitative RT-PCR was performed with primers against STAT3 target genes. Results from RT-PCR were normalized to GAPDH and expressions of STAT3 targets in pBabe control cells were set to 1-fold. Results are averages of at least three separate experiments, with each one performed in triplicate.
(PKCα/β inhibitor), U0126 (MEK/ERK inhibitor), and LY294002 (PI3kinase inhibitor) (Figure 4) (5). As previously shown, CTAR1 (lane 2) induced EGFR expression, Bcl-3 expression, and tyrosine / serine phosphorylation of STAT3 compared to parental C33A cells (lane 1). Elevated levels of AKT and ERK phosphorylation were also detected in CTAR1-expressing C33A cells. The PKCδ inhibitor, Rottlerin, inhibited CTAR1-induced serine phosphorylation but not tyrosine phosphorylation of STAT3 at 5μM and more significantly at 40μM (lane 3 and 4). At 40μM, Rottlerin also reduced CTAR1-induced Bcl-3 protein expression. Treatment with Rottlerin slightly decreased the effects of CTAR1 on EGFR. In contrast, inhibition of MEK/ERK with U0126 or PI3kinase with LY294002 effectively decreased CTAR1-induced ERK phosphorylation and AKT phosphorylation, respectively, but did not block CTAR1-induced serine phosphorylation of STAT3 (lane 6 and 7). Treatment of CTAR1-expressing C33A cells with PKCα/β inhibitor, Gö6976, did not decrease serine phosphorylation of STAT3, indicating that CTAR1-induced serine phosphorylation of STAT3 was PKCδ-specific and independent of PKCα/β (lane 5). These results indicate that PKCδ, but not PI3K, ERK, or PKCα/β, is responsible for LMP1-CTAR1-induced serine phosphorylation of STAT3. Importantly, inhibition of PKCδ activity also blocked phosphorylation of ERK (lane 3 and 4) but did not affect activation of PI3kinase/Akt. This suggests that in addition to serine phosphorylation of STAT3, PKCδ is also responsible for the CTAR1-induced and endogenous ERK phosphorylation in C33A cells.

**PKCδ is important for CTAR1-mediated transformation of Rat-1 cells.** To test the requirement for PKCδ in LMP1-mediated transformation, the effect of its inhibition was assessed using a focus formation assay in Rat-1 fibroblast cells (Figure 5A). Subconfluent
Rat-1 cells were transduced with LMP1-CTAR1 (1-220)-containing retrovirus at three
different dilutions (10⁻¹, 10⁻², and 10⁻³). CTAR1 induced many foci at the 10⁻¹ dilution which
was decreased with the $10^{-2}$ and $10^{-3}$ retrovirus dilutions. Treatment with 1μM Rottlerin blocked focus formation at all three dilutions. Western blot analysis indicated that ERK phosphorylation was significantly decreased by Rottlerin treatment in Rat-1 cells transduced with $10^{-1}$ or $10^{-2}$ dilutions of retrovirus that also had the most significant reduction of focus formation (Figure 5B). Immunoblotting with total-ERK antibody indicated that the total

![Image of Western Blot](image)

**Figure 5. PKCδ inhibitor, Rottlerin, inhibited CTAR1-induced focus formation.** A) Rat-1 cells were transduced with pBabe and CTAR1(1-220) at three different dilutions, maintained for 10 days, stained with crystal violet, and observed for focus formation. B) Whole cell protein lysates were prepared from a duplicate focus formation experiment of A). Western blot analysis was performed using antibodies against total ERK and phosphorylated ERK. GAPDH was detected as a loading control.
level of ERK was not affected and GAPDH expression was assessed as an internal control. This result indicates that PKCδ is required for LMP1-mediated transformation of Rat-1 cells. As ERK activation has been previously shown to be required for LMP1-mediated Rat-1 transformation, the block of transformation by rottlerin inhibition of PKCδ confirms that PKCδ mediates the activation of ERK by LMP1-CTAR1.

**LMP1-CTAR1 induces Tyrosine-311 phosphorylation of PKCδ.** PKCs mature and are activated through a series of phosphorylation steps (14, 39). To analyze the activation of PKCδ by CTAR1 in C33A cells, whole cell lysates of CTAR1-expressing C33A cells were assessed by western blotting using antibodies against total PKCδ and two specific phosphorylations that have been suggested to be important for PKCδ activity, Threonine 505 and Tyrosine 311.

![Figure 6](image)

**Figure 6. Tyrosine-311 phosphorylation of PKCδ is elevated in CTAR1 C33A cells.** C33A cells stably expressing pBabe or CTAR1 were assayed for total level of PKCδ, Thr505 phosphorylation of PKCδ, and Tyr311 phosphorylation of PKCδ by western blot analysis. GAPDH expression was detected as a loading control.
and Tyrosine 311 (24, 27, 51, 52). The total protein level of PKCδ was similar in both pBabe- and CTAR1-expressing C33A cells (Figure 6). Overexpression of CTAR1 induced Tyr-311 phosphorylation of PKCδ but reduced Thr-505 phosphorylation of PKCδ. These data suggest that CTAR1-mediated PKCδ activity is not dependent on the phosphorylation at Thr-505 but that Tyr-311 phosphorylation of PKCδ might contribute to its activity in CTAR1-expressing C33A cells.

DISCUSSION

These data presented in this report reveal that LMP1-CTAR1 can activate both EGFR and STAT3 independently of serum growth factors, and that EGFR can be further activated by EGF treatment to induce tyrosine phosphorylation of STAT3 and expression of STAT3 targets. More importantly, these studies also identified PKCδ as the likely serine kinase of STAT3, as well as the upstream effector of ERK activation mediated by LMP1-CTAR1. The ability of PKCδ inhibitor Rottlerin to inhibit CTAR1-induced transformation points to the importance of this kinase in LMP1-mediated oncogenesis.

One possible cause for this serum-independent activation of EGFR is CTAR1-mediated induction of EGFR ligands. It has been shown that LMP1 transactivates TGFα and enhances TGFα secretion in T cells (26). However, in CTAR1-expressing C33A cells, expression of TGFα was not altered, yet, transcription of another EGFR ligand, epiregulin, was increased. Interestingly, in an LMP1 transgenic mouse model with epidermal hyperplasia, hyperphosphorylation of EGFR occurs concomitantly with induced TGFα, HB-EGF, and epiregulin expression, suggesting that LMP1 can activate EGFR activation through induction of EGFR ligands, such as epiregulin (3). Taken together, the serum-independent
activation of EGFR observed in this study could provide a mechanism for the increased growth characteristics of LMP1-expressing epithelial cells in serum-free environment (34).

These data also indicate that CTAR1-induced EGFR responded to EGF treatment which greatly increased the tyrosine phosphorylation of STAT3. This phosphorylation was blocked by treatment with the EGFR inhibitor AG1478, indicating this was an EGFR-specific effect. It is well established that EGFR can activate the JAK/STAT pathway and can also physically interact and phosphorylate STAT3 directly. This activation is important for inhibition of apoptosis, cell growth, and cell proliferation in cancer cells of epithelial origins (19, 43, 44). EGFR expression and STAT3 activation are also elevated in NPC, suggesting that LMP1 contributes to NPC development through its effects on EGFR, STAT3 activation, and regulation of STAT3 target genes (2). In response to EGF, CTAR1 cells had greatly increased mRNA for specific STAT3 target genes, including cyclin D1 and fos, which have been shown to be induced by LMP1 (Figure 3) (49, 54). In breast carcinoma, the EGFR signaling pathway induces epithelial-mesenchymal transition (EMT) of cancer cells through STAT3-mediated TWIST gene expression, and subsequently E-cadherin downregulation (28). LMP1 has also been shown to induce TWIST expression and EMT in NPC cells (16).

These findings suggest that by inducing EGFR expression, LMP1 mediates the EGFR-STAT3 signaling pathway to regulate cellular transcriptional and biologic properties.

Previous studies have identified several phosphotyrosine residues in the C-terminal tail of EGFR to be important for recruitment of STAT3 to EGFR and activation of STAT3, including Tyr992, Tyr1045, Tyr1086, and Tyr1068 (47). It is possible that CTAR1-mediated serum-independent activation of EGFR is responsible for the low levels of tyrosine phosphorylation of STAT3 observed in serum-starved CTAR1 cells (Figure 1). Additional
phosphorylation induced by EGF treatment likely enhances the interaction between STAT3 and EGFR, resulting in increased STAT3 phosphorylation/activation (Figure 1).

CTAR1 also induced serum-independent serine phosphorylation of STAT3, which was not changed by treatment with EGF or its inhibitor, AG1478, suggesting that the CTAR1-induced serine phosphorylation of STAT3 was not dependent on EGFR signaling (lane 4-7 of Figure 1). STAT3 serine phosphorylation can be induced by several serine-threonine kinases including ERK, PI3K, and PKCδ (5). However, specific inhibition of these serine kinases indicated that only the PKCδ-inhibitor Rottlerin reduced serine phosphorylation of STAT3 (Figure 4). This result demonstrates that PKCδ, but not PKCα/β, ERK, or PI3K, is responsible for CTAR1-induced serine phosphorylation of STAT3. LMP1 has not been previously shown to activate the PKCδ-dependent signaling pathways, although it does regulate annexin 2 and ezrin through PKCα/β (9, 30). Interestingly, PKCδ, which is known to activate the MAPK pathway through different mechanisms, also blocked both CTAR1-induced and endogenous ERK phosphorylation (18). These findings suggest that PKCδ may function as a master regulator for both LMP1-mediated STAT3 and ERK activation.

Inhibition of LMP1-CTAR1-mediated activation of ERK blocked rodent fibroblast transformation (32, 42). The inhibition of focus formation and ERK phosphorylation by the PKCδ inhibitor in Rat-1 cells indicates that PKCδ is required for LMP1-mediated transformation, potentially by regulating ERK activation (Figure 5). PKCδ-mediated serine phosphorylation of STAT3 has been shown to be important for keratinocyte proliferation, and LMP1 activation of STAT3 is required for the enhanced growth properties of LMP1 transgenic lymphocytes (12, 45). However, the role for PKCδ-mediated serine
phosphorylation of STAT3 in LMP1-mediated transformation and NPC development has not been determined.

Phosphorylation patterns of PKCδ in response to different stimuli are complex (18, 39). Phosphorylation at the activation-loop site is the important first step for maturation and activation of PKCs (14). Based on sequence homology, phosphorylation of Thr505 at PKCδ’s activation-loop is thought to be important for its activity (27, 39). However, increased Thr505 phosphorylation of PKCδ in CTAR1-expressing C33A cells compared to vector control cells was not detected (Figure 5). It has been shown that bacteria-generated PKCδ retains catalytic activity despite lack of phosphorylation at Thr505 (51). Instead, increased levels of Tyr311 phosphorylation of PKCδ was detected in CTAR1-expressing C33A cells. A study using mass spectrometry showed that Tyr311 phosphorylation is critical for increased activity of PKCδ in response to H2O2 (24). Interestingly, LMP1 has been shown to induce production of H2O2 in EBV-negative nasopharyngeal epithelial cell line (55). This suggests that LMP1, through CTAR1, may activate PKCδ through inducing production of H2O2. PKCδ can be regulated by PI3K through PDK1 (27). However in these studies, PI3K-specific inhibitor did not reduce CTAR1-induced serine phosphorylation fo STAT3 indicating that PI3K-PDK1 signaling is not responsible for PKCδ-mediated phosphorylation of STAT3 in CTAR1-expressing cells (Figure 3). A recent study also suggested that EGFR can mediate tyrosine phosphorylation of PKCδ in H2O2-stimulated cells (36). It will be interesting to determine the exact phosphorylation profile of PKCδ and identify potential additional kinases that contribute to PKCδ activation in LMP1/CTAR1-expressing cells.
In summary, this study reveals that LMP1-CTAR1 mediates STAT3 signaling pathways through EGFR and PKCδ. The CTAR1-activated EGFR responds to EGF stimulation to increase tyrosine phosphorylation of STAT3 and regulate expression of STAT3 target genes. CTAR1 additionally induces serine phosphorylation of STAT3 and ERK activation through PKCδ. These combined effects of LMP1 on EGFR and STAT3 likely increase the transcription of additional cellular genes. The further study of the effect of LMP1 on these pathways will enhance our understanding of the mechanisms through which EBV contributes to oncogenesis.

ACKNOWLEDGEMENT

We thank H. Shelton Earp for providing anti-EGFR rabbit antiserum. This work was supported by NIH grants CA32979, CA19014 to N.R.-T.
REFERENCES


CHAPTER FIVE

General Conclusions
Considering that EBV infects more than 90% of the world’s adult population, the human immune system is really underappreciated for doing an amazing job in keeping this virus in check. The majority of the primary and persistent EBV infections are asymptomatic for a lifetime without causing health problems. The most common concern for EBV infection is the development of infectious mononucleosis (IM) when the primary infection occurs in adolescence or early adulthood. But IM is normally a self-limited illness and the mild symptoms caused by IM usually recede after several weeks.

However, due to the ubiquitous nature of EBV infection, the number of serious diseases caused by EBV simply cannot be ignored. Two major EBV-associated cancers, nasopharyngeal carcinoma and Hodgkin disease, although have relatively low incidence-rate in the US, still have 11,000 and 7300 cases per year in the US, respectively (53). Moreover, EBV infection is also associated with some forms of approximately 60,000 new cases of non-Hodgkin lymphoma (NHL) in US each year, including Burkitt’s lymphoma. Burkitt’s lymphoma is especially prevalent in persons younger than 20-year-old, where approximately 40% of NHL cases are Burkitt’s lymphoma in western countries (67). In some cases EBV-associated malignancies also exhibit specific patterns of geographical distribution, potentially due to environmental, genetic, and other factors that may have predisposing effects on EBV-associated diseases. For example, EBV-associate endemic Burkitt’s lymphoma has an incidence rate at 10-15 cases per 100,000 human population in New Guinea and equatorial regions of Africa, and accounts for 20-50% of all pediatric malignancies in certain areas (45, 64). In Chinese Cantonese males who live in Hong Kong or Guangzhou, the incidence rates can climb up to over 100 cases per 100,000 population per year (14). These facts make EBV
a significant threat for human health and further understanding of EBV-mediated
tumorigenesis is necessary.

EBV-associated malignancies mainly occur in two cell types, B cells and epithelial
cells. Persistent infection of EBV in these two cell types is characterized based on three
different latency types of gene expression, latency type I, II, and III. EBV’s major
oncoprotein, latent membrane protein 1 (LMP1), is expressed in type II and type III latency
and is consistently found in several EBV-associated cancers. Many studies have focused on
LMP1 as it is essential for EBV-mediated B-cell transformation and it alone can transform
rodent fibroblasts (22, 63). Although LMP1’s functions have been mainly mapped to two
signaling domains CTAR1 and CTAR2, it has been shown that CTAR1 is required for
LMP1-mediated transformation while CTAR2 is dispensable (34, 35). Therefore, most of
the studies in this dissertation have focused on CTAR1’s contribution to LMP1-mediated
gene regulation and transformation.

It has been shown that LMP1, through CTAR1, upregulates EGFR expression in
epithelial cells and EGFR is induced in most EBV-associated NPCs (38, 52, 69). This
transcriptional upregulation has been found to be mediated by a novel NF-κB complex
comprised with p50 homodimer and Bcl-3 (58, 59). In chapter 2, we revealed that LMP1-
CTAR1 also induces the expression of Bcl-3 at the transcriptional level and this upregulation
is mediated by CTAR1-activated STAT3. Inhibition of CTAR1-induced STAT3
phosphorylation results in the downregulation of Bcl-3 and EGFR, indicating that CTAR1
activates STAT3 to directly and indirectly affect cellular gene expressions in epithelial cells.
It has been shown that LMP1 activates STAT3 to initiate an autoregulatory loop in which
activated STAT3 regulates EBNA1 and LMP1 expression (5, 6). Moreover, STAT3 has also
been shown to be responsible for LMP1-induced expression of MUC1 (25). This finding suggests that by activating STAT3 pathway, LMP1 is able to have a global effect on expression of both viral and cellular genes. The detection of induced STAT3 activation in NPC specimens and LMP1-transgenic mice suggests that activation of STAT3 is an important signaling event LMP1 manipulates to control cell transformation (2, 51). A recent study further supports this implication by showing that STAT3 is directly involved with cell proliferation and invasiveness of EBV-infected NPC cell lines (33). With improved technologies, it is much easier to identify targets of transcriptional factors (TFs) at the genome-wide level (10). Chromatin-immunoprecipitation-sequencing (ChIP-seq) and ChIP-on-chip analyses have been applied widely to study transcriptional regulatory networks. With its importance in regulating various cell functions, STAT3 has been subject to ChIP-seq analysis in embryonic stem (ES) cells and comprehensive mapping of STAT3-targeted genes is being explored (7, 15). This accumulating information may be useful to identify potential targets of LMP1-activated STAT3 and their functions in LMP1-mediated transformation. Conversely, it may be a good idea to apply these techniques to identify potential targets of STAT3, or even other LMP1-activated TFs, in the expression of LMP1. It may dramatically increase our understanding of LMP1’s roles in transcriptional regulation.

At roughly the same time of our study, another report also confirmed the induction of Bcl-3 in LMP1-expressing cells (43). Despite the fact that this study suggested that LMP1 induces Bcl-3 through CTAR2-mediated NF-κB pathway, it is apparent that protooncogene Bcl-3 is downstream target of LMP1 and its induction may contribute to LMP1-mediated transformation. Bcl-3 has been shown to complexes with p52- or p50-homodimers to transactivate gene expression, including cyclin D1 (48, 66). This provides an additional
mechanism for LMP1-induced cyclin D1, which was suggested to be regulated by c-Jun/JunB (54, 55). Another potential function of Bcl-3 is to suppress p53 activity by inducing the expression of Hdm2 (21). LMP1 also regulates p53 activity through the induction of A20, indicating that p53 is an important target of LMP1-mediated oncogenesis (12).

As mentioned in chapter 2, Bcl-3 has been reported to be phosphorylated by GSK3 and it leads to proteasome-mediated degradation of Bcl-3 (62). LMP1 has also been shown to inactivate GSK3 potentially through activation of Akt (34). It is therefore possible that LMP1 could upregulate Bcl-3 post-transcriptionally by inactivating its negative regulator GSK3. Our preliminary data showed that although the treatment of GSK3 inhibitors further increased Bcl-3 protein level in cells expressing CTAR1, inhibition of GSK3 alone was not sufficient to induce Bcl-3 expression (data not shown). Moreover, the presence of CTAR1 did not increase Bcl-3’s stability upon treatment with protein synthesis inhibitors (data not shown). This indicates that LMP1-CTAR1 increases Bcl-3 expression solely through transcriptional upregulation. Interestingly, a recent study showed that GSK3 inhibitors blocked STAT3 DNA binding activity and the expression of STAT3-induced Bcl-3, suggesting an autoregulatory mechanism for LMP1-regulated Bcl-3 expression (1). Nuclear translocation of Bcl-3 has been shown to be regulated by K63-linked ubiquitination, which can be blocked by the deubiquitinase CYLD (36). LMP1 has been shown to induce K63-linked ubiquitination, albeit through CTAR2-associated RIP (18). Whether CTAR1 induces nuclear translocation of Bcl-3 observed in our study through a similar mechanism remains to be determined.
In chapter 3, we investigated the roles of NF-κB pathways in LMP1-CTAR1-mediated EGFR upregulation using mouse embryonic fibroblasts (MEFs). It has been shown previously that LMP1 induces EGFR expression through TRAF-binding motifs and canonical NF-κB pathways may play a role in the event (38). Surprisingly, using MEFs defective for different NF-κB effectors, we found that CTAR1 induces EGFR expression in MEF cells and the induction is completely abrogated in the absence of functional NIK.

Induction of EGFR in IKKα−/−, IKKβ−/−, and IKKγ−/− cells suggests that LMP1-mediated EGFR induction is not dependent on either canonical or noncanonical NF-κB pathway. A previous study suggested that LMP1-CTAR1 activates an atypical IKKβ-dependent/IKKγ-independent NF-κB pathway in addition to IKKβ/IKKγ-dependent canonical and NIK/IKKα-dependent noncanonical pathways (32). This argues that the function of individual IKKs may be critical for specific LMP1-mediated gene regulation. Our study revealed that LMP1 induces EGFR expression through another “atypical” NF-κB signaling event which is NIK-dependent. The canonical and noncanonical NF-κB pathways may also contribute to LMP1-mediated EGFR upregulation, however, as IκBα and dominant-negative constructs of IKKα/IKKβ decreased EGFR expression and a relatively mild induction of EGFR was observed in IKKα−/− and IKKβ−/− cells (38, 59). Moreover, the extent of EGFR induction mediated by LMP1 may also be affected by the basal level of EGFR detected in each MEF mutant cell. It will be interesting to see if functions of any of these NF-κB effectors are affected endogenously in C33A cells, the cell line with extremely low basal level of EGFR for studies of LMP1-mediated EGFR upregulation.

It is not yet clear how NIK affects CTAR1-mediated EGFR upregulation. One possibility is that NIK may contribute to EGFR induction by activating STAT3 through
phosphorylation as reported recently (41). This hypothesis is consistent with the reduction of STAT3 serine phosphorylation in CTAR1-expressing NIK-defective MEFs and CTAR1-expressing C33A cells transfected with NIK (K-A) construct. However, overexpression of NIK failed to increase serine phosphorylation of STAT3 in epithelial cell lines and NIK-defective MEFs. This indicates that although NIK is critical for LMP1-mediated EGFR upregulation, other factors associated with NIK may be also required. Furthermore, it will be interesting to see if STAT3-regulated Bcl-3 expression, as described in chapter 2, plays a role in CTAR1-mediated EGFR induction in MEF cells. The result that NIK (K-A), but not DNNIK and DNNIK<sup>aly</sup>, constructs exhibited dominant-negative effects on CTAR1-induced EGFR was consistent with the lack of CTAR1-mediated EGFR upregulation in NIK<sup>aly/aly</sup> MEF cells. It also indicates that association with other proteins may be important for NIK’s activity to mediate EGFR induction, since aly-mutation is known to abrogate NIK’s interaction with its binding partners, including IKKα and TRAFs (11). Additionally, we also showed that CTAR1 was able to induce EGFR expression in TRAF6-defective, but not TRAF2- or TRAF3-defective cells. It will be interesting to see if the interactions between NIK and TRAF2 or TRAF3 are important for LMP1-mediated EGFR upregulation. It has been shown that TRAF3 can function as a negative regulator of NIK by inducing NIK ubiquitination and degradation. Therefore, LMP1-CTAR1 could potentially activate the NIK-mediated signaling pathway by interacting with and sequestering TRAF3, which would indicate that the lack of CTAR1-induced EGFR in TRAF3-defective cells is due to separate mechanisms.

The NF-κB member p50 forms a transactivating complex with Bcl-3 and this complex is responsible for LMP1-mediated EGFR upregulation (58, 59). The activation of
p50 is also found in LMP1-expressing cells or NPC (27, 46, 58). In chapter 3, we showed that CTAR1-induced p50 activation is consistent among most MEF mutant cells that have induced EGFR, including IKKβ-, IKKγ-, and TRAF6-defective MEFs. CTAR1 failed to activate p50 in TRAF2-defective cells, indicating that TRAF2 may contribute to LMP1-mediated EGFR upregulation through regulating p50 activation. However, in NIK- or TRAF3-defective MEFs where CTAR1-induced EGFR expressions were abrogated, activation of p50 was still detected. This suggests that although the p50 homodimer can transcriptionally induce EGFR, its importance varies among mutant MEFs we used in this study. It has been shown that the presence or absence of specific TRAFs can tilt the importance to other TRAFs in LMP1-mediated signaling pathways (8, 31). The increased p50 activation in TRAF3-defective cells could reflect the role of TRAF2 in mediating p50 activation, while also indicates that there are TRAF3-dependent pathways regulating EGFR induction. We also showed that CTAR1-induced p50 activation is mediated by a proteasome-dependent pathway (26). It will be interesting to see if LMP1 activates p50 via other possible pathways, such as importin-dependent protein transportation (9).

In addition to p50-homodimer/Bcl-3 regulation, EGFR can be transcriptionally regulated by several other mechanisms. For example, the p53 homologue p63 represses the transcriptional expression of EGFR (44). EGFR expression is also inhibited by microRNA-7 (23, 65). Given LMP1’s ability to regulate multiple signaling pathways and its newly-found roles in regulating miRNA expression, it is possible that LMP1 also regulates these mechanisms to affect EGFR level or cellular gene expression in general (13, 56).

EGFR upregulation has been well documented in NPC and inhibition of EGFR activity has detrimental effects on cell growth of NPC cells (47, 68, 70). In addition to
EGFR upregulation, we revealed in chapter 4 that LMP1-CTAR1 also activates EGFR both in the absence of serum and upon treatment of EGF. CTAR1-activated EGFR may be responsible for the regulation of certain LMP1-responsive cellular genes both intrinsically and in response to external stimulation, such as EGF. It is not yet clear how LMP1, through CTAR1, activates EGFR in the absence of growth factors. However, the induction of EGFR ligand epiregulin in CTAR1-expressing C33A cells may contribute. Epiregulin expression has also been found to be increased in LMP1 transgenic mice, supporting this hypothesis (4).

Several recent studies have shed some light on a new ErbB-activating mechanism involved with exosome-mediated shedding of ErbBs, including EGFR and HER2 (29, 49, 50). Full-length and proteolytically-processed EGFRs can be secreted through small membrane vesicles called exosomes in human keratinocyte cell line. These secreted EGFRs retain ability to bind to ligands and potentially retain its tyrosine kinase activity. Additionally, EGFR shedding is correlated with a highly malignant cell phenotype, indicating that this phenomenon might contribute to EGFR-induced cell proliferation and invasion. Interestingly, LMP1 has recently been shown to not only present in exosomes of NPC cells, but also promote exosome release of a potent growth factor, FGF-2 (3, 24, 37). Exosome-mediated secretion of LMP1 has recently been suggested to be responsible for the detection of LMP1 in the sera and saliva of a majority of NPC patients (17). One EGFR ligand, HB-EGF, has been shown to undergo PKCδ-induced ectodomain shedding (19, 61). In this study we showed that LMP1 can induce downstream signaling pathways through PKCδ. Metalloproteinases are important for the process of shedding membrane-anchor proteins, and LMP1 is known to induce expression of several MMPs, including MMP1, 2, 3, and 9 (16, 19, 28, 40, 42, 57, 61). These findings suggest that LMP1 potentially activates
EGFR in a serum-independent manner by regulating protein trafficking pathways involving exosome productions. Studies designed to test this hypothesis are currently under development.

Another important finding in chapter 4 was the identification of PKCδ as the upstream effector of LMP1-CTAR1-mediated activations of STAT3 and ERK, and show that PKCδ activity is required for CTAR1-mediated transformation in rodent fibroblasts. These findings have brought us one step closer to determine how LMP1 induces two of its downstream pathways, STAT3 and MAPKK/ERK activations. Although we identified increased phosphorylation of Tyr311 of PKCδ to be one marker of PKCδ activation, due to the complex nature of PKCδ’s phosphorylation and regulation, it remains to be determined how LMP1 regulates PKCδ activity in epithelial cells (20). Notably, EGFR has been shown to be able to regulate PKCδ through specific phosphorylations (39). Therefore, it will be interesting to see if it is an interplay between LMP1-regulated EGFR and PKCδ activation.

A couple of recent studies showed that PKCδ activates p65 to induce expression of downstream targets in response to TNFα or phorbol 12-myristate 13-acetate (PMA, also called TPA) (30, 60). Considering that LMP1 is a potent activator of NF-κB signaling and the common application of TPA in studying EBV lytic replication from latently-infected cells, it will be interesting to investigate PKCδ’s role in NF-κB activity in both lytic and latent infection of EBV.

In summary, these studies have helped us further understand different signaling pathways which LMP1, especially through CTAR1, utilizes to control expression of cellular genes that may contribute to LMP1- and EBV-mediated tumorigenesis (Figure 1). Using LMP1-mediated EGFR upregulation as the model, we found that LMP1-CTAR1 activates
STAT3 to induce expression of the critical member of EGFR’s transcriptional regulator, Bcl-3. CTAR1 activates STAT3 through PKCδ and potentially NIK, which is also required for CTAR1-mediated EGFR induction. STAT3 can be further activated through CTAR1-activated EGFR in response to EGF to induce downstream target genes, including Fos and cyclin D1. PKCδ was also found to be an upstream regulator of ERK activation and was required for CTAR1-mediated transformation of rodent fibroblasts. Moreover, CTAR1-mediated EGFR upregulation is dependent on TRAF2 and TRAF3 but not TRAF6, and TRAF2 may contribute to EGFR induction by regulating p50 activation.

The further understanding of the complexity of LMP1’s effects on signaling pathways will help clarify the contribution of LMP1 to EBV pathogenesis. Since I started graduate study five years ago, significant progress has been made in the last five years in the field of EBV research. The first viral miRNA was identified in EBV, followed by approximately 40 publications to date discussing the relationship between EBV and miRNA (both viral and cellular). More positive correlations were established between EBV infection and autoimmune disease such as MS. Promising results came out of phase I and phase II trials of EBV vaccines. A connection was made between the invasiveness of EBV-associated malignancies and EBV’s ability to regulate the composition of cellular junction proteins. And multiple signaling pathways have been identified during different stages of EBV infection. It will be an honor to consider this dissertation has made a contribution, however small, to our knowledge of EBV pathogenesis and the never-ending fight to stop diseases associated with EBV.
Figure 1. CTAR1-mediated signaling pathways for EGFR upregulation. LMP1-CTAR1 engages multiple signaling pathways to upregulate EGFR expression. CTAR1 induces expression of Bcl-3 through activation of STAT3, which is mediated by PKCδ and possibly NIK. NIK is required for CTAR1-mediated EGFR induction, which is not dependent on either canonical or noncanonical NF-κB pathways. In addition to STAT3 activation, CTAR1 may also mediate ERK activation through PKCδ. CTAR1 induces p50 activation in a proteasome-independent but TRAF2-dependent manner. CTAR1-activated EGFR can phosphorylate STAT3 and induce expressions of downstream targets of STAT3 in response to EGF.
REFERENCES


