Spring 1-1-2011

The Transmembrane Domains of Latent Membrane Protein -1 Play Important Roles in Viral Signaling and Complex Formation

Christopher Martin Wrobel
University of Colorado Boulder, cmwrobel@alumni.calpoly.edu

Follow this and additional works at: https://scholar.colorado.edu/mcdb_gradetds

Part of the Molecular Biology Commons, and the Virology Commons

Recommended Citation

https://scholar.colorado.edu/mcdb_gradetds/50

This Dissertation is brought to you for free and open access by Molecular, Cellular, and Developmental Biology at CU Scholar. It has been accepted for inclusion in Molecular, Cellular, and Developmental Biology Graduate Theses & Dissertations by an authorized administrator of CU Scholar. For more information, please contact cuscholaradmin@colorado.edu.
THE TRANSMEMBRANE DOMAINS OF LATENT MEMBRANE PROTEIN -1 PLAY IMPORTANT ROLES IN VIRAL SIGNALING AND COMPLEX FORMATION

by

CHRISTOPHER MARTIN WROBEL

B.S. California Polytechnic State University, San Luis Obispo 2004

A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado in partial fulfillment
of the requirement for the degree of
Doctor of Philosophy
Department of Molecular,
Cellular, and Developmental Biology
2011
This thesis entitled:
The Transmembrane Domains Of Latent Membrane Protein -1 Play Important Roles In Viral Signaling And Complex Formation
written by Christopher Martin Wrobel
has been approved for the Department of Molecular, Cellular, and Developmental Biology

(Jennifer Martin, Thesis Advisor)

(Corrie Detweiler, Head of Thesis Committee)

Date:___________________

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.
The Transmembrane Domains Of Latent Membrane Protein-1 Play Important Roles In Viral Signaling And Complex Formation

Thesis directed by Assistant Professor Jennifer M. Martin

Epstein-Barr virus (EBV) is an oncogenic Human Herpesvirus that infects B lymphocytes. EBV’s Latent Membrane Protein-1 (LMP-1) is a constitutively active Tumor Necrosis Factor Receptor analog that activates a myriad of signaling pathways. LMP-1 activates NF-κB signaling, cytostasis, and suppresses interferon alpha (IFNα) signaling. This thesis characterizes LMP-1 signaling and dissects LMP-1 complex formation in infected cells.

Structure function analysis revealed that the first transmembrane domain of LMP-1 is required for suppression of IFNα signaling. The LMP-1 C-terminus, dispensable for suppression of IFNα signaling in B cells, is required for IFNα suppression in 293T cells. These findings indicate that the mechanisms of suppression IFNα signaling in these two cell types are different. LMP-1 signaling activity correlates with its ability to oligomerize and localize in membrane microdomains. A high molecular weight species of LMP-1 was identified by non-reducing SDS-PAGE analysis and shown to be a cysteine 238 disulfide linked homo-dimer. Dimeric LMP-1 co-purifies with TRAF3 and dimeric LMP-1 is restricted to lipid rafts. Ability to crosslink interacting LMP-1 monomers requires the LMP-1 transmembrane domain and correlates with signaling activity. These findings support the model in which complex formation is key for LMP-1 signaling activity. I explored the hypothesis that LMP-1 signals to its various effectors not by forming one large multimeric signaling complex but forming multiple distinct complexes.

Analysis of extracts from EBV-infected cells by BN-PAGE demonstrated LMP-1 forms multiple
high molecular weight native complexes. The larger subset (>669 kDa) of these complexes is enriched in lipid rafts, as is actively signaling LMP-1. The smaller subset of complexes (<669kDa) is found primarily in detergent soluble membranes, as are nonfunctional LMP-1 variants. Recombinant purified LMP-1 and LMP-1 mutants lacking transmembrane helices are unable to form the highest molecular weight complexes (>669 kDa). These results demonstrate that the intact transmembrane domain of LMP-1 is not only important in IFNα signaling suppression, but plays an essential role in LMP-1 homo-oligomerization and higher order complex formation. Understanding the mechanisms by which LMP-1 activates signaling is necessary for understanding the oncogenicity of LMP-1 and for development of effective therapeutics to disrupt this signaling activity.
DEDICATION

I would like to dedicate this thesis to my Mom for always supporting me and encouraging me to follow my dreams.
ACKNOWLEDGEMENTS

I would like to thank all of the people that helped me guide me through life so that I could make it here. First and foremost, I would like to thank my Mom for most obviously birth and for doing a wonderful job raising me. My high regard for education came from growing up under her watchful eye. She has aided me in times of trouble and celebrated my victories. The emphasis on education in my house made me dream high and reach for the stars. I would like to thank my sister, she has been supportive in all I have tried to do and I am fortunate to be able to call her family.

I would like to thank all the teachers/mentors I have had from grade school all the way through the end of graduate school. My passion from learning started and was rejuvenated by all of them. Encouragement from my teachers has been never-ending and has made me want to continually encourage and teach others. Most importantly they have given me the skills to continue to educate myself I never have to stop learning.

I would like to thank the Martin Lab and the scientific community in MCDB, both past and present. They have been instrumental in helping me grow as both a scientist and as a person. Particularly, I have learned much from the Germans (Teresa, Jakob, Steffi, and Lydia), Sandi Clement, Miguel Gonzalez and his wife Norma, and Sarah McQuate.

Finally, I would like to thank my thesis advisor, Jennifer Martin, and her dog Bode Miller. Her passion for science and enthusiasm for research has kept me going through many of the challenging times in graduate school. I am happy to call her both a mentor and a friend. It has been an honor and privilege for me to do science with her and I am grateful for all the time and attention she has spent helping me on my journey through Graduate School.
TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION ................................................................................................ 1

Herpesviruses ............................................................................................................................. 1

Epstein-Barr Virus .................................................................................................................... 2

EBV’s Latent Proteins .................................................................................................................. 4

EBERs ......................................................................................................................................... 4

EBV Nuclear antigens ................................................................................................................ 6

Latent Membrane Proteins ....................................................................................................... 7

EBV infection in vitro .................................................................................................................. 8

EBV infection in vivo ................................................................................................................... 11

B cell activation and differentiation to memory ......................................................................... 11

EBV-triggered B cell activation and differentiation into memory ............................................... 12

The Lytic Cycle ......................................................................................................................... 12

Tumorigenesis ........................................................................................................................... 13

Latent Membrane Protein-1 ...................................................................................................... 15

LMP-1 transmembrane domains .............................................................................................. 17

LMP-1 C-terminal mediated signaling ...................................................................................... 19

Goals of Thesis Project ............................................................................................................. 21

CHAPTER 2: THE FIRST TRANSMEMBRANE DOMAIN OF LMP-1 IS ESSENTIAL FOR SUPPRESSION IFNα SIGNALING ................................................................. 22

Introduction ................................................................................................................................. 22

Results ........................................................................................................................................ 24

Does LMP-1 indirectly suppress phosphorylation of Tyk2? ....................................................... 24
Does LMP-1 regulate Tyk2 activity via activation of cellular phosphatases? ..........24

Does LMP-1 inhibit IFNα signaling via a direct interaction with the IFN receptor? ......25

Structure function analysis of IFNα signaling suppression ..................................................28

Sequence motifs in the cytoplasmic N-terminus of LMP-1 are not required for inhibition of IFNα signaling. .................................................................................................28

The hydrophobic transmembrane domain of LMP-1 contributes specifically to inhibition of IFNα signaling. .............................................................................................................32

The first transmembrane domain of LMP-1 is critical for inhibition of IFNα signaling...34

FWLY sequence motif in the first transmembrane domain of LMP-1 does not mediate suppression of IFNα signaling. .........................................................................................37

Alteration of the leucine heptad motifs in the first and sixth TMD of LMP-1 results in intermediate suppression IFNα signaling and NF-κB signaling. .................................................42

Suppression of IFNα signaling by LMP-1 differs in 293T cells compared to DG75 cells. ...........................................................................................................................................46

Suppression of IFNα signaling is separable from lipid raft association and TRAF binding by LMP-1..........................................................................................................................52

Discussion .................................................................................................................................55

Sequence requirements for suppression of IFNα signaling ........................................57

Material and Methods .............................................................................................................59

Cells...................................................................................................................................59

Plasmids................................................................................................................................59

Cytokines/Antibodies ........................................................................................................60

Transfections .....................................................................................................................61

Immunoprecipitation ..........................................................................................................61

SDS-PAGE and Western Analysis: ...................................................................................61

Lipid Raft Flotation assays: ...............................................................................................62
CHAPTER 3: ANALYSIS OF LMP-1 DIMER FORMATION ............................................. 63

Introduction ............................................................................................................................. 63

Results ....................................................................................................................................... 65

A SDS-resistant, higher molecular weight form of LMP-1 is found in B cell extracts. ...65
The higher molecular weight LMP-1 species is a dimer. .................................................. 65
Cysteine 238 is required for formation of the LMP-1 homo-dimer. .............. 67
Cysteine 238 is not required for activation of NF-κB by LMP-1...................... 69
Formation of higher molecular weight LMP-1 species upon crosslinking sulfhydryl
groups in LMP-1............................................................................................................... 73
TRAF3 selectively co-purifies with dimeric LMP-1.............................................. 77
Homo-oligomerization correlates with disulfide-crosslinking.......................... 79
The dimeric form of LMP-1 is restricted to lipid rafts. ................................. 81

Discussion ................................................................................................................................. 84

Materials and Methods ........................................................................................................... 88

Cells. .................................................................................................................................. 88
Antibodies and reagents. ............................................................................................... 88
Plasmids.............................................................................................................................. 89
Transfections.................................................................................................................... 90
Membrane isolation......................................................................................................... 90
BMH Crosslinking ............................................................................................................ 91
TMEA and EGS Crosslinking ..................................................................................... 91
CuP crosslinking.............................................................................................................. 92
CHAPTER 4: LMP-1 FORMS MULTIPLE HIGH MOLECULAR WEIGHT COMPLEXES

Introduction ............................................................................................................................. 95

Results ....................................................................................................................................... 97

Latent Membrane Protein-1 forms multiple high molecular weight complexes ............... 97
HMW LMP-1 complexes immunoreactive contain monomeric LMP-1. ......................... 99
Native LMP-1 complexes resolved by BN-PAGE resemble those resolved by size exclusion chromatography ................................................................. 101
Native LMP-1 complexes are unequally distributed between detergent resistant membranes and soluble fractions ................................................................. 105
LMP-1 alone is not sufficient for formation of very HMW native complexes ............ 107
The LMP-1 TMD is required for formation of HMW native complexes ..................... 109

Discussion ............................................................................................................................... 111

Materials and Methods ......................................................................................................... 113

Cells ................................................................................................................................ 113
Antibodies and reagents ................................................................................................. 113
Plasmids ......................................................................................................................... 114
Transfections .................................................................................................................. 114
NEM treatment .................................................................................................................. 114
SDS-PAGE and Western blotting ................................................................. 115
Blue Native PAGE ....................................................................................... 115
2D Gel Electrophoresis ............................................................................... 116
Size Exclusion Chromatography ............................................................... 117
Baculovirus generation and Flag-LMP-1-6xHis purification ...................... 117

CHAPTER 5: SUMMARY AND FINAL DISCUSSION ........................................... 119

TM1 of LMP-1 and NF-κB activation are required for suppression of IFNα signaling 121
LMP-1 forms a high molecular weight dimer ............................................... 125
High Molecular Weight Native LMP-1 Complexes .................................... 130
Conclusion ................................................................................................... 134
Works Cited ................................................................................................. 137
TABLES

Table 5-1 Summary of sembed ignaling activities of TMD mutants of LMP-1..........................131
FIGURES

Figure 1-1  EBV episome and latent open reading frames .................................................................5

Figure 1-2  Immortalization of B cells. ...................................................................................................9

Figure 1-3  Comparison of LMP-1 and TFNR CD40 signaling ............................................................16

Figure 1-4  Schematic of LMP-1 signaling. ..........................................................................................18

Figure 2-1  Classical IFNα signaling pathway. ..........................................................................................23

Figure 2-2  Both SHP-1 Phosphatase and IFNAR do not specifically interact with LMP-1 by co-
immunoprecipitation. .....................................................................................................................................26

Figure 2-3  The cytosolic N-terminus of LMP-1 is not responsible for suppression of IFNα
signaling by LMP-1 .......................................................................................................................................30

Figure 2-4  Schematic of TMD deletion mutants. ......................................................................................33

Figure 2-5  The transmembrane domains of LMP-1 are required for ISRE suppression by LMP-1
........................................................................................................................................................................35

Figure 2-6  TM1 of LMP-1 is required for ISRE suppression by LMP-1 .....................................................36

Figure 2-7  Schematic of LMP-1 mutants with altered motifs in LMP-1 TMD. ........................................38

Figure 2-8  The previously characterized FWLY signaling motif in the first transmembrane
domain of LMP-1 is not required for ISRE suppression but does retains NF-κB
signaling. .........................................................................................................................................................39

Figure 2-9  FWLY motif in the first transmembrane domain of LMP-1 is not required for NF-κB
signaling. .........................................................................................................................................................43

Figure 2-10  Previously characterized leucine heptad signaling motif in the first transmembrane
domain of LMP-1 is not required for ISRE suppression by LMP-1............................................................44

Figure 2-11  Schematic of C-terminal LMP-1 mutants. ............................................................................47

Figure 2-12  Mutants lacking C-terminal activation regions (CTARs) of LMP-1 cannot activate
NF-κB signaling. ............................................................................................................................................49

Figure 2-13  LMP-1 mutants differentially activate ISRE signaling in 293T and DG75 cell lines.
.....................................................................................................................................................................51
Figure 2-14  LMP-1 first TMD mutants cannot migrate to lipid rafts or bind TRAFs. ............54

Figure 3-1  B cells expressing LMP-1 contain an SDS-resistant, homo-dimeric form of LMP-1. ..........................................................66

Figure 3-2  Cysteine 238 is required for formation of LMP-1 homo-dimers.........................68

Figure 3-3  Cysteine 238 is not essential for activation of NF-κB ........................................70

Figure 3-4  Cysteine-disulfide crosslinking occurs post cell lysis. ........................................72

Figure 3-5  Generation of high molecular weight forms of LMP-1 following crosslinking ......74

Figure 3-6  Dimeric LMP-1 selectively co-purifies with TRAF3. ........................................78

Figure 3-7  Cysteine-disulfide crosslinked LMP-1 species are not found in cells expressing a non-oligomerizing LMP-1 variant (LMP-1/TMD5,6). ........................................80

Figure 3-8  Disulfide-crosslinked dimeric LMP-1 is restricted to detergent resistant membranes. ..................................................................................................................82

Figure 4-1  LMP-1 forms multiple high molecular weight complexes. ..........................98

Figure 4-2  Complexes resolved by BN-PAGE contain LMP-1 ........................................100

Figure 4-3  High molecular weight complexes contain monomeric LMP-1......................102

Figure 4-4  LMP-1 complexes can be fractionated using size exclusion chromatography......103

Figure 4-5  HMW LMP-1 native complexes are in enriched in DRMs. ............................106

Figure 4-6  Recombinant LMP-1 is not sufficient to form all the highest molecular weight complexes..................................................................................................................108

Figure 4-7  LMP-1 complexes in transfected B cells are comparable to complexes identified in EBV+ Lymphoblastoid cell lines.........................................................110

Figure 5-2  Model illustrating the hypothetical relationship between LMP-1 trimerization, multimerization, and formation of LMP-1 disulfide-linked dimers...................128

Figure 5-3  Hypothetical relationships between LMP-1 complex formation and signaling complexes..........................................................................................................132

Figure 5-4  LMP-1 TMD mediates LMP-1 complex formation leading to greater signaling potential..........................................................135
CHAPTER 1: INTRODUCTION

The Epstein-Barr virus is a Human Herpesvirus associated with tumorigenesis. EBV is associated with Burkitt’s lymphoma, Nasopharyngeal carcinoma, and Hodkin’s disease. The viral signaling protein, LMP-1, is necessary in EBV’s ability to establish infection and immortalize primary cells in culture. The work in this thesis examines the signaling propagated and native complexes formed by EBV’s LMP-1 to characterize EBV’s oncogenic potential.

Herpesviruses

The Human Herpesvirdiae family of viruses are large linear double-stranded DNA viruses. A hallmark of this virus family is its long latent phase during which it does not replicate. The name “herpeseviruses” is derived from Greek word ‘herpein’ meaning “to creep” and refers to the long latent phase of viral life cycle. Characteristics of this family of viruses include a 120-220 nm enveloped particle with glycoprotein spikes, a 120-230 kb double stranded linear DNA genome, and amorphous tegument (Morgan, Rose, Holden, & Jones, 1959). Herpesviruses encode their own genes for DNA synthesis. Viral progeny bud through the plasma membrane of the host, coating the progeny virus capsids with a lipid envelope derived from the host membrane. Latency is disrupted by lytic viral production and results in the death of the host cell.

Eight human herpesviruses have been discovered: Herpes simplex virus 1 and 2 (HSV-1 and HSV-2), Varicella Zoster virus (VSV)(Chicken Pox virus), Epstein-Barr virus (EBV), Cytomeglovirus (CMV), Human Herpesvirus 6 and 7 (HHV6 and HHV7) and Kaposi’s Sarcoma-associated virus (KSHV). Herpesviruses are segregated into alpha, beta, and gamma based on host range, genome sequence similarity, and characteristics of infection (Roizman, et
Alphaherpesviruses are the most well known of the herpesvirus family and contain the viruses that are the cause of cold sores, chicken pox, and shingles (i.e. HSV1,2 and VSV). Betaherpesviruses include CMV, HHV6, HHV7, and gammaherpesviruses include the lymphocryptic viruses such as EBV and KSHV. EBV infects and establishes latency in B lymphocytes. EBV expresses between one and nine proteins during EBV’s latent phase. The thesis research described here focuses on the mechanism of signaling of an essential latent viral protein from EBV, LMP-1. The subject of this thesis is on EBV’s Latent Membrane Protein-1 (LMP-1) and the complexes and signaling propagated from that viral protein.

**Epstein-Barr Virus**

The Epstein-Barr Virus (EBV) is a human herpesvirus and infects over 90% of the adult population worldwide. EBV infects primary human B cells and is the causative agent of infectious mononucleosis. Transmission of EBV by saliva has earned it the nickname “the kissing disease”. Primary infection of EBV is usually asymptomatic and usually occurs in childhood. Individuals not infected as children may generate a large immune response upon primary infection later in life resulting in acute infectious mononucleosis. Fatigue during mononucleosis is caused by the overactive stimulation of the immune system and the proliferation of EBV-infected B cells. The immune system of a recovered patient can successfully detect and eliminate EBV-infected cells and produce of EBV-specific inactivating antibodies.

EBV is composed of a torid-shaped protein core wrapped with viral dsDNA linear genome of 172 kilobases. EBV’s DNA content is 60 mole percent guanine or cytosine. The viral nucleocapsid is composed of 162 capsomeres proteins that form an icosahedron about 100 nanometers in diameter. A tegument of globular proteins surrounds the DNA genome and is in
between the nucleocapsid and the viral envelope. The outer envelope is composed of lipids from the host cell membrane with numerous glycoprotein spikes. EBV has one predominant glycoprotein expressed on the outer envelope, gp350/220.

Gp350/220 is a ligand for CD21 (CR2) on human cells. CD21, a cell surface receptor on B cells, signaling is triggered upon binding to complement. This signaling is necessary for EBV gene expression. EBV targets B cells by gp350/220 and gp42 binding to major histocompatibility class II (MHC-II) proteins, respectively (J. Fingeroth, et al., 1984; Q. Li, et al., 1997; Tanner, Weis, Fearon, Whang, & Kieff, 1987). Viral entry can occur by two mechanisms. EBV virions can be endocytosed and fusion of the viral envelope with the endosomal membrane releases the viral capsid into the cytoplasm. Alternatively, the bound virus can enter by fusion of the viral envelope with the plasma membrane (J. D. Fingeroth, Diamond, Sage, Hayman, & Yates, 1999). After fusion viral DNA is transported to the nucleus by an unknown mechanism most likely involving movement along cytoskeletal elements. EBV gene expression induces the infected cell to enter the cell cycle.

Once in the nucleus EBV’s genome is maintained as an episome. The genome is circularized by the long tandem repeats in 16-20 hours after initial infection (Alfieri, Birkenbach, & Kieff, 1991; Hurley & Thorley-Lawson, 1988). The genome encodes over 100 open reading frames (ORFs), most of which are replication machinery expressed during the lytic cycle (Kieff, Fields, Knipe, & Howley, 1996). EBV’s genome maintenance and replication are dependent the plasmid origin of replication (OriP). OriP consists of tandem repeats and a dyad symmetry element. These two elements form binding sites that allow the viral protein EBNA1 to tether the EBV episome to the genome. Tethering of the episome to the genome is essential in
maintenance and replication of the viral genome (J Yates, Warren, Reisman, & Sugden, 1984). OriP also ensures replication of the viral episome during cellular DNA synthesis.

**EBV’s Latent Proteins**

EBV, like other herpesviruses, spends most of its life cycle in the latent phase during which the viral genome replicates once per cell cycle (with the host DNA) and viral gene expression is limited to key latent viral genes. In this phase EBV maintains its genome and minimizes signaling activity to avoid the body’s immune surveillance. Up to 9 viral proteins and 2 RNAs are expressed by EBV during the latent phase (Figure 1-1) (Kieff, et al., 1996). These gene products include two EBV RNAs (EBERs), six EBV nuclear antigens known as EBNAs (EBNA1, EBNA2, EBNA3A, EBNA3B, and EBNA3C, and EBNA-LP), and three latent membrane proteins (LMP-1, LMP-2A and LMP-2B).

**EBERs**

EBERs are non-coding RNAs and are the most abundantly expressed EBV transcripts in latently infected cells. EBERs 1 and 2 play key roles in maintenance of malignant phenotypes in Burkitt’s lymphoma tumor cells (Komano, Maruo, Kurozumi, Oda, & Takada, 1999). High levels of EBER expression have been found in EBV-positive Burkitt’s lymphoma tumor biopsies (Takada & Nanbo, 2001). The primary sequences of the EBERs are strongly conserved among different EBV strains. The EBERs induce autocrine production of IL-10 supporting...
Figure 1-1 EBV episome and latent open reading frames.
Schematic of the 172 kilobase EBV episome and latently expressed ORFs and RNAs. Names of the gene products show the approximate positions of the ORFs on the genome. *EBER*: Epstein-Barr encoded RNA; *EBNA*: Epstein-Barr nuclear antigen; *LMP*: latent membrane protein. Asterisks indicate proteins essential for immortalization of primary B cells *in vitro*. Arrows indicate promoters of the latent genes, EBNA proteins are expressed from either the C or W promoter (Cp or Wp, respectively). *TR*: Terminal repeats are required circulation of the linear genome. *OriP* is the plasmid origin of replication to which EBNA1 binds to tether the episome to chromosomal DNA.
growth of Burkitt’s lymphoma (Kitagawa, et al., 2000; Samanta, Iwakiri, & Takada, 2008). The EBERs bind the active double stranded RNA bound form of Protein Kinase R (PKR) and inhibit its phosphorylation blocking IFNα-induced apoptosis (Nanbo, Inoue, Adachi-Takasawa, & Takada, 2002; Takada & Nanbo, 2001).

**EBV Nuclear antigens**


EBNA1 associates with chromosomes during mitosis and binds with affinity to a specific viral DNA sequence within OriP (Jones, Hayward, & Rawlins, 1989). EBNA1 is composed of 4 domains: a basic amino terminus, a glycine-alanine copolymer that makes beta sheets, a short domain of basic amino acids, and a long hydrophilic tail rich in basic and acidic amino acids. EBNA1 is essential for maintenance, replication, and segregation of the viral genomes during latency (Jones, et al., 1989; J. Yates, Warren, & Sugden, 1985). EBNA1 also regulates its own transcription as well as transcription of LMP-1(Kieff, et al., 1996; Nonkwelo, Skinner, Bell, Rickinson, & Sample, 1996).

EBNA2 is acidic and is composed of a negatively charged amino terminus, polyproline domain, conserved domain, divergent domain, and a charged carboxy-terminus. EBNA2

EBNAs 3A-3C inhibit transcription by binding to CBF-1/RBP-Jκ (Krauer, Kienzle, Young, & Sculley, 1996; Robertson, Lin, & Kieff, 1996). These hydrophobic EBNAs localize to large intranuclear clumps that fill the nucleus (Jiang, et al., 1991).

**Latent Membrane Proteins**

EBVs genome encodes three latent membrane proteins, which function as B cell receptor mimics (LMP-1, LMP-2A, and LMP-2B). LMP-2A and B are not essential for transformation by EBV (Longnecker, 2000). LMP-2A and LMP-2B proteins are expressed from separate mRNAs that differ in their first exon arrangements. LMP2A and LMP2B both contain an identical 12-pass transmembrane domain but LMP2A encodes an addition of a cytoplasmic N-terminal domain with an SH2 binding domain and ITAM motif. These N-terminal motifs are required for LMP2A’s function as a viral B cell receptor mimic. LMP-2A and 2B have also been shown to down regulate surface expression of the interferon-alpha and gamma receptors by promoting their degradation (Shah, et al., 2009).

Latent Membrane Protein-1 (LMP-1) is known as the EBV oncoprotein. LMP-1 mimics the tumor necrosis factor receptor (TNFR), CD40, by activating both NF-κB and JNK signaling.
A N-terminally truncation version of LMP-1 (expressed from a separate mRNA), lyLMP-1 is up regulated in the lytic cycle and doesn’t share LMP-1 signaling activities (Erickson & Martin, 2000). LMP-1 is the subject of this thesis and will be discussed in detail later in this chapter.

**EBV infection in vitro**

EBV efficiently immortalizes primary B cells in culture. Human B cells isolated from peripheral blood cannot survive in vitro unless first infected with EBV (Figure 1-2). Immortalization of primary human B cells by EBV is efficient. At least 50% of B cells can be infected with EBV and 30% become immortalized (Henderson, Miller, Robinson, & Heston, 1977). This efficiency indicates that a wild type virus infects and immortalizes a wild type B cell (i.e. immortalization does not occur as a result of mutation in the viral cellular genome).

Immortalization of cells in vitro requires expression of four EBV proteins: EBNA2, EBNA3A, EBNA3C and LMP-1 (Hammerschmidt & Sugden, 1989; Kaye, Izumi, & Kieff, 1993; Tomkinson, Robertson, & Kieff, 1993). It is important to distinguish that cells immortalized in vitro do not differentiate into memory B cells (as occurs in vivo). Rather, EBV transforms the naïve B cell into a proliferating lymphoblast in vitro (Henle, Diehl, Kohn, Zur Hausen, & Henle, 1967; Pope, Horne, & Scott, 1968; Thorley & Mann, 1985). Infection of primary B cells induces RNA synthesis, immunoglobulin secretion, DNA synthesis, B cell activation markers, and cell division (Thorley & Mann, 1985).
**Figure 1-2 Immortalization of B cells.**
Illustration depicts life cycle of EBV in B cells in vitro and in vivo. *Top:* Differentiation of uninfected resting naïve B cell into memory B cell. Stimulation of B-lymphocytes with antigen triggers activation into lymphoblasts. Activated lymphoblasts proliferate and from a germinal center. Rescue of the B cell from apoptosis and differentiation into memory requires stimulation by CD40 ligand and antigen (see text). *Middle:* EBV life cycle in vivo. EBV transforms stimulates naïve B-lymphocytes into proliferating B-lymphoblasts to become proliferating lymphoblasts, which then form a germinal center. These infected cells are rescued from apoptosis by two viral proteins LMP-1 and LMP-2A. LMP-1 mimics activation of CD40, while LMP-2A mimics stimulation of the B cell receptor with antigen. Rescued cells either become plasma cells (hypothesized to be virus producing/lytic) or differentiate into memory B cells. *Bottom:* Model of EBV life cycle in vitro. Infection of primary B-lymphocytes in culture by EBV results in transformation to B-lymphoblasts and these cells are immortal. Some immortalized cell lines can be stimulated to enter the lytic cycle with chemicals or crosslinking of surface immunoglobulin (see text). In vitro transformed cells continue to proliferate ad infinitum and are immortal. The dashed lines represent entry into EBV’s lytic cycle, an event that happens rarely in vitro and spontaneously in vivo.
EBV infection in vivo

EBV is transmitted in saliva and the site of primary infection is the nasopharengeal epithelial mucosa. Epithelial cells are infected with EBV, the virus replicates lytically and progeny virus infects naïve B cells in the underlying mucosa. EBV proteins trigger host B cell signaling pathways in the infected B cell with the goal of promoting their differentiation into the memory B cell pool (Babcock, Decker, Volk, & Thorley-Lawson, 1998; Thorley-Lawson & Babcock, 1999). Infected B cells are activated and proliferate in a manner that mimics B cell activation/proliferation by antigen (Figure 1-2). EBV triggered activation/differentiation is constitutive whereas B cell receptor activation/differentiation requires antigen and T-cell help.

B cell activation and differentiation to memory

Antigen stimulation in healthy individuals results in B cell activation, proliferation, and differentiation. These proliferating lymphoblasts form sites of T-cell exclusion and high-density proliferation known as germinal centers. Proliferating lymphoblasts undergo apoptosis unless external signals are received from helper T-cells (Figure 1-2). Apoptosis is avoided when the proliferating lymphoblast receives two signals from helper T-cells. The first signal is the secondary stimulation with antigen presented by the helper T-cell to the B cell receptor on the lymphoblast. This signal ensures the B cell can still recognize its target antigen. The second signal is the stimulation of the B cell TNFR CD40 by CD40 ligand (CD40L) on the surface of helper T-cells. CD40 is a B cell surface receptor of the TNFR superfamily that plays an essential role in B cell proliferation, survival, and differentiation to memory (Xie, Kraus, Stunz, & Bishop, 2008). Stimulation by CD40 ensures that the B cell is presenting a foreign (not “self”) antigen. CD40 and B cell receptor activation triggers downstream signaling important for B cell
maturation. Signals by both CD40 and the B cell receptor are necessary for survival of germinal center B cells. Once selected, cells either differentiate into antibody secreting lymphoblasts (plasma cell) or memory B cells. Plasma cells have a short lifespan whereas the long-lived memory B cells are non-dividing until stimulated by antigen to differentiate into a plasma cell.

**EBV-triggered B cell activation and differentiation into memory**

The environment within infected B cells very closely mirrors that of an antigen-stimulated B cell. Infection transforms B lymphocytes to proliferating B lymphoblasts. Proliferating B lymphoblasts migrate to lymph nodes and form germinal centers. Signals generated by viral proteins prevent apoptosis of infected cells. LMP-1 provides signaling normally generated by activation of CD40 and CD40L, and LMP2A provides signals normally generated by B cell receptor activation. LMP-1 rescues germinal center B cells from apoptosis in the absence of antigen. These rescued B cells then differentiate into memory or become antibody producing plasma cells. LMP-1 positive memory B cells can be reactivated upon stimulation with antigen. EBV’s infection of memory B cells allows EBV to remain latent in the long-lived infected memory B cells and allows it to remain relatively undetected by the immune system.

**The Lytic Cycle**

EBV is primarily latent in B cells but reactivation is necessary for production and spread of progeny virus. Reactivation of the virus and entry to the lytic cycle are proposed to occur upon stimulation of the B cell receptor by antigen or differentiation of the infected cell into a plasma cell (Laichalk & Thorley-Lawson, 2005). Plasma cells normally return to the site of initial stimulation with antigen to secrete antibody. Virus producing plasma cells are also
hypothesized to return the initial site of viral infection (oropharyngeal mucosa) to shed their virus. Viral shedding at this site allows infection to be maintained in the host by re-infection of new naïve B cells through viral shedding into salvia for infection of new individuals.

Activation of infected memory B cells result in reactivation of the virus and entry lytic cycle. In vitro, few infected B cell lines can be induced to enter the lytic cycle. Permissive cells can be induced into the lytic cycle through cross-linking of surface immunoglobulin or by treatment with phorbol esters, calcium ionphores, sodium butyrate, and 12-0-tetradecyloxyphorbal-13-acetate (TPA) (Faggioni, et al., 1986; zur Hausen, O'Neill, Freese, & Hecker, 1978). Chemical and surface immunoglobulin induction of the lytic cycle results in increased transcription of two essential immediate early genes of EBV’s lytic cycle (BZLF1 and BRLF1) resulting in reactivation of EBV’s lytic cycle.

Most EBV encoded genes are lytic cycle proteins (replication proteins, structural proteins, and envelope glycoproteins). EBV replicates its genome during the lytic cycle by rolling circle replication after an initial amplification of EBV episomes following infection. Viral envelope glycoproteins are predominately found on the surface of infected cells during the lytic cycle. Replicated DNA is packaged into viral capsids that bud through multiple membrane compartments before finally budding through the plasma membrane. Lytic cycle production of viral progeny ultimately results in host cell death.

Tumorgenesis

EBV is associated with many human cancers (Burkitt’s Lymphoma, nasopharyngeal carcinoma, Hodgkin’s lymphoma, Non-Hodgkins’s lymphoma, gastric carcinomas). The EBV genome does not integrate into the host genome and EBV does not overexpress dominant oncogenes. The long latency period between primary infection and tumor development (5-30
years) is evidence that EBV is not sufficient for tumorigenesis. EBV’s association with cancer is evidenced by clonal copies of EBV’s genome found in biopsies of EBV-associated tumors. EBV infected cells isolated from lymphomas and injected into nude and SCID mice develop lymphomas (Okano, et al., 1990; Walter, et al., 1992). This demonstrates that immunosuppression and EBV infection are required for tumorgenesis in this animal model.

EBV can efficiently transform human B cells in culture. Despite EBV’s ability to immortalize B cells, most individuals do not develop cancer. Immunocompetent individuals do not develop EBV-associated tumors because the number of infected cells is kept low by immune surveillance. Immunosuppression is a major factor in EBV associated tumorgenesis.

EBV-positive endemic Burkitt’s lymphoma (BL) is endemic to equatorial Africa as is malarial infection (Burkitt, 1958; God & Haque, 2010). EBV is found in 98% of African Burkitt’s lymphoma. BL is characterized by a chromosomal translocation of c-myc gene on chromosome 8 to the immunoglobulin locus on chromosome 14 resulting in “deregulation” of c-myc. Immunosuppression associated with malarial infection results in uncontrolled proliferation of EBV-infected B cells. Tumors usually form around the facial bones, jaws, ovaries, or breasts. Individually, the contributing factors (Malaria and EBV infection) do not lead to tumorgenesis, but synergistically provide an environment for tumorgenesis (Dalldorf & Barnhart, 1972; God & Haque, 2010). Immunodeficiency associated Burkitt’s lymphoma does not require EBV infection for tumorgenesis, although EBV is associated with 30-60% of AIDS related Burkitt’s lymphoma (Carbone, Cesarman, Spina, Gloghini, & Schulz, 2009; God & Haque, 2010; Ramos da Silva & Elgui de Oliveira, 2011). Unlike endemic Burkitts lymphoma, immunodeficiency associated Burkitt’s lymphoma occurs in adults HIV/AIDS patients. EBV is associated with
many AIDS related lymphomas where the percentage of cases associated with viral infection varies from 60% to 100% (Carbone, et al., 2009).

EBV is also associated with Hodkin’s disease and Nasopharyngeal carcinoma. Hodkin’s disease is commonly associated with EBV infection and is identified by tumor cells that express cellular markers of germinal center B cells. Biopsies from Hodkin’s disease patients reveal cells with mutations in the immunoglobulin variable region. These cells would normally undergo apoptosis in a healthy individual. EBV genomes are found in some, but not all Hodkin’s disease. LMP-1 and LMP-2A’s role in rescuing germinal center B cells could explain EBV’s role in Hodkin’s disease. Nasopharyngeal carcinoma is common in the southern regions of China and is tumor of the nasopharynx. EBV is highly associated with this carcinoma but strong evidence supports a role for additional factors such as environment, diet, and ethnic background.

**Latent Membrane Protein-1**

Latent Membrane Protein-1 (LMP-1) is a latently expressed viral membrane protein. LMP-1 transforms rodent cells to anchorage independent growth and is required for EBV immortalization of primary human B cells (Kaye, et al., 1993; D. Wang, Liebowitz, & Kieff, 1985). LMP-1 functions in part as a constitutive viral mimic of CD40 (Uchida, et al., 1999) (Figure 1-3). Like CD40, the cytoplasmic C-terminus of LMP-1 interacts with a variety of cellular signaling proteins via conserved C-terminal activating regions (CTARS) 1, 2, and 3 (Brodeur, Cheng, Baltimore, & Thorley-Lawson, 1997; Gires, et al., 1997; Izumi, Kaye, & Kieff, 1997) and sends constitutive signals via CTARs 1, 2, and 3 to NF-κB, JNK/AP-1, and
Figure 1-3  Comparison of LMP-1 and TFNR CD40 signaling.  

CD40 signaling: CD40 ligand is shown on the surface of a helper T-cell, binding to the extracellular domain of CD40. Active CD40 is trimerized in lipid rafts. Signaling to NF-κB and JNK activate survival.  

LMP-1 signaling: LMP-1 oligomerizes (shown as a monomer in the picture for simplicity) in lipid rafts and constitutively activates downstream NF-κB signaling via TRAFs.
STAT transcription factors resulting in B cell survival and proliferation (A. Eliopoulos & L. Young, 1998; Eliopoulos, et al., 2003; Gires, et al., 1999; Huen, Henderson, Croom-Carter, & Rowe, 1995). LMP-1 and CD40 share no overt homology other than the small CTAR domains. LMP-1, unlike CD40, is oncogenic in part due to its constitutive activity.

LMP-1 is 386 amino acids long and has three structural domains defined by their function: a cytoplasmic N-terminus (aa 1-24), six membrane-spanning domains (aa 24-186), and an acidic cytoplasmic C-terminus (aa 187-386)(Figure 1-4). Although LMP-1 has a predicted molecular weight of 42 kDa, it migrates at 63 kDa on SDS-PAGE due to its highly acidic C-terminus. LMP-1 turns over rapidly and has a half-life between 3 to 6 hours (Martin & Sugden, 1991b).

The cytosolic N-terminus of LMP-1 is essential for proper membrane insertion of the LMP-1 transmembrane domain and subsequent turnover (Coffin , Erickson, Hoedt-Miller, & Martin, 2001; Martin & Sugden, 1991b). Functional analysis of the N-terminus has not demonstrated any known signaling activity (Coffin , et al., 2001).

LMP-1 transmembrane domains

The transmembrane domain (TMD) of LMP-1 has six predicted transmembrane domain segments as predicted by hydrophobicity plot (Hudson, Farrell, & Barrell, 1985). The reverse turn between the first and second transmembrane domain has a chymotrypsin cleavage site whose extracellular location that has been experimentally confirmed (Hennessy, Fennewald, Hummel, Cole, & Kieff, 1984; Liebowitz, Wang, & Kieff, 1986; Martin & Sugden, 1991b). The TMD is the LMP-1 “activation” domain. The TMD of LMP-1 is necessary and sufficient for
LMP-1 C-terminal signaling results in stimulation of NF-κB, JNK, and STAT pathways. LMP-1 CTARs 1, 2, and 3 are sequences that bind TRAFs (TRAFs 1, 2, 3, 5), TRADD, and JAK to stimulate downstream signaling. LMP-1 activates both the canonical and non-canonical NF-κB pathways. LMP-1 TMD confers association with lipid rafts, homo-oligomerization (LMP-1 in the figure is shown as a monomer for simplicity), and cytostatic signals. The TMD sequence requirements for each of these activities are unknown.
oligomerization and association with lipid rafts (Clausse, et al., 1997; Higuchi, Izumi, & Kieff, 2001). LMP-1 signaling activity correlates with its association with lipid rafts (Higuchi, et al., 2001), and LMP-1 TMD replaced with a heterologous TMD is deficient in C-terminal signaling (A Kaykas, Worringer, & Sugden, 2001). Lipid rafts are classically defined as sphingolipid and cholesterol enriched membrane microdomains that provide scaffolding and bring signaling components together to promote more efficient signaling. Association with detergent resistant membranes following fractionation by a sucrose step gradient is used to measure LMP-1 association with lipid rafts.

LMP-1 is constitutively active unlike the TNFR it mimics, CD40. CD40 binds ligand as a trimer in lipid rafts to activate signaling. The requirements for LMP-1 signaling to date include homo-oligomerization and raft association. The first membrane-spanning segment (TM1) of LMP-1 is important for its activity to migrate to lipid rafts (Coffin, et al., 2001; J. Lee & Sugden, 2007; Yasui, Luftig, Soni, & Kieff, 2004). Two motifs in TM1, the leucine heptad motif and an FWLY sequence, contribute to LMP-1 lipid raft association and downstream NF-κB activation.

**LMP-1 C-terminal mediated signaling**

LMP-1 functions in part as a constitutive CD40 mimic (Gires, et al., 1997; Kilger, Kieser, Baumann, & Hammerschmidt, 1998; Mosialos, et al., 1995). The cytoplasmic C-terminus of LMP-1 is required for downstream activation to NF-κB, JNK, and STAT pathways through binding TRAFs by three CTARs (Figure 1-4). As a constitutively active TNFR mimic, LMP-1 activates a variety of pathways (NF-κB/JNK) linked to B cell survival and proliferation. Unlike the TNFRs, where activity is ligand dependent, LMP-1 signaling is ligand-independent and oncogenic. LMP-1 and CD40 bind to many of the same Tumor Necrosis Factor Receptor
Associated Factors (TRAFs) via CTARs in their cytoplasmic C-termini and activate many of the same pathways (i.e. NF-κB and JNK) to elicit similar, but not identical, phenotypic changes in cells (Bishop, et al., 2007; Brodeur, et al., 1997; O Devergne, et al., 1996; A. G. Eliopoulos & L. S. Young, 1998; Huen, Henderson, Croom-Carter, & Rowe, 1995; Kulwichit, et al., 1998; M. Sandberg, Hammerschmidt, & Sugden, 1997). LMP-1 activates these pathways by binding of TRAFs to its CTARs.

The C-terminus of LMP-1 is necessary for activation of both NF-κB and JNK signaling pathways. NF-κB activation by LMP-1 is mediated by TRAF1/2 and TRAF3/TRADD heterodimers that bind to LMP-1 CTAR 1 and 2, respectively (H. Li & Chang, 2003) (Figure 1-4). TRAF3 and TRAF5 bind to LMP-1 the (PxQxT) binding sequence of CTAR1 (Brodeur, et al., 1997). CTAR1 is also required for phosphoinositatal-3-kinase (PI3K) activation and Akt signaling (Dawson, Tramountanis, Eliopoulos, & Young, 2003). CTAR2 binds TRAF2/TRADD heterodimers resulting in JNK activation. TRAF3 overexpression results in inhibition of LMP-1 NF-κB signaling. LMP-1 interacts with JAK3 through CTAR3 and activates downstream STAT signaling (Gires, et al., 1999).

LMP-1 is also coupled to non-TRAF associated signaling pathways via its hydrophobic transmembrane domain (TMD). Cells expressing have underphosphorylated Tyk2 janus kinase and are unresponsive to interferon alpha (Geiger & Martin, 2006). LMP-1 expression also activates cellular stress pathways leading to activation of the unfolded protein response (D. Lee, Lee, & Sugden, 2008). Overexpression of the LMP-1 TMD (as little as 3 fold higher than found in immortalized 721 cells) results in cell cycle arrest (Floettmann, Ward, Rickinson, & Rowe, 1996; Hammerschmidt, Sugden, & Baichwal, 1989; A Kaykas & Sugden, 2000).
Understanding the mechanisms of LMP-1 signaling is challenging because of the many different signaling outputs. To date, the study of LMP-1 signaling has been tackled using a structure/function approach. The signaling activities of LMP-1 mutants are measured using reporter assays or biochemical assays and are compared to wildtype LMP-1. However, the stoichiometry and mechanism by which LMP-1 homo-oligomerizes is unknown. The assembly of LMP-1 signaling complexes in membrane microdomains is likely required for active signaling, but nature of these complexes is unknown. My research is focused on understanding the mechanisms of signaling activation by LMP-1’s TMD.

**Goals of Thesis Project**

The majority of studies of LMP-1 signaling have centered on understanding the LMP-1 cytoplasmic C-terminal signaling domain. Our lab discovered that LMP-1 suppresses IFNα signaling and that this suppression activity is independent of the LMP-1 C-terminus. The goal of my thesis work was to dissect the mechanism by which LMP-1 interacts with the IFNα pathway, with a focus on the contribution of the TMD to this process. Integral to this project was an analysis of the nature of LMP-1 signaling complexes in the cell, with the goal of determining whether distinct signaling complexes are linked to specific singling outcomes.
CHAPTER 2: THE FIRST TRANSMEMBRANE DOMAIN OF LMP-1 IS ESSENTIAL FOR SUPPRESSION IFNα SIGNALING

Introduction

The janus kinase signaling intermediate, Tyk2, is underphosphorylated in LMP-1 positive cells resulting in suppression of interferon alpha (IFNα) signaling (Geiger & Martin, 2006). This chapter describes experiments to elucidate the mechanism and domains by which LMP-1 suppresses IFNα signaling.

The host’s immune system is activated upon viral infection. This occurs in two phases: a nonspecific innate immunity phase followed by a specific adaptive immunity phase. IFNα, part of the innate immune phase, is produced and detected within 24 hours after EBV infection (Kikuta, Mizuno, Yano, & Osato, 1984). Stimulation by IFNα causes transcription of interferon-stimulated genes (ISGs), resulting in anti-proliferative and anti-viral effects in the cell (Figure 2-1). The IFNα receptors (IFNAR) 1 and 2 dimerize upon binding extracellular IFNα. The receptor-associated janus kinases (JAKs), Jak1 and Tyk2, undergo auto-phosphorylation and phosphorylate the receptor. The phosphorylated receptor acts as a docking site for signal transducers and activators of transcription (STAT) proteins 1 and 2, which are substrates for the associated JAK kinases. Phosphorylated STATs are then released from the receptor, dimerize, and recruit interferon regulatory factor-9 (IRF-9) in the cytoplasm. This complex, known as interferon stimulated gene factor 3 (ISGF3), translocates to the nucleus and binds
Figure 2-1 Classical IFNα signaling pathway.
IFNα causes IFNAR subunits to dimerize leading to phosphorylation of the associated janus kinases (Tyk2 and Jak1) and STAT1 and STAT2. The phosphorylated STATs dimerize and recruit IRF9 forming the transcription factor ISGF3. The latter migrates into the nucleus and activates the transcription of IFN-stimulated genes that have ISRE sequences in their promoter.
IFN-stimulated response elements (ISREs) upstream of interferon-stimulated genes (ISGs) (Platanias, 2005). Our lab has demonstrated that LMP-1 interacts with the Tyk2 signaling intermediate (Geiger & Martin, 2006). In LMP-1 positive cells, Tyk2 is underphosphorylated and IFNα-stimulated ISRE signaling is suppressed. Suppression of IFNα signaling by LMP-1 relies on mechanisms that do not require the cytoplasmic C-terminus of LMP-1.

In this chapter, I extend these findings to show that the first transmembrane domain of LMP-1 is crucial for suppression of IFNα signaling. The contribution of the first transmembrane domain of LMP-1 to suppression of IFNα signaling is separable from previously characterized sequence motifs in the first transmembrane domain and from association of LMP-1 with lipid rafts and NF-κB activation.

**Results**

**Does LMP-1 indirectly suppress phosphorylation of Tyk2?**

**Does LMP-1 regulate Tyk2 activity via activation of cellular phosphatases?**

LMP-1 could suppress IFNα signaling by direct or indirect mechanisms. Phosphorylation of Tyk2 is important in IFNα stimulated signaling. Phosphorylation of Tyk2 results in activation allowing phosphorylation of STATs necessary for formation of transcription factor complexes that induce ISG expression. LMP-1 could accomplish this signaling by a number of mechanisms. LMP-1 could prevent the phosphorylation of Tyk2 or recruit a phosphatase to dephosphorylate active Tyk2 thereby blocking IFNα signaling. A candidate
approach was used to establish whether if LMP-1 recruits Tyk2-specific phosphatases to
downregulate ISRE signaling.

SHP-1 phosphatase has been implicated in JAK dephosphorylation. The mechanism by
which SHP-1 mediates JAK dephosphorylation has yet to be elucidated; either SHP-1
dephosphorylates upstream signaling molecules or is involved in dephosphorylation of the JAKs
themselves (Tonks & Neel, 1996). Interaction between SHP-1 and LMP-1 was tested by co-
immunoprecipitation from 721 cells, an LMP-1 positive EBV-immortalized cell line.
Immunoprecipitation of SHP-1 or LMP-1 from 721 cells failed to demonstrate a detectable
interaction between the two proteins (Figure 2-2). It is however possible that LMP-1 may
regulate SHP-1 transcription or that a SHP-1/LMP-1 interaction may be undetectable by co-
immunoprecipitation analysis. In addition to SHP-1, co-immunoprecipitation analysis failed to
reveal an interaction between LMP-1 and PTP1B or SHP2 tyrosine phosphatases (data not
shown). Negative interaction results between LMP-1 candidate phosphatases directed my
research to explore possible interactions between LMP-1 and the IFNAR1.

**Does LMP-1 inhibit IFNα signaling via a direct interaction with the interferon receptor?**

The interaction between LMP-1 and Tyk2 was identified by mass spectrometry analysis
of purified LMP-1 from 721 cell lysates. The LMP-1/Tyk2 interaction was difficult to
demonstrate by immunoprecipitation analysis of infected cells but was detectable when cells
overexpressing both LMP-1 and Tyk2 were isolated and analyzed by co-immunoprecipitation
(Geiger & Martin, 2006). The difficulty of demonstrating the interaction between Tyk2 and
Figure 2-2 Both SHP-1 Phosphatase and IFNAR do not specifically interact with LMP-1 by co-immunoprecipitation.

A&B) 721 cells were lysed and immunoprecipitated for SHP-1 or LMP-1. Immunoprecipitates or whole cell lysates (WCL) were resuspended in 4xSDS sample buffer and resolved by SDS-PAGE and blotted for SHP-1 or LMP-1. C) 721 cells were lysed and immunoprecipitated for IFNAR1. Immunoprecipitates or whole cell lysates (WCL) were resuspended in 4xSDS sample buffer and resolved by SDS-PAGE and blotted for LMP-1. A-C) Mock immunoprecipitation was carried out by the same protocol but without the addition of antibody. Arrow to the left marks the migration of SHP-1 or LMP-1.
LMP-1 could be an indication that the interaction between Tyk2 and LMP-1 is indirect. Demonstrating the interaction between LMP-1 and Tyk2 is indirect requires finding the intermediate required for LMP-1 and Tyk2 interaction. The IFNAR1 is an integral membrane protein like LMP-1 and has been shown to directly interact with Tyk2. Maturation and expression of the IFNAR1 on the plasma membrane is dependent on association of the IFNAR1 with Tyk2 (Ragimbeau, et al., 2003). LMP-1 could interact with the IFNAR1 blocking the phosphorylation of Tyk2 leading to downregulation of ISGs. LMP-1 was immunoprecipitated from 721 cell lysates and co-immunoprecipitates were analyzed for IFNAR1 by Western blot. No specific interaction between LMP-1 and IFNAR1 was detectable (Figure 2-2). The reciprocal co-immunoprecipitation for LMP-1 did not demonstrate IFNAR1 present in the immunoprecipitates (data not shown). LMP-1 expression in 721 cells is quite high, 5x10^4 molecules of LMP-1/cell (M. L. Sandberg, Kaykas, & Sugden, 2000), compared to the IFNAR, which has an average of 50-5000 receptors per cell (Ragimbeau, et al., 2003). Detection of any potential interaction between LMP-1 and the IFNAR1 is problematic due to the very low levels of IFNAR1 relative to LMP-1. LMP-1 non-specifically associates with agarose beads used for co-immunoprecipitation of the IFNAR1 (Figure 2-2). Non-specific binding of LMP-1 with the agarose beads is most likely due to interactions with the large hydrophobic transmembrane domains of LMP-1. Altering immunoprecipitation conditions to reduce nonspecific binding of LMP-1 to the beads resulted in complete signal loss making it difficult to determine if these two membrane proteins interact (not shown). Interaction between LMP-1 and IFNAR1 could not be demonstrated. Such results demonstrate a need to measure a potential interaction between two membrane proteins by a method other than immunoprecipitation. Inability of demonstrating an interaction between the IFNAR1 and LMP-1 by immunoprecipitation directed me to focus my
efforts on structure function analysis of LMP-1 and the discovery of the sequence of LMP-1 required for suppression of IFNα signaling.

Structure function analysis of IFNα signaling suppression

**Sequence motifs in the cytoplasmic N-terminus of LMP-1 are not required for inhibition of IFNα signaling.**

A structure function approach was undertaken to analyze the sequence requirement for LMP-1 mediated suppression of IFNα signaling. Our lab demonstrated that the C-terminal activation regions of LMP-1 are not required for the suppression of IFNα signaling (Geiger & Martin, 2006). I continued structure function analysis of domains important in suppression of IFNα signaling by LMP-1.

Our has lab demonstrated that the specific sequence of cytoplasmic N-terminus of LMP-1 is not important in previously known signaling activities of LMP-1. This research was done by replacement analysis of cytoplasmic N-terminal domain of LMP-1. The N-terminus of LMP-1 is composed of a 20 amino acid domain containing a potential proline/arginine rich Src Homology 3-domain (SH3) binding consensus sequence (RGPPGPRPRPGRPP). The presence of the SH3 domain binding consensus sequence suggests the N-terminus of LMP-1 functions as an effector domain in LMP-1 signaling. SH3 domains are conserved sequences that interact with proline rich peptides to form protein aggregates that serve in protein recruitment for cell-to-cell communication and signal transduction. To analyze the cytoplasmic N-terminus of LMP-1 for signaling functions an LMP-1 chimera was used. In this chimera (ASGPR/LMP-1) the first 20 N-terminal amino acids of LMP-1 were replaced with the cytoplasmic N-terminal domain of the
asialoglycoprotein receptor HI (ASGPR)(Figure 2-3) (Coffin , et al., 2001). Replacement of the N-terminal amino acids of LMP-1 did not affect LMP-1 activation of NF-κB signaling. The primary sequence of the substituted amino-terminus was not critical (as long as it was positively charged) for LMP-1 oligomerization, TRAF binding, and NF-κB signaling (Coffin , et al., 2001). Results from this study suggested that the cytoplasmic N-terminus of LMP-1 ensures proper orientation and topology of the transmembrane domains of LMP-1 in the membrane.

Regulation of IFNα signaling by LMP-1 differs mechanistically from regulation of NF-κB by LMP-1. Activation of NF-κB requires the cytoplasmic and TRAF binding domain. In contrast, this signaling domain is not involved in regulation of IFNα signaling. It was possible, that in addition to its role as a topological domain, the cytoplasmic N-terminal domain of LMP-1 functioned as an effector domain in suppression of IFNα signaling. I asked if whether the cytoplasmic N-terminus of LMP-1 functions as an effector domain in suppression of IFNα signaling by comparing the activity of ASGPR/LMP-1 to that of LMP-1 in human B lymphoma cells (DG75 cells)(Figure 2-3). In this assay activation of IFNα-stimulated transcription was measured by reporter assay. Cells were transfected with a luciferase reporter under the control of 5 tandem interferon stimulated response elements (ISREs), a β-galactosidase reporter, and RSV-driven LMP-1 expression vectors. Transfected cells were treated with 200 U/ml IFNα for 7 hours 2 days post-transfection and lysates were assayed for luciferase accumulation, β-galactosidase activity, and lysates were Western blotted for LMP-1 expression (Figure 2-3).
Figure 2-3  The cytosolic N-terminus of LMP-1 is not responsible for suppression of IFNα signaling by LMP-1.

A) Schematic of N-terminal replacement mutant ASGPR-LMP-1. Chimera of LMP-1 constructed by replacement of LMP-1 first N-terminal amino acids and replacing them with myc tag and amino acids 16-25 of the asialoglycoprotein (ASGPR) receptor H1. Detail shows a sequence comparison of LMP-1 N-terminus compared to substituted amino acids. (Coffin, et al., 2001) B) DG75 cells were transfected with 1μg of LMP-1, LMP-1/TMD5,6, ASGPR/LMP-1, or Empty Vector and ISRE driven luciferase vectors with LacZ expression vectors for quantification of ISRE activity. Two days post transfection cells were stimulated with IFNα for 7 hours before harvest and assayed for luciferase and B-galactosidase activity. Fold activation is compared to unstimulated empty vector control set to 1 with correction for B-galactosidase values. Assays were done in duplicate. Schematics of each mutant are shown below the graph. C) Lysates from A were resuspended 1:10 with 4xSDS sample buffer and resolved by SDS-PAGE and Western blotted for cytoplasmic C-termini of LMP-1. Numbers above blots correspond to samples on the graph in part A. Arrows to the left of the blots indicate migration of given mutant.
ASGPR/LMP-1 inhibited IFNα stimulated ISRE activity in a manner that was indistinguishable from LMP-1, demonstrating that the cytoplasmic N-terminus of LMP-1 does not contribute an effector function to the regulation of IFNα signaling by LMP-1. LMP-1/TMD5,6, a mutant of LMP-1 lacking the first four transmembrane domains of LMP-1, does not suppress IFNα like LMP-1 and was used as a negative control in this experiment. These results, together with previous results demonstrating cytoplasmic C-terminus of LMP-1 is not important for suppression of IFNα signaling (Geiger & Martin, 2006), demonstrate that the hydrophobic TMD of LMP-1 (and not its cytoplasmic N- or C-termini) is critical for negative regulation of IFNα signaling by LMP-1.

The hydrophobic transmembrane domain of LMP-1 contributes specifically to inhibition of IFNα signaling.

Previous results suggested that the hydrophobic TMD of LMP-1 plays a critical role in suppression of IFNα signaling by LMP-1 (Figure 2-3) (Geiger & Martin, 2006). It is possible that the hydrophobic TMD of LMP-1 contributes to suppression of IFNα signaling by specifically interacting with cellular proteins and/or lipids in the bilayer. Alternatively, the hydrophobic TMD could function simply to anchor LMP-1 in a membrane environment. To confirm that the LMP-1 TMD is important in suppression of IFNα signaling, I asked whether a heterologous multi-spanning TMD from another integral membrane protein (EBV’s LMP2A) could substitute for the LMP-1 TMD in suppression of IFNα signaling. The IFNα suppression activity of a chimera encoding the N-terminal cytoplasmic domain of LMP-1, the first six membrane spanning segments of the EBV LMP2A protein, and the cytoplasmic C-terminus of LMP-1 (LMP-1(Δ25-180)/LMP2A(126-280)(Figure 2-4) (AKaykas & Sugden, 2000) was
Figure 2-4 Schematic of LMP-1 TMD deletion mutants.

Top Row: **LMP2A**- Schematic of LMP2A and its N-terminus, 12 membrane spanning domains and cytoplasmic C-terminus. **LMP-1**- Wildtype LMP-1 with its N-terminus, 6 membrane spanning domains and cytoplasmic C-terminus. The TMDs of LMP-1 have been numbered above each respective domain. **LMP-1(Δ25-180)/LMP2A(126-280)** – Chimeric protein containing the cytoplasmic domains of LMP-1 and the transmembrane domains of LMP2A. Specifically, the first 25 cytosolic amino acids of LMP-1 were fused to the first six transmembrane domains of LMP2A connected to the last 206 amino acids of the cytoplasmic C-terminus of LMP-1.

Bottom row: **LMP-1/TMD1,6** – The cytoplasmic N-terminal domain and first TMD of LMP-1 were fused to the 6th TMD and cytoplasmic C-terminus, deleting the 2nd-5th TMDs. **LMP-1/TMD1,2** – The cytoplasmic N-terminus, 1st, and 2nd TMDs were fused to the cytoplasmic C-terminus of LMP-1, a deletion of TMDs 3-6. **LMP-1/TMD5,6** – The 5th and 6th TMD and cytoplasmic C-terminus of LMP-1. A Deletion of the N-terminus and TMDs 1-4 of LMP-1. Wildtype LMP-1 is displayed in the box for reference.
compared to that of LMP-1 (Figures 2-5). The LMP-1/LMP2A chimera had no inhibitory effect on IFNα stimulation of ISRE activity, demonstrating that the LMP-1 TMD is necessary to block IFNα stimulation. This result demonstrates that the LMP-1 TMD inhibits suppression of IFNα signaling not by just being a hydrophobic membrane anchor, but that the sequence of the LMP-1 TMD is important in suppression of IFNα signaling.

**The first transmembrane domain of LMP-1 is critical for inhibition of IFNα signaling.**

The hydrophobic LMP-1 TMD plays a role in suppression of IFNα signaling beyond that of a simple membrane anchor. Individual TMD sequence requirements for IFNα suppression were explored using as series of deletion mutants. The cytoplasmic N and C-termini of LMP-1 were fused to combinations of LMP-1 transmembrane domain segments to ensure proper orientation in the membrane and facilitate immunological detection using C-terminal specific antibodies. LMP-1/TMD1,2 lacks TMDs 3-6 and retains reduced NF-κB signaling activity and fractionates to detergent resistant membranes (Coffin, Geiger, & Martin, 2003), LMP-1/TMD1,6 lacks TMDs 2-5 and previously has not been characterized for LMP-1 signaling activities, LMP-1/TMD5,6 (lyLMP-1) lacks TMDs 1-4 and does not retain LMP-1 signaling activities (Erickson & Martin, 1997; Geiger & Martin, 2006) (Figure 2-4). TMD mutants of LMP-1 were assayed for activity by ISRE reporter activity 7 hours after stimulation with IFNα as in Figure 2-6. LMP-1/TMD1,2 and LMP-1/TMD1,6 were indistinguishable from wild type LMP-1 in their ability to suppress IFNα signaling. In contrast, LMP-1/TMD5,6 had no effect on IFNα signaling activity (Figure 2-6). Analysis of TMD mutants demonstrates that not all TMDs of LMP-1 contribute
Figure 2-5 The transmembrane domains of LMP-1 are required for ISRE suppression by LMP-1.
293T cells were transfected with 25ng of pSG5-LMP-1(Δ25-180)/LMP2A(126-280), pSG5-LMP-1 cDNA, or Empty Vector and ISRE driven luciferase vectors with LacZ expression vectors for quantification of ISRE activity. Cell lines were transfected two days ahead of time and stimulated with IFNα for 7 hours before harvest and assayed for luciferase and β-galactosidase activity. Fold activation are compared to unstimulated empty vector control set to 1 with correction for B-galactosidase values. Assays were done in duplicate. Schematics of each mutant are shown below the graph.
Figure 2-6 The first transmembrane domain of LMP-1 is required for ISRE suppression by LMP-1. DG75 cells were transfected with 1μg of LMP-1, LMP-1/TMD1,6, LMP-1/TMD1,2, or Empty Vector and ISRE driven luciferase vectors with LacZ expression vectors for quantification of ISRE activity. ISRE activity was calculated as in Figure 2-5. Assays were done in duplicate. Schematics of each mutant are shown below the graph.
equally in suppression of IFNα signaling. These results suggest that the first transmembrane domain of LMP-1 and not its 6th, plays a critical role in suppression of IFNα signaling.

**FWLY sequence motif in the first transmembrane domain of LMP-1 does not mediate suppression of IFNα signaling.**

The first transmembrane domain of LMP-1 is crucial for efficient NF-κB activation by LMP-1 (Coffin, et al., 2003; Ajamete Kaykas, Worrringer, & Sugden, 2002; J. Lee & Sugden, 2007; Vishal Soni, Yasui, Cahir-McFarland, & Kieff, 2006; Yasui, et al., 2004). A FWLY motif in the first TMD, has been reported to contribute to lipid raft association and NF-κB signaling (Vishal Soni, et al., 2006; Yasui, et al., 2004). In addition to this motif, a leucine heptad in the first transmembrane domain has been linked to LMP-1 activity (Ajamete Kaykas, et al., 2002; J. Lee & Sugden, 2007). The essential nature of TM1 for activation of NF-κB begged the question as to its involvement in suppression of IFNα signaling.

The contribution of the FWLY sequence in TM1 was tested first. The FWLY in TM1 was mutated to either A38ALA41 in LMP-1 or to L38 LLL 41 in LMP-1/TMD1,6 to generate LMP-1(A38ALA41) and LMP-1/TMD1,6(L38 LLL 41) (Figure 2-7). Interferon-stimulated ISRE activity in 293T cells transfected with LMP-1/A38ALA41 or LMP-1/TMD1,6(L38 LLL 41) expression vectors was indistinguishable from LMP-1 (Figure 2-8A). 293T cells were used instead of DG75 cells because the DG75 cells had lost their responsiveness to IFNα treatment. Preliminary work with 293T cells demonstrated LMP-1, LMP-1/TMD1,2, LMP-1/TMD1,6, and LMP-1/TMD5,6 suppressed IFNα signaling similarly in 293T cells and DG75 cells (not shown). Treatment with IFNα resulted in a ~16 fold stimulation of ISRE activity which was inhibited ~75% by LMP-1, LMP-1(A38ALA41), and LMP-1/TMD1,6(L38 LLL 41). In contrast, LMP-
Figure 2-7 Schematic of LMP-1 mutants with altered motifs in LMP-1 TMD.
A) Wildtype LMP-1 is shown in the box for reference. Detail shows the sequence of the first TMD of LMP-1. Bolded amino acid residues show amino acid residues changed in TMD mutants. B) LMP-1(A38ALA41) – LMP-1 with F38WLY41 in the first TMD to A38ALA41. C) LMP-1/TMD1,6(L38LLL41) – LMP-1/TMD1,6 (see figure 2-4) with the F38WLY41 motif in the first TMD mutated to L38LLL41. D) LMP-1(TMD1L-A) – LMP-1 with the 7 leucines in the first TMD changed to alanines. C) LMP-1(TMD6L-A) – LMP-1 with 7 leucines in the 6th TMD changed to alanines. B-E) Bold amino acids indicate changed from wild type LMP-1. Detail of amino acid sequence displays sequence of the mutant.
Figure 2-8 The previously characterized FWLY signaling motif in the first transmembrane domain of LMP-1 is not required for ISRE suppression but does retain NF-κB signaling. A) 293T cells were transfected with 25ng of LMP-1, LMP-1/TMD5,6, LMP-1/TMD1,6, LMP-1/TMD1,6(L38LLL41), pCMV-LMP-1(A38ALA41) or Empty Vector and ISRE driven luciferase vectors with LacZ expression vectors for quantification of ISRE activity. ISRE activity was calculated as in Figure 2-5. B) 293T cells were transfected with 25ng of LMP-1, LMP-1/TMD5,6, LMP-1/TMD1,6, LMP-1/TMD1,6(L38LLL41), pCMV-LMP-1(A38ALA41) or Empty Vector and NF-κB driven luciferase vectors with LacZ expression vectors for quantification of ISRE activity. Cell lines were transfected two days ahead of and assayed for luciferase and B-galactosidase activity. Fold activation are compared to unstimulated empty vector set to 1 and corrected for with B-galactosidase values. A&B) Assays were done in duplicate. Schematics of each mutant are shown below for both graphs. C) Lysates from B were resuspended 1:10 with 4xSDS sample buffer and resolved by SDS-PAGE and Western blotted for LMP-1 expression. Numbers above blots correspond to samples on the graph in part B. Arrow to the right of blot indicates migration of full length LMP-1
1/TMD5,6 did not suppress ISRE stimulation but rather, had a stimulatory effect (this stimulation by LMP-1/TMD5,6 was reproducible). Thus, the FWLY motif in TM1 of LMP-1 does not contribute to inhibition of IFNα signaling by LMP-1.

The ability of the FWLY mutants to regulate IFNα signaling was compared to their NF-κB activating activity. 293T cells were transfected with LMP-1 expression vectors, a β-galactosidase reporter (to monitor transfection efficiency), and a luciferase reporter plasmids with a minimal promoter and κB binding sites were assayed 48 hours post-transfection for luciferase activity. LMP-1, LMP-1/TMD1,6, LMP-1(A38ALA41), and LMP-1/TMD1,6(L38LLL41) all showed similar ~30 fold activation of NF-κB signaling (Figure 2-8). These results suggested that not only does the FWLY motif play no role in suppression of IFNα signaling; it likewise does not activate NF-κB. This was unexpected because the FWLY motif in the first TMD was identified because of its essential role in NF-κB signaling (Yasui, et al., 2004). The expression level of LMP-1 dictates the level to which NF-κB signaling is activated. The relationship between LMP-1 expression levels and NF-κB activation is complex. NF-κB activation by LMP-1 is “dose-dependent” at lower levels of LMP-1 expression. NF-κB activation is actually diminished in cells expressing high levels of LMP-1 (M. L. Sandberg, et al., 2000). Expression levels of LMP-1(A38ALA41) and wild-type LMP-1 differed dramatically in the experiment shown in Figure 2-8C.

Since expression levels can affect NF-κB reporter activity dramatically, LMP-1 (A38ALA41) and LMP-1 expression vectors were titrated to find a range of overlapping expression levels for comparison of NF-κB activation. Two days post transfection cells were assayed for NF-κB signaling by luciferase assay and Western blot for quantification of LMP-1 expression. LMP-1 (A38ALA41) retained 76% of wild-type LMP-1 NF-κB activation when
expressed at comparable levels (Figure 2-9). The activity of LMP-1/TMD1,6 was ~25% that of wildtype LMP-1 when compared at equivalent expression levels. As observed for LMP-1/TMD1,2 deletion mutant, LMP-1/TMD1,6 can activate NF-κB to similar levels activated by wildtype LMP-1 if expressed at high enough levels in the cell (Figure 2-8) (Coffin, et al., 2003). Together results from deletion mutants (LMP-1/TMD1,2; LMP-1/TMD5,6; and LMP-1/TMD1,6) demonstrate that TM1 plays a role in both NF-κB activation and IFNα stimulation by LMP-1 and that TMD6 is not critical for either activity. In addition, the FWLY motif in TM1 does not contribute to the function of TM1 in activation of either signaling pathway.

**Alteration of the leucine heptad motifs in the first and sixth TMD of LMP-1 results in intermediate suppression IFNα signaling and NF-κB signaling.**

TM1 of LMP-1 encodes leucine rich sequences that closely resemble a leucine heptad (zipper) motif. Leucine heptads in TMD can mediate protein interactions in artificial membrane systems (Gurezka, Laage, Brosig, & Langosch, 1999). Recombinant virus encoding leucine to alanine substitutions in the leucine heptad LMP-1 TM1 is half as efficient in B cell transformation than wild type EBV (J. Lee & Sugden, 2007). To determine if this leucine-rich domain played a role in suppression of IFNα signaling alanine to leucine substitution mutants were analyzed. Leucine heptad sequences in TM1 and TM6 were replaced by alanines (LMP-1(TMD1L-A); LMP-1(TMD6L-A);(Ajamete Kaykas, et al., 2002)(Figure 2-7) and analyzed for ISRE activation and NF-κB signaling in 293T cells. LMP-1 and LMP-1/TMD1L-A inhibited IFN-signaling by 86% and 57%, respectively (Figure 2-10). These results are consistent with results in Figure 2-6 identifying TM1 as critical for regulation of IFNα signaling by LMP-1 and
Figure 2-9 FWLY motif in the first transmembrane domain of LMP-1 is not required for NF-κB signaling.

A) 293T cells were transfected with various amounts of LMP-1 (25, 75ng), LMP-1/TMD5,6 (25ng), LMP-1/TMD1,6 (25ng), pCMV-LMP-1(A38ALA41) (3ng) or Empty Vector and NF-κB driven luciferase vectors with LacZ expression vectors for quantification of ISRE activity. Cell lines were transfected two days ahead of and assayed for luciferase and B-galactosidase activity. Fold activation are compared to unstimulated empty vector set to 1 and corrected for with B-galactosidase values. Assays were done in duplicate. Schematics of each mutant are shown below the graph. Lysates from samples were resuspended 1:10 with 4xSDS sample buffer and resolved by SDS-PAGE and Western blotted for LMP-1 expression. Blots were quantified by chemiflourescence using a Storm Imager. (*) or (**) represent samples with equivalent protein expression, respectively, based on Western blots quantified by storm imager.
Figure 2-10  Previously characterized leucine heptad signaling motif in the first transmembrane domain of LMP-1 is not required for ISRE suppression by LMP-1.

A) 293T cells were transfected with 25ng of pSG5-LMP-1, pSG5-LMP-1(TMD1L-A), pSG5-LMP-1(Δ25-180)/LMP2A(126-280), pSG5-LMP-1/TMD6L-A, pRSV-LMP-1 or Empty Vector and ISRE driven luciferase vectors with LacZ expression vectors for quantification of ISRE activity. ISRE activity was calculated as in Figure 2-4. Assays were done in duplicate. Schematics of each mutant are shown below the graph. B) 293T cells were transfected with 25ng of 25ng of pSG5-LMP-1 cDNA, pSG5-LMP-1(TMD1L-A), pSG5-LMP-1(Δ25-180)/LMP2A(126-280), pSG5-LMP-1(TMD6L-A), or Empty Vector and ISRE driven luciferase vectors with LacZ expression vectors for quantification of ISRE activity. ISRE activity was calculated as in Figure 2-8. Assays were done in duplicate. Schematics of each mutant are shown below the graph. C) Lysates from B were resuspended 1:10 with 4xSDS sample buffer and resolved by SDS-PAGE and Western blotted for LMP-1 expression. Numbers above blots correspond to samples on the graph in part B.
suggest a possible contribution of the leucine heptad motif (i.e. via protein/protein interaction or protein/lipid interaction) to inhibition of IFNα signaling.

Substitution of leucines in TMD6 also had an inhibitory effect on IFNα signaling suppressing ISRE signaling (74% inhibition relative to empty vector). Substations of leucines in either TM1 or TM6 suppressed IFNα signaling more than LMP-1(Δ25-180)/LMP2A(126-280) (our negative control)(Figure 2-10), which I have shown doesn’t block IFNα signaling (Figure 2-5). The leucine substitution mutants, LMP-1/TMD1L-A and LMP-1/TMD6L-A, exhibited intermediate stimulation of NF-κB signaling indistinguishable from that reported in the literature (Figure 2-10)(J. Lee & Sugden, 2007). Together, these results support a role for TM1, but not TM6, in IFNα suppression and NF-κB activation.

Suppression of IFNα signaling by LMP-1 differs in 293T cells compared to DG75 cells.

Analysis of LMP-1 mutants indicated thus far that suppression of IFNα is correlates with NF-κB activation. Mutants with diminished NF-κB activation do not suppress IFNα signaling. Preliminary published work from the lab demonstrated that suppression of IFNα signaling by LMP-1 was independent of C-terminal signaling domains (CTARs), which are required for NF-κB activation. These results suggested strongly that suppression of IFNα signaling by LMP-1 occurred by a mechanism distinct from that involved in NF-κB activation (Geiger & Martin, 2006). To confirm and extend this observation, the ability of a CTAR1,2 mutant (LMP-1/ΔCTAR1,2) to signal to NF-κB and suppress IFNα signaling was compared in 293T cells. LMP-1/ΔCTAR1,2 was constructed by cleavage of the last 55 amino acids from the cytoplasmic C-terminus with point mutations in the first CTAR domain to abolish TRAF binding (Figure 2-11). As predicted from the lack of CTAR domains LMP-1/ΔCTAR1,2 could not activate NF-κB
**Figure 2-11 Schematic of C-terminal LMP-1 mutants.**

*LMP-1* – Wild type LMP-1 is shown in the box for reference. Detail shows the TRAF binding sequence in CTAR1. (P$_{204}$QQAT$_{208}$) as well as the location of CTAR2. *LMP-1/ΔCTAR1* – LMP-1 with the P$_{204}$QQAT$_{208}$ in the CTAR1 of LMP-1 with two residues at position 204 and 206 changed to alanines (A$_{204}$QAAT$_{208}$) so that TRAF binding motif (PXQXT) was not conserved. *LMP-1/ΔC55* - LMP-1 mutant lacking the last Δ55 amino acids of the LMP-1 cytoplasmic C-terminus. Deleted for CTAR2. *LMP-1/ΔCTAR1,2* – A combination of LMP-1/ΔC55 and LMP-1/ΔCTAR1, mutant was constructed from LMP-1/ΔC55 and site directed mutagenesis was used to engineer P$_{204}$QQAT$_{208}$ to A$_{204}$QAAT$_{208}$. Deleted for both CTARs 1 and 2.
A LMP-1/ΔCTAR1,2 when expressed at comparable levels, was less active than the nonfunctional LMP-1/TMD5,6 mutant. These results confirmed that the CTAR1 mutant that behaved like wild-type LMP-1 in suppressing IFNα signaling in DG75 cells was deficient in NF-κB activation and suggested these activities are separable.

Initial experiments exposing the structure function relationship of LMP-1 and suppression of IFNα were conducted in DG75 cells (Geiger & Martin, 2006) (Figures 2-5,2-6,2-7). Subsequent experiments were carried out in 293T cells because of their high sensitivity to IFNα. 293T cells are readily stimulated by IFNα (20-200 fold vs 4 to 7 fold in DG75 cells)(Figure 2-12, Figure 2-3). In addition, DG75 cells “lost” their IFNα response in culture. Our work demonstrating IFNα suppression by LMP-1, LMP-1/TMD1,6, LMP-1/TMD1,2, LMP-1/TMD5,6 in 293T mimicked the results I observed in DG75 cells (data not shown).

To confirm IFNα signaling was CTAR independent in 293T cells as well as in DG75 cells, the IFNα suppression activity of LMP-1/ΔCTAR1,2 was assessed in 293T cells. To our surprise, LMP-1/ΔCTAR1,2 was deficient in IFNα suppression activity and was indistinguishable from the nonfunctional LMP-1/TMD5,6 negative control in these cells (Figure 2-13). The activity of either CTAR1 or CTAR2 was required for LMP-1 suppression of IFNα signaling in this background. In contrast, the CTAR mutants (LMP-1/ΔCTAR1 (LMP-1 mutant with point mutations that disrupts TRAF binding to CTAR1), LMP-1/CΔ55 (LMP-1 lacking the last 55 amino acids of the cytoplasmic C-terminus and lacks CTAR2), and LMP-1/ΔCTAR1,2 (a combination of both ΔCTAR1 and CΔ55 mutants) were indistinguishable from wild type LMP-1 in IFNα suppression in DG75 cells (Geiger & Martin, 2006). Thus, suppression of IFNα signaling by LMP-1 in 293T cells correlated with strong NF-κB activation.
Figure 2-12 Mutants lacking C-terminal activation regions (CTARs) of LMP-1 cannot activate NF-κB signaling.

A) 293T cells were transfected with LMP-1(25, 50, 100ng), LMP-1/TMD5,6(50ng), LMP-1/ΔCTAR1,2(25, 50, 100ng) or Empty Vector and NF-κB driven luciferase vectors with LacZ expression vectors for quantification of NF-κB activity. NF-κB activity was calculated as in Figure 2-9. Assays were done in duplicate. Schematics of each mutant are shown below the graph. B) Lysates from A were resuspended 1:10 with 4xSDS sample buffer and resolved by SDS-PAGE and Western blotted for LMP-1 expression. Numbers above blots correspond to samples on the graph in part B. Arrow to the right of the blot indicates migration of full length LMP-1.
Figure 2-13 LMP-1 mutants differentially activate ISRE signaling in 293T and DG75 cell lines.

A) 293T cells were transfected with LMP-1(50ng), LMP-1/ΔCTAR1,2(100ng), LMP-1/ΔCTAR1(50ng), LMP-1/ΔC55(50ng), LMP-1/TMD5,6(50ng), LMP-1 (50ng) with IKKBΔ34(250ng) and dN-NIK(250ng), or Empty Vector and ISRE driven luciferase vectors with LacZ expression vectors for quantification of ISRE activity. ISRE activity was calculated as in Figure 2-5. Assays were done in duplicate. B) DG75 cells were transfected with 1μg of LMP-1, LMP-1/ΔCTAR1,2, LMP-1/ΔC55, LMP-1/TMD1,6, or Empty Vector and ISRE driven luciferase vectors with LacZ expression vectors for quantification of ISRE activity. ISRE activity was calculated as in Figure 2-4. Assays were done in duplicate. Schematics of each mutant are shown below the graph.
Suppression of IFNα signaling by LMP-1 could result from NF-κB dependent and NF-κB independent processes. The NF-κB dependent component could be supplied by LMP-1 in 293 cells (293T cells have extremely low basal NF-κB activity) and by the high basal NF-κB activity in DG75 cells. Interestingly, co-transfection of NF-κB inhibitor vectors (inhibitor of the canonical pathway, IκBΔ34, and inhibitor of the non-canonical pathway, dnNIK) appeared to have no affects on IFNα suppression by LMP-1 (not shown). I was unable however to confirm expression of these inhibitors due to quality of the reagents. CTAR activity is required in 293T cells and not in DG75 cells. Numerous attempts were made to “resurrect” the failing DG75 cells to continue characterization of suppression of IFNα by LMP-1 (New cell lines were obtained from other labs and ATCC, alternate transfection methods, and new sources of IFNα were all tried).

**Suppression of IFNα signaling is separable from lipid raft association and TRAF binding by LMP-1.**

LMP-1 partitions with detergent resistant membrane fractions (lipid rafts) when lysed in a low percentage of TritonX-100 and floated through a sucrose step gradient (H Ardila-Osorio, et al., 1999; H Ardila-Osorio, et al., 2005; Coffin, et al., 2003; Higuchi, et al., 2001; J. Lee & Sugden, 2007; Yasui, et al., 2004). Association of LMP-1 with lipid rafts correlates with its ability to activate NF-κB. The LMP-1 TMD, in particular TM1, is essential for raft association (Coffin, et al., 2003; Higuchi, et al., 2001; A Kaykas, et al., 2001; J. Lee & Sugden, 2007; Yasui, et al., 2004). To ask whether suppression of IFNα signaling correlated with association of DRMs, the raft association of LMP-1/TMD1,6 was determined. Both LMP-1/TMD1,2 and LMP-1/TMD1,6 retain the ability to activate NF-κB and to suppress IFNα signaling. Our lab
has previously demonstrated that TMD1,2 fractionates to lipid rafts (or detergent resistant membranes(DRMs)) in transfected 293T cells. It was predicted that TM1 mutants retaining both the leucine heptad and FWLY motifs in the first TMD would migrate to lipid rafts similarly to LMP-1. DG75 cells were transfected with LMP-1/TMD1,6 or LMP-1 expression vectors and analyzed by sucrose step gradient. Gradient fractions were removed from the top of the gradient to the bottom and analyzed by Western for LMP-1 and Lyn kinase (a raft marker). Analyses of LMP-1/TMD1,6 in B cells shows that this mutant, unlike LMP-1/TMD1,2 does not fractionate to detergent resistant membranes like LMP-1(Figure 2-14). In fact, LMP-1/TMD1,6 behaves like LMP-1/TMD5,6 in this assay (Coffin, et al., 2003). These results demonstrate that association with lipid rafts by LMP-1 is separate from its suppression of IFNα signaling. The difference between LMP-1/TMD1,6 and LMP-1/TMD1,2 to migrate to lipid rafts is surprising and could indicate a role for TM2 in LMP-1 lipid raft association.

LMP-1 activates NF-κB signaling activity by binding to tumor necrosis factor receptor associated factors (TRAFs) to propagate downstream signaling (Bishop, et al., 2007; Brodeur, et al., 1997; O. Devergne, et al., 1996; Kulwichit, et al., 1998; Raab-Traub, Damania, & Pipas, 2009; M. Sandberg, et al., 1997; Uchida, et al., 1999; Xie, et al., 2008). Both LMP-1/TMD1,2 and LMP-1/TMD1,6 fail to co-immunoprecipitate with TRAFs (Figure 2-14B). LMP-1 co-immunoprecipitates with TRAF3 (M. Sandberg, et al., 1997). TRAF3 is undetectable in LMP-1/TMD1,6 and LMP-1/TMD1,2 immunoprecipitates. Interestingly, both LMP-1/TMD1,2 and LMP-1/TMD1,6 can activate NF-κB signaling despite the lack of the ability to bind TRAFs. These results bring new insights to understanding IFNα signaling as it relates to LMP-1s other signaling activities. They also raise more questions that need to be addressed about the
Figure 2-14  LMP-1 first TMD mutants cannot migrate to lipid rafts or bind TRAFs.
A) LMP-1/TMD1,6 does not migrate to lipid rafts like LMP-1. DG75 cells were transfected with 0.5 ug of LMP-1 or LMP-1/TMD1,6. Cells were harvested, lysed, and placed at the bottom of a sucrose step gradient and spun overnight at 100,000xG. 200 ul Fractions were collected from the top (Fraction 1) of the gradient toward the bottom of the tube (Fraction 12/Pellet). Fractions were resuspended in 400ul of 4xSDS sample buffer except the pellet, which was resuspended in 600ul of 4xSDS sample buffer. Samples were resolved by SDS-PAGE and western blotted with antibodies specific for the LMP-1 C-terminus. Blots were stripped and reprobed for Lyn kinase as a marker for detergent resistant membranes (DRMs). B) LMP-1/TMD1,6 and LMP-1/TMD1,2 cannot bind TRAFs. DG75 cells were transfected with LMP-1/TMD1,6, LMP-1/TMD1,2 or LMP-1. Cells were harvested 48 hours post transfection. Lysates were immunoprecipitated for the LMP-1 C-terminus and blotted for LMP-1 or TRAF3. Immunoprecipitates or whole cell lysates (WCL) were resuspended in 4xSDS sample buffer and resolved by SDS-PAGE and Western blotted for LMP-1 or TRAF3. (+) represents immunoprecipitated for LMP-1 with LMP-1 antibody. (−) represents mock immunoprecipitation control, same protocol. Arrows to the right of blots indicate migration of LMP-1, LMP-1 TMD mutants, or TRAF3.
mechanism by which the cytoplasmic C-terminus activates NF-κB and the mechanism by which LMP-1 partitions itself to these detergent resistant membranes.

**Discussion**

Work described in this chapter aimed at studying the mechanism of LMP-1 suppression of IFNα signaling. Cells expressing LMP-1 are resistant to activation of signaling in response to IFNα treatment and this suppression correlates with decreased tyrosine phosphorylation of the janus kinase Tyk2. Although LMP-1 has been shown to interact with Tyk2 by co-immunoprecipitation analysis, the interaction is difficult to detect and therefore I believe it is indirect. For this reason, mechanistic studies were first focused on finding cellular proteins that bridged the Tyk2/LMP-1 interaction. The most logical candidate was the IFNα receptor (IFNAR1) itself, for the following reasons. First, suppression of IFNα signaling by LMP-1 is specific, LMP-1 does not effect signaling in response to IFNγ, and IFNα and IFNγ bind to distinct cell surface receptors. Second, LMP-1 and the IFNAR1 are both localized in the membrane, so an interaction between the two is not unreasonable. An interaction between IFNAR1 and LMP-1 could not be demonstrated. Isolation of integral membrane proteins (like LMP-1 and IFNAR1) from the membrane is not trivial and it is difficult to demonstrate specific interaction given the hydrophobic nature both proteins. The IFNAR1 is present in most cells at very low levels (50-5000 receptors/cell (Ragimbeau, et al., 2003)), whereas the number of LMP-1 molecules/cell is relatively high (can be 100,000/cell in EBV immortalized cells). I also attempted to screen a panel of tyrosine phosphatases for interactions with LMP-1. It was reasoned that because the effect of LMP-1 on Tyk2 dephosphorylation, was likely indirect, perhaps LMP-1 was activating a Tyk2 phosphatase to exert its effect on signaling. Tyk2 is
dephosphorylated in B cells via the 4 phosphatases, CD45 (an integral membrane protein), SHP1, SHP1, and PTP1B. Co-immunoprecipitation analyses were inconclusive for IFNAR1. These results demonstrate the need for a cell-based assay to identify potential interactions between two membrane proteins. In this regard a modified yeast two hybrid assay designed to measure interactions between proteins (membrane proteins included) in yeast is being established. Such a system is perfectly suited for detecting potential interactors that mediate suppression of IFNα signaling by LMP-1. This system, in addition to its use in detecting interactions between LMP-1 and candidate membrane proteins such as CD45 and the IFNAR1, could be useful in screens for (as yet unknown) interactors in LMP-1 signaling.

One can imagine many mechanisms by which LMP-1 could inhibit IFNα signaling via interfering with the receptor itself. Another latent membrane protein of the Epstein-Barr virus, LMP2A interferes with IFN signaling by promoting the degradation of the IFNα and IFNγ receptors (Shah, et al., 2009). LMP-1 could promote a similar activity but has not yet been tested. LMP-1, via directly binding Tyk2, could act as a Tyk2 “sink”. IFNAR requires Tyk2 for maturation and surface expression on the plasma membrane (Ragimbeau, et al., 2003). Critical for testing these hypotheses is a decent IFNAR1 antibody. This is reagent is difficult to procure as most antibodies only readily recognize the immature unglycosylated IFNAR1. LMP-1 could conceivably bind to Tyk2 and indirectly prevent IFNAR1 surface expression. In addition to its anti-viral effects, IFNα exerts anti-proliferative effects on cells (Platanias, 2005). Previously, our lab demonstrated that LMP-1 levels increased over time with prolonged exposure to IFNα suggesting that LMP-1 protects cells against the anti-proliferative effects of IFNα. These “high expressing” LMP-1 cells could be used to screen for candidate genes upregulated by LMP-1 that result in suppression of IFNα signaling, and for characterizing downstream signaling
consequences of IFNα selection (i.e. IFNα signaling intermediates that allow survival of cells with higher levels of LMP-1).

**Sequence requirements for suppression of IFNα signaling**

Structure function analysis of LMP-1 in B cells demonstrated that the cytoplasmic N-terminus of LMP-1 is not required to suppress IFNα signaling (Figure 2-3). Deletion analysis of the LMP-1 TMD demonstrated that LMP-1/TMD1,2 and LMP-1/TMD1,6 could suppress IFNα signaling but LMP-1/TMD5,6 could not, identifying a role for the first transmembrane domain of LMP-1 in suppression IFNα signaling (Figure 2-3, 2-6). The TM1 mutants (LMP-1/TMD1,2 and LMP-1/TMD1,6) both weakly activate NF-κB signaling through their cytoplasmic C-terminal tails and have intermediate IFNα suppression. Characterization of LMP-1/TMD1,6 demonstrated that LMP-1/TMD1,6 can activate NF-κB despite its inability to fractionate to DRMs and bind TRAFs. NF-κB activation by LMP-1/TMD1,6 is TRAF-independent and occurs by a different mechanism than LMP-1.

I showed suppression of IFNα signaling was dependent on the first transmembrane domain of LMP-1 in B cells (Figure 2-6), whereas LMP-1 CTARs were dispensable in suppression of IFNα signaling in DG75 cells (Geiger & Martin, 2006). These results suggested strongly that suppression of IFNα signaling (C-terminal independent) was separable from activation of NF-κB (C-terminal dependent). However, structure function requirements for IFNα suppression in 293T cells were dramatically different. CTAR mutants lacking both the first and second CTARs were defective in suppression of IFNα signaling in 293T cells (Figure 2-13). One major difference between 293T cells and DG75 cells is their “basal” levels of active NF-κB. DG75 cells (B cells) have high basal levels of active NF-κB, whereas NF-κB activity is
undetectable in 293T cells. These results argue that NF-κB contributes to LMP-1 suppression of IFNα signaling and that CTAR-dependent NF-κB activation is not required for IFNα suppression in DG75 cells because these cells have high basal NF-κB activity. In contrast, CTAR-dependent NF-κB activation is required for IFNα suppression because these cells have little or no active NF-κB. Thus, the mechanism by which LMP-1 suppression of IFNα signaling is likely to have an NF-κB component. The behavior of TM1 substitution mutants (correlation between NF-κB activation and IFNα suppression) supports this model.

I also characterized the leucine heptad mutants in the context of suppression of IFNα signaling. Leucine heptad mutants of LMP-1 have alanines substituted in the leucine heptad present in the 1st or 6th transmembrane domain. These mutants showed decreased IFNα signaling suppression. Previously characterization of the leucine heptad in TM1 demonstrated that this mutant is unable to traffic to lipid rafts like wild type, does not activate NF-κB like wild-type, is not distributed in the cell similar to wild-type LMP-1 by immunofluorescence, and does not transform B cells like wild type LMP-1 (J. Lee & Sugden, 2007). Partial suppression of IFNα signaling was observed, much like the partial activation NF-κB activity that was observed. This is to be expected after the following results demonstrating that NF-κB activation is required for suppression of IFNα signaling in 293T cells. Contribution of the leucine heptad motif in this context is difficult because of observed correlation to NF-κB signaling. It does however suggest that there was partial suppression upon stimulation of some activation of NF-κB these mutants retain signaling necessary for suppression of IFNα signaling.

Structure function analysis of LMP-1 reported in this chapter demonstrates the importance of TM1 of LMP-1 in LMP-1 function. The TM1 is important in NF-κB activity,
IFNα suppression, and lipid raft association. These observations, together with the requirement of the C-terminal for IFNα suppression in 293T cells, support a model in which LMP-1 suppression of IFNα signaling is dependent on NF-κB signaling. NF-κB signaling can be induced by LMP-1 or can pre-exist in cells (i.e. cells with high basal NF-κB). Studies aimed at unraveling the mechanism of IFNα suppression by LMP-1 must, therefore, include an analysis of the role of NF-κB activation in regulation of IFN-stimulated gene expression.

**Material and Methods**

**Cells**

DG75 cells are an EBV-negative B lymphoma cell line. 721 cells are an *in vitro* transformed EBV-positive lymphoblastoid cell line. All B cell lines were grown in RPMI 1640 medium supplemented with 10% bovine calf serum (R10C). 293T cells are an embryonic kidney carcinoma cell line positive for large T antigen grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% bovine calf serum (D10C). All cells were grown at 37°C in high humidity and 5% CO2.

**Plasmids**

pRSV-LMP-1 (LMP-1), pRSV-LMP-1/TMD5,6 (LMP-1 deletion lacking TMDs 1-4), pRSV-LMP-1/TMD1,2 (LMP-1 deletion lacking TMDs 3-6), LMP-1/ΔCTAR1 (a mutation altering the P204XQXT208 motif in the CTAR1 binding domain of LMP-1 to A204QAAT208), LMP-1/ΔC55 (LMP-1 mutant lacking the last 55 amino acids of the LMP-1 C-terminus), LMP-1/ΔCTAR1,2
and pRSV-LacZ are pRC-RSV (Empty Vector) based expression vectors (Invitrogen) and have been described previously (Coffin, et al., 2003; Geiger & Martin, 2006). pCMV-LMP-1/(A_{38}A_{41}) (LMP-1 with the F_{38}W_{41} motif in TM1 changed to A_{38}A_{41}). pRSV-LMP-1/TMD1,6 (LMP-1 deletion lacking TMDs 2-5, with LMP-1 residue 51 (alanine) fused in frame to residue 166 (threonine); pRSV-LMP-1/TMD1,6(L_{38}L_{41}) (pRSV-LMP/TMD1,6 with the F_{38}W_{41} motif in the first TMD of LMP-1 to L_{38}L_{41}). pSG5-LMP-1(TMD1L-A); SubLZILMP-1, pSG5-(LMP-1/TMD6L-A); SubLZ6LMP-1; LMP-1(Δ25-180)/(LMP2A(126-280); NLLMP-1 were gifts from Bill Sugden (University of Wisconsin-Madison). pCMV-IKKβA34-FLAG (kinase dead) and pCMV-dn-NIK-FLAG were gifts from Ellen Cahir-McFarland.

Cytokines/Antibodies

IFNα/D (Universal type 1 IFN) was from either R&D systems or Sigma based upon availability, both cytokines stimulated cells similarly. Anti-LMP-1 antiserum is an affinity-purified rabbit polyclonal serum raised against the LMP-1 C-terminus (residues 188-352) fused to glutathione-S-transferase. Lyn kinase (sc-7274) and TRAF3 (sc-948G) antibodies are from Santa Cruz Biotechnology. HRP-conjugated, goat anti-rabbit (W401B), goat anti- mouse (W402B) and donkey anti-goat (V805A) antibodies are from Promega. Anti-LMP-1 antibodies were used at a dilution of 1:2500 Anti-Lyn at 1:1000, Anti-TRAF3 at 1:200, and HRP labeled secondary antibodies at a dilution of 1:2500. IFNAR antibodies were used at a dilution of 1:500 and were a gift from Pierre Eid (Laboratoire d'oncologie virale). SHP-1[Y476] antibody was a Rabbit monoclonal and PTP1B [AE4-2J] was a mouse monoclonal from Abcam.
Transfections

10^7 cells/cuvette were transfected by electroporation in 0.4 cm gapped cuvettes using a Bio-Rad gene pulsar. DG75 cells were electroporated in 0.35 ml R10C at 0.250 kV and 960 µF. 293T cells were transfected using Mirus TransIT-293 transfection according to manufacturer’s instructions. Transfected cells were assayed two days post-transfection.

Immunoprecipitation

721 cells were lysed in lysis buffer (50mM TrisHCL pH7.5, 150mM Sodium chloride, 2mM EDTA, 1%NP-40) used as starting material for immunoprecipitation analysis. Antibody was added to the precleared starting material, incubated for 30 minutes on ice, and immune complexes were recovered with Protein-G agarose (Roche Diagnostics). Beads were washed according to manufacturers directions and heated to 85°C for 15 minutes in 4X nonreducing SDS-sample buffer.

SDS-PAGE and Western Analysis:

Cells were harvested and lysed in 4xSDS-sample buffer, boiled, and resolved on 10% acrylamide gels. Proteins were transferred to Immobilon (Millipore), and stained with the appropriate antisera as follows: Blots were blocked in PBS/1% milk/0.05% Tween 20, and incubated with primary antibody and secondary antibody (1:2500 HRP-conjugated anti-rabbit antibody, 1:2500 anti-mouse HRP-conjugated antibody or 1:2500 anti-rabbit HRP-conjugated antibody (Promega)). Blots were visualized by ECL according to manufacturers instructions (GE Healthcare).
**Lipid Raft Flotation assays:**

DG75 cells were transfected two days prior to harvest. 2x10^7 cells were homogenized on ice with a Dounce homogenizer in 1.0 ml of MNE buffer (25mM MES, 150mM NaCl, 5mM EDTA) with 0.2% Triton X-100. Extracts were diluted 1:1 in 80% sucrose MNE, overlaid with 2.0 ml of 30% sucrose MNE and 1 ml of 5% sucrose MNE, and centrifuged at 4°C for 18 hours in a SW50.1 rotor at 200,000xG. Gradients were harvested from the top of the gradient in 400ml fractions and mixed with 200ml of 4x Sample Buffer (100mM TrisHCl pH6.8, 5%SDS, 100mM DTT, 10% Glycerol, 0.02g Bromophenol Blue) for SDS-PAGE analysis. The pellet was suspended in 600ml 4x Sample Buffer and sonicated. 8ml of each fraction were analyzed by SDS-PAGE and Western analysis.

**ISRE and NFkB reporter assays:**

DG75 and 293T cells were transfected as described above in duplicate with LMP-1 mutants, pRSV-LacZ, and either pISRE-Luc or pkB-Luc. All transfections were performed with equivalent mg of DNA by addition of an expression vector control plasmid (pRC-RSV). 1.5 days post transfection cells were treated with or without IFNα (for ISRE activation) or untreated (for NF-κB activation) for 6-7 hours and extracts were assayed for luciferase and β-galactosidase accumulation using the Tropix Dual Light assay kit from Applied Biosystems. Luciferase activity was normalized within each transfection to β-galactosidase activity. Transfections were performed in duplicate and assayed for luciferase and β-galactosidase activity in triplicate.
CHAPTER 3: ANALYSIS OF LMP-1 DIMER FORMATION

Introduction

LMP-1 functions in part as a constitutively active CD40 mimic (Gires, et al., 1997; Kilger, et al., 1998; Mosialos, et al., 1995). CD40 is a B cell surface receptor of the Tumor Necrosis Factor Receptor (TNFR) superfamily that plays an essential role in B cell proliferation, survival, and differentiation to memory (Xie, et al., 2008).

Activated TNFRs, such as CD40, signal as TRAF-binding trimers from lipid raft microdomains (A.-O. Hueber, Bernard, Herincs, Couzinnet, & He, 2002; A. O. Hueber, 2003; Muppidi, Tschopp, & Siegel, 2004; Vidalain, et al., 2000). TNFR activation is the result of a combination of factors including ligand-induced trimerization, ligand-induced conformational changes, and formation of receptor aggregates (Siegel, Chan, Chun, & Lenardo, 2000). Receptor multimerization has been shown to occur in response to binding of trimerized ligand and prior to ligand binding via a pre-ligand-binding domain (PLAD) (Chan, et al., 2000; Clancy, et al., 2005; Papoff, et al., 1999; Siegel, Frederiksen, et al., 2000). Ligand-induced CD40 homo-dimer formation has been reported in a variety of EBV-positive and negative B cells and correlates with activation of phosphatidylinositol-3-kinase (Reyes-Moreno, Girouard, Lapointe, Darveau, & Mourad, 2004). CD40 signals more robustly as a trimer, although both dimeric and trimeric CD40 can trigger NF-κB signaling in response to activation by ligand (Werneburg, J., Dang, Kehry, & Crute, 2001). The extent of CD40 multimerization can determine the nature of the biological response (Baccam & Bishop, 1999; Fanslow, et al., 1994; Pound, et al., 1999).

Studies of LMP-1 activation and signaling have proven difficult because of the constitutively nature of LMP-1 signaling. A number of studies show a correlation between LMP-1 oligomerization and/or raft association and activation of NF-κB signaling (H Ardila-
Osorio, et al., 1999; Hector Ardila-Osorio, et al., 2005; Coffin, et al., 2003; Floettmann, Eliopoulos, Jones, Young, & Rowe, 1998; Floettmann & Rowe, 1997; Hatzivassiliou, Miller, Raab-Traub, Kieff, & Mosialos, 1998; Higuchi, et al., 2001; J. Lee & Sugden, 2007; Yasui, et al., 2004). Evidence for LMP-1 homo-oligomerization largely comes from co-immunoprecipitation analyses of LMP-1 in solubilized cells extracts and from measuring signaling output following forced aggregation of LMP-1 chimeras (Floettmann, et al., 1998; Floettmann & Rowe, 1997; Gires, et al., 1997; Hatzivassiliou, et al., 1998). Evidence for lipid raft association is based primarily on results from two kinds of assays: co-fractionation of LMP-1 with detergent-resistant membranes isolated from lysed cells (Clausse, et al., 1997; Higuchi, et al., 2001) and from co-localization of LMP-1 with raft markers (i.e. GM1) visualized by confocal microscopy (A Kaykas, et al., 2001). Little is known about the stoichiometry of LMP-1 homo-oligomers and the mechanism of their formation. Furthermore, the existence of LMP-1 homo-oligomers in the cell prior to cell lysis, has yet to be demonstrated.

LMP-1 is constitutively active unlike the protein it mimics, CD40. Insight into the mechanisms of LMP-1 constitutive activation is key for designing strategies to interfere specifically with LMP-1 signaling. Understanding LMP-1 activation mechanism will require in depth analyses of the mechanism of LMP-1 oligomerization and raft association. Work in this chapter describes the identification and characterization of a cysteine-disulfide linked dimeric form of LMP-1 in transfected B cells and EBV-immortalized lymphoblastoid cells. The nature of this dimeric form of LMP-1 and its relation to LMP-1 signaling are explored, as are the implications for the stoichiometry of the LMP-1 homo-oligomer in LMP-1 signaling complexes.
**Results**

A SDS-resistant, higher molecular weight form of LMP-1 is found in B cell extracts. LMP-1 activation of downstream signaling pathways is proposed to require homo-oligomerization. Evidence from co-immunoprecipitation analyses supports this model (Floettmann, et al., 1998; Gires, et al., 1997), but the nature of the LMP-1 oligomer is unknown.

To ask whether multimeric LMP-1 could be detected in cell extracts from B cells expressing LMP-1, the migration of LMP-1 in nonreducing SDS-polyacrylamide gels was analyzed. 721 cells, an EBV-positive lymphoblastoid cell line were grown and harvested for SDS-PAGE gel analysis. The LMP-1 apparent molecular weight on reducing SDS-polyacrylamide gels is ~60 kDa. Both the ~60 kDa species and an additional higher molecular weight LMP-1 species migrated between the 85 kDa and 190 kDa markers were observed (Figure 3-1A). The observed SDS-resistance and sensitivity to reduction is consistent with a cysteine-disulfide linkage between two LMP-1 monomers or between LMP-1 and an interacting protein.

**The higher molecular weight LMP-1 species is a dimer.**

Current models of LMP-1 signaling predict that LMP-1 signals as a constitutive trimer, akin to activated CD40. The higher molecular weight LMP-1 species described above could be a homo-dimer, a homo-trimer, or LMP-1 crosslinked to a cellular interactor of similar size, such as TRAF3. To distinguish these possibilities, the migration of LMP-1 and an LMP-1 deletion
Figure 3-1 B cells expressing LMP-1 contain an SDS-resistant, homo-dimeric form of LMP-1.

A) Membranes isolated from EBV-immortalized 721 cells were solubilized in 4X nonreducing SDS-sample buffer and heated to 85°C for 15 minutes in the absence or presence of 50 mM DTT. Samples were resolved on 10% or 7.5% SDS-polyacrylamide gels and analyzed by Western blot for LMP-1 using anti-LMP-1 antibodies.

B) DG75 cells were transfected with LMP-1 or LMP-1/CΔ55 and harvested 48 hours later. Membranes were solubilized in 4X nonreducing SDS-sample buffer and heated to 85°C, in the presence or absence of 50 mM DTT, for 15 minutes. Samples were resolved on 7.5% SDS-polyacrylamide gels and analyzed by Western blot for LMP-1. Treatment with DTT is noted above each blot, and the expressed protein is noted below the blots (B). The migration of protein standards (in kDa) are indicated to the left of each blot, arrows mark the migration of LMP-1 and LMP-1/CΔ55, and arrowheads mark the migration of the higher molecular weight LMP-1 and LMP-1/CΔ55 species.
mutant lacking the last 55 amino acids from the carboxy-terminus were compared. LMP-1/CΔ55 was chosen because it encodes the entire hydrophobic transmembrane domain of LMP-1 and therefore retains the ability to homo-oligomerize (Coffin, et al., 2001; Coffin, et al., 2003). The higher molecular weight LMP-1 species in extracts from cells expressing LMP-1 migrated at a molecular weight of ~125 kDa on 10% nonreducing SDS-polyacrylamide gels (Figure 3-1B), approximately twice that of the ~60 kDa LMP-1 monomer. Importantly, a higher molecular weight form of the truncated LMP-1 mutant (LMP-1/CΔ55) was also observed, and migrated with a molecular weight of ~85 kDa, approximately twice that of the LMP-1/CΔ55 monomer (~45 kDa). These results suggest that the slower migrating form of LMP-1 represents an LMP-1 homo-dimer.

**Cysteine 238 is required for formation of the LMP-1 homo-dimer.**

LMP-1 possesses four cysteine residues: residues 78, 84, 116, and 238; referred to here as cysteines 1, 2, 3, and 4 respectively. Cysteine’s 1-3 are located in the hydrophobic transmembrane domain while cysteine 4 is located in the cytoplasmic carboxy-terminus (Figure 3-2A). Cysteine to alanine substitutions were introduced into the LMP-1 coding sequence with the goal of identifying which of the cysteine residue(s) were required for the disulfide linkage. Substitution of cysteines 1-3, either individually or in combinations (LMP-1/CsubA1, LMP-1/CsubA1,2, LMP-1/CsubA3 and LMP-1/CsubA1-3) had no effect on the formation of the dimeric form of LMP-1 (Figure 3-2B). In contrast, LMP-1 mutants with cysteine 4 replaced with alanine (LMP-1/CsubA4), or substitutions of all four cysteines with alanines (LMP-1/CsubA1-4), were unable to form the higher molecular weight species. These results suggest
Figure 3-2  Cysteine 238 is required for formation of LMP-1 homo-dimers.  
A) Schematic of LMP-1 showing the relative position of its 4 cysteines (LMP-1 residues 78 (1), 84 (2), 116 (3), and 238 (4)).  The relative positions of LMP-1 CTAR1 and CTAR2 motifs are also noted.  B) DG75 cells were transfected with pCMV-LMP-1 or pCMV-LMP-1 substitution mutants harboring cysteine to alanine substitutions.  Cysteine 78 is substituted with alanine in CsubA1; cysteines 78 and 84 are substituted with alanines in CsubA1,2; cysteine 116 is substituted with alanine in CsubA3; cysteines 78, 84, and 116 are substituted with alanines in CsubA1-3; cysteine 238 is substituted with alanine in CsubA4; cysteines 78, 84, 116, and 238 are substituted with alanines in CsubA1-4.  Membranes were solubilized in nonreducing 4X SDS-sample buffer and heated to 85°C, with and without 50 mM DTT, for 15 minutes.  Samples were resolved on 10% SDS- polyacrylamide gels and analyzed by Western blot for LMP-1.  Data for LMP-1, CsubA4 and Csub1-4 are from the same blot; data for CsubA1, CsubA1,2, CsubA3 and CsubA1-3 are from different blots.  Treatment with DTT is noted above the blots, and the expressed LMP-1 protein is noted beneath each blot.  The migration of protein standards (in kDa) are shown on the left of the blots, and the arrows on the right marks the migration of monomeric LMP-1 and the arrowhead marks the migration of dimeric LMP-1.
strongly that the cytoplasmic cysteine 238 is required for the formation of the dimeric form of LMP-1.

**Cysteine 238 is not required for activation of NF-κB by LMP-1.**

To test the requirement of cysteine 238 in LMP-1 signaling, NF-κB activation by LMP-1/C_{sub}A4 was compared to NF-κB activation by LMP-1 using an NF-κB reporter assay. HEK 293T cells co-transfected with LMP-1 expression vectors and an NF-κB luciferase reporter were assayed for luciferase accumulation (Figure 3-3). LMP-1 stimulated NF-κB activity 13.8 fold relative to empty vector. LMP-1/C_{sub}A4 activated NF-κB to wildtype LMP-1 levels (12.99 fold) whereas the negative control, LMP-1/TMD5,6, was inactive. LMP-1/TMD5,6 (lyLMP-1) is a nonfunctional N-terminally truncated form of LMP-1 lacking the cytoplasmic N-terminus and first 4 transmembrane domains of LMP-1 (Baichwal & Sugden, 1989; Hudson, et al., 1985; D. Wang, et al., 1988). These results clearly demonstrate that cysteine 238 is not essential for LMP-1 CTAR dependent signaling to NF-κB.

**Disulfide crosslinking of cysteine 238 is blocked by N-ethylmaleimide.** The lack of requirement of cysteine 238 in activation of NF-κB by LMP-1 suggested that formation of active LMP-1 signaling complexes does not depend on the formation of a disulfide crosslink at cysteine 238. The observed disulfide crosslinking could occur fortuitously if the β carbons of two cysteine 238 residues in adjacent LMP-1 monomers are in close enough proximity (within 3.4 to 4.6 angstroms) to allow disulfide bond formation (Balaji, Mobasser, & Rao, 1989; Careaga & Falke, 1992). We asked whether the observed disulfide-linked LMP-1 dimers were formed before or after cell lysis. To address this question, 721 cells were treated with the membrane permeable alkylating agent N-ethylmaleimide (NEM) prior to harvest. NEM irreversibly and
Figure 3-3  Cysteine 238 is not essential for activation of NF-κB.
HEK 293T cells were cotransfected with the indicated LMP-1 expression vectors, an NF-κB luciferase reporter (p1242) and a b-galactosidase reporter (pRSV-lacZ). Cells were harvested 48 hours later and assayed for luciferase and b-galactosidase accumulation and LMP-1 expression. A) Data are shown as fold NF-κB activation relative to pRC-RSV (empty vector). B) Extracts from "A" were resolved on 10% SDS-polyacrylamide gels and analyzed by Western blot for LMP-1; numbers below each lane correspond to the numbers below the introduced expression vector in "A". Lane 1, pRC-RSV; lane 2, pRSV-LMP-1; lane 3, pRSV-LMP-1/TMD5,6; lane 4, pRSV-LMP-1/CsubA4. Data are representative of three experiments.
specifically alkylates free sulfhydryl groups at neutral pH and does not disrupt pre-existing disulfide bonds (Gorin, Martic, & Doughty, 1966). Live cells were treated with 20 mM NEM for 60 minutes prior to harvest and analyzed by nonreducing SDS-PAGE and Western analysis. NEM pretreatment blocked the formation of the LMP-1 dimer, indicating that disulfide bond formation occurred post cell lysis (Figure 3-4A). To determine the time course of disulfide bond formation after cell lysis, 721 cells were treated with and without 20 mM NEM for 60 minutes and lysates were incubated at 4°C for the indicated times (Figure 3-4B). Dimeric LMP-1 is detectable 40 minutes post lysis and peak levels are detected by 24 hours. As in Figure 3-4A, formation of the dimeric form of LMP-1 is completely blocked by pretreatment with NEM. These results demonstrate that LMP-1 monomers are not covalently linked via disulfide bonds within the cell prior to lysis. Rather, disulfide crosslinking occurs following cell lysis and the extent of crosslinking increases with time following lysis (Figure 3-4B). These results are consistent with the relative red/ox state of the cytoplasm versus the cell lysate. Prior to cell lysis, the LMP-1 C-terminus is localized within the reducing environment of the cytosol. The oxidizing environment encountered post lysis, during experimental manipulation of the cell lysate, promotes covalent linkage between cysteine residues in close enough proximity to allow disulfide bond formation (i.e. between interacting LMP-1 monomers within an LMP-1 oligomer). These results are consistent with the relative red/ox state of the cytoplasm versus the cell lysate and consistent with the formation of LMP-1 oligomers within the cell.

To test the hypothesis that LMP-1 dimers pre-exist in the cell prior to lysis, 721 cells were treated with the cell permeable sulfhydryl-reactive bifunctional crosslinker bismaleimidohexane (BMH). BMH is a bifunctional sulfhydryl-reactive crosslinker with a 13.0-angstrom spacer arm. 721 cells pre-treated with BMH before lysis and analyzed by SDS-PAGE
Figure 3-4  Cysteine-disulfide crosslinking occurs post cell lysis.
A) 721 cells were treated with or without 20 mM NEM for 60 minutes, solubilized in nonreducing SDS-sample buffer, heated to 85°C in the presence or absence of 50 mM DTT for 15 minutes and analyzed by 10% SDS-PAGE and Western blot for LMP-1. Treatment with NEM and DTT is noted above the blot. B) 721 cells pretreated with or without 20 mM NEM for 60 minutes were lysed in phosphate buffer containing 1% Triton X-100. Lysates were incubated for up to 40 hours at 4°C, diluted 1:1 with 4X nonreducing sample buffer containing NEM and analyzed by SDS-PAGE and Western blot for LMP-1. Treatment with or without NEM is noted at the top of each gel. Length of incubation is noted below the blot. Migration of protein standards (in kDa), and of monomeric (arrow) and dimeric (arrowhead) LMP-1, are shown on the left and right of each blot, respectively.
gel analysis showed the LMP-1 dimer, whereas samples treated with DMSO alone didn’t show a high molecular weight species (Figure 3-5A). This result demonstrates that LMP-1 is within close enough proximity to dimerize and form oligomers intracellularly, and that appearance of the dimer post lysis is not from nonspecific aggregation or excessive oxidation of cysteines post lysis. Dimeric LMP-1 was detected in extracts from BMH-treated cells and not in untreated extracts (Figure 3-5A). These results demonstrate that the interaction between LMP-1 monomers detected post lysis by crosslinking exist within the cell prior to lysis and can be stabilized by the addition of a membrane permeable sulfhydryl specific crosslinker.

Formation of higher molecular weight LMP-1 species upon crosslinking sulfhydryl groups in LMP-1.

The extent of observed cysteine 238 disulfide crosslinking depends on oxidation conditions (Figure 3-4) and therefore crosslinking may be incomplete. To test this, treatment under stronger oxidizing conditions that favor disulfide bond formation that would enhance the formation of the higher molecular weight LMP-1 species or result in additional high molecular weight LMP-1 species was carried out. Membranes from DG75 cells expressing LMP-1 or LMP-1/CΔ55 were treated with the redox catalyst Cu(II)(1,10-phenanthroline)₃ (CuP) in the presence of ambient oxygen as oxidization agent (Careaga & Falke, 1992). Nonreducing SDS-PAGE analysis revealed no additional high molecular weight forms of LMP-1 upon treatment with CuP and oxygen, although there did appear to be an increase in crosslinking efficiency (Figure 3-5B). Occasionally a very high molecular weight LMP-1 species that did not enter the gel was observed in both CuP treated and untreated membranes. This species comprised a very small percentage of the total LMP-1 signal (not shown). The predominant high molecular weight LMP-1/CΔ55 species in CuP-treated membranes did not differ in molecular weight from
Figure 3-5 Generation of high molecular weight forms of LMP-1 following crosslinking.
A) 721 cells were treated not with 1mM BMH at 37°C for 1 hour. The reaction was quenched in 180mM DTT. Cells were resuspended in 4xSample buffer with 100 mM DTT and resolved on 10% SDS-polyacrylamide gel and analyzed by Western Blot for LMP-1. B) DG75 cells were transfected with pCMV-LMP-1 or pCMV-LMP-1/CΔ55. 48 hours later, membranes were treated with or without 0.2 mM CuP and ambient O2 followed by solubilization in 4X nonreducing SDS-sample buffer. Samples were heated to 85°C, with and without 50 mM DTT, for 15 minutes and then resolved on 10% SDS-polyacrylamide gels and analyzed by Western blot for LMP-1. DTT and CuP treatment is noted above the blot, and the expressed LMP-1 protein is noted below. C) 721 cells were lysed in Syn7 buffer and soluble material was incubated with increasing concentrations of EGS or TMEA as described in Materials and Methods. Following crosslinking, extracts were diluted in 4X SDS-sample buffer containing 50 mM DTT, heated to 85°C, resolved on 10% SDS-polyacrylamide gels and analyzed by Western blot for LMP-1 using anti-LMP-1 monoclonal antibodies (CS1-4). Lanes 1 and 5, no crosslinker (DMSO control); Lanes 2-4, crosslinked with 0.5 mM, 1.0 mM, and 1.5 mM EGS, respectively; lanes 6-8, crosslinked with 125 µM, 250 µM, and 375 µM TMEA, respectively. Migration of protein standards (in kDa), and of monomeric (arrow), dimeric and higher molecular weight LMP-1 species (arrowheads) are shown to the left and right of the blots, respectively.
A. 

BMH: - +

kDa

148 98 64 50

LMP-1

B. 

CuP: + - +

DTT: - + - +

kDa

150 100 75 50

LMP-1 LMP-1/CΔ55

C. 

+ EGS + TMEA

kDa

250 148 98 64 50

LMP-1

*B is work of Rebecca Nix; C is the work of Alfredo Cervantes
that in untreated membranes. These results suggest that the cysteine residues linking LMP-1 monomers are already involved in a disulfide linkage and that there are no additional cysteine residues in LMP-1 or LMP-1 associated proteins that are in close enough proximity to be efficiently crosslinked upon oxidation.

Disulfide bonding between adjacent cysteine 238 residues depends upon their close proximity to one another (within 3-5 angstroms). LMP-1 molecules within oligomeric complexes are likely to make intermolecular contacts mediated by non-covalent interactions that occur over greater distances. Treatment of lytes cells with both bifunctional and trifunctional crosslinkers was done to see if additional higher molecular weight LMP-1 species could be obtained. The amine-specific bifunctional crosslinker EGS, which has a spacer arm of 16.1 angstroms, and the sulfhydryl-specific crosslinker TMEA, which has a spacer arm of 10.3 angstroms were used. TMEA was chosen because it is trifunctional and thus has the potential to crosslink three adjacent sulfhydryls (i.e. three cysteine 238 residues in a potential trimer).

Solubilized extracts of 721 cells were incubated with EGS or TMEA and analyzed by SDS-PAGE and Western blotting (Figure 3-5C). Crosslinking with the sulfhydryl-specific crosslinker TMEA was efficient in that the majority of LMP-1 was shifted to a higher molecular weight species. The predominant species detected following TMEA treatment was dimeric LMP-1. No evidence of a trimeric form of LMP-1 was detected, although there appeared to be additional, lower abundance, higher molecular weight species than observed with CuP crosslinking. The predominant species observed in samples crosslinked with the amine-specific crosslinker EGS was of very high molecular weight (greater than 250 kDa and beyond the resolution of the gel). Unlike TMEA treatment, very little of the dimeric LMP-1 species was observed in EGS crosslinked samples. These results demonstrate that LMP-1 forms oligomeric complexes that
can be captured in SDS-soluble dimeric units upon crosslinking of sulfhydryls and in a high molecular weight multimeric form(s) by crosslinking of amine groups.

**TRAF3 selectively co-purifies with dimeric LMP-1.**

A large body of evidence suggests TRAF-mediated LMP-1 signaling requires constitutive homo-oligomerization of LMP-1 (Floettmann, et al., 1998; Floettmann & Rowe, 1997; Gires, et al., 1997; Hatzivassiliou, et al., 1998). Detection of disulfide-linked LMP-1 dimers provides strong evidence that individual LMP-1 molecules in the membrane are in close enough proximity to form crosslinks between adjacent cysteine 238 residues. Disulfide-linked LMP-1 dimers may represent two LMP-1 molecules in an individual TRAF-binding LMP-1 oligomer. Alternatively, the crosslinked dimer may reflect the interaction between two adjacent TRAF-binding LMP-1 trimers in a higher order oligomer. This hypothesis was tested by asking whether TRAF3 selectively associates with disulfide-linked dimeric LMP-1 in detergent extracts of EBV-positive 721 cells. TRAF3 was immunoprecipitated from 721 cell lysates and the immunoprecipitates analyzed by nonreducing SDS-PAGE and Western blot for LMP-1 (Figure 3-6). The monomeric and dimeric forms of LMP-1 were present in the starting material in a 1:1 ratio. TRAF3 immunoprecipitates were dramatically enriched for dimeric LMP-1, while LMP-1 was not detected in the negative HA immunoprecipitation control. Dimeric LMP-1 in the starting material and in the TRAF3 immunoprecipitate was reduced to the monomeric form upon treatment with DTT. The presence of TRAF3 in the immunoprecipitate was confirmed by Western blot (not shown). These results suggest that the dimeric form of LMP-1 is representative of the TRAF-binding oligomeric form of LMP-1 in EBV-immortalized B cells.
Figure 3-6  Dimeric LMP-1 selectively co-purifies with TRAF3.
Triton-solubilized 721 cell extracts were immunoprecipitated with TRAF3 (TRAF3 IP) or HA (HA IP) antibodies. Triton-solubilized starting material (SM) and immunoprecipitates were diluted in 4X nonreducing SDS-sample buffer and heated to 85°C in the presence or absence of 50 mM DTT, resolved on 10% SDS-polyacrylamide gels and analyzed by Western blot for LMP-1. The HA antibody was used as an irrelevant antibody control for the TRAF3 immunoprecipitation. Treatment with DTT is noted above the blot. Migration of protein standards (in kDa), and of monomeric (arrow), dimeric and higher molecular weight LMP-1 species (arrowheads) are shown to the right and left of the blots, respectively. The asterisks (*) mark the migration of the precipitating antibody.
**Homo-oligomerization correlates with disulfide-crosslinking.**

If formation of the dimeric form of LMP-1 is a reflection of LMP-1 oligomeric status in the membrane, then dimeric forms of non-oligomerizing LMP-1 mutants of LMP-1 should be undetectable via nonreducing SDS-PAGE. This prediction was tested by comparing homo-oligomerization and disulfide-crosslinking properties of LMP-1 and the nonfunctional LMP-1 mutant LMP-1/TMD5,6 (lyLMP-1) (See Chapter 2, Figure 2-8). LMP-1/TMD5,6 cannot oligomerize with itself (submitted) or with LMP-1 (Yasui, et al., 2004). DG75 cells were co-transfected with expression vectors encoding LMP-1 and LMP-1\textit{myc} or LMP-1/TMD5,6 and LMP-1/TMD5,6\textit{myc}. LMP-1\textit{myc} and LMP-1/TMD5,6\textit{myc} were immunoprecipitated from solubilized membranes and analyzed by SDS-PAGE and Western blot for LMP-1 and LMP-1/TMD5,6. As expected, LMP-1 is detected in the myc immunoprecipitate from LMP-1 and LMP-1\textit{myc} co-expressing cells and LMP-1/TMD5,6 is not found in the myc immunoprecipitate from LMP-1/TMD5,6 and LMP-1/TMD5,6\textit{myc} co-expressing cells (Figure 3-7A). Importantly, although dimeric LMP-1 is detected in extracts from LMP-1 expressing cells, dimeric LMP-1/TMD5,6 was not present in extracts from LMP-1/TMD5,6 expressing cells(Figure 3-7B). These results and the finding that LMP-1/ LMP-1/\textDelta 55 (oligomerizing deletion mutant) can be resolved in a disulfide-linked dimeric form (Figure 3-1) suggest that a homo-oligomerization correlates with disulfide linked dimer formation. Taken together with the finding that dimeric LMP-1 co-immunoprecipitates with TRAF3 (Figure 3-6), support a model in which the dimeric form of LMP-1 represents the active, TRAF binding LMP-1 oligomer.
Figure 3-7  Cysteine-disulfide crosslinked LMP-1 species are not found in cells expressing a non-oligomerizing LMP-1 variant (LMP-1/TMD5,6).

A) DG75 cells were co-transfected with pCMV-LMP-1 and pCMV-LMP-1 \textit{myc} or pCMV-LMP-1/TMD5,6 and pCMV-LMP-1/TMD5,6 \textit{myc} and harvested 48 hours later. Triton-solubilized extracts were immunoprecipitated with myc antibodies (myc IP), immunoprecipitates resolved on 10\% SDS-polyacrylamide gels and analyzed by Western blot for LMP-1; \textit{SM}, starting material for immunoprecipitation; \textit{myc IP}, anti-myc immunoprecipitate; 721, 721 cell extract included as LMP-1 standard. The blots on the left and right show results for extracts of cells expressing LMP-1 and LMP-1/TMD5,6, respectively. Migration of protein standards are shown on the left and arrows point to LMP-1, LMP-1\textit{myc}, LMP-1/TMD5,6, LMP-1/TMD5,6\textit{myc} proteins (right). B) DG75 cells were transfected with pCMV-LMP-1 or pCMV/LMP-1/TMD5.6, harvested 48 hours later and solubilized in 4X nonreducing SDS-sample buffer. Extracts were heated to 85\degree C and resolved on 10\% SDS-polyacrylamide gels and analyzed by Western blot for LMP-1 and LMP-1/TMD5,6. The migration of protein standards (in kDa) is shown to the left of the blot, and migration of monomeric LMP-1 and lyLMP-1 (arrows) and dimeric LMP-1 (arrowhead) is shown to the right of the blot.

*Work performed by Sandra Balser*
The dimeric form of LMP-1 is restricted to lipid rafts.

The association between oligomerization and disulfide linked dimer formation (Figure 3-2, 3-7) and the co-immunoprecipitation of TRAF3 with the dimeric form of LMP-1 (Figure 3-1) suggests that the dimeric form of LMP-1 is a “marker” for active, TRAF binding LMP-1 oligomer. LMP-1 constitutive activation of signaling has been linked to its ability to localize in lipid raft microdomains (H Ardila-Osorio, et al., 1999; Hector Ardila-Osorio, et al., 2005; Coffin, et al., 2003; Higuchi, et al., 2001; J. Lee & Sugden, 2007; Yasui, et al., 2004). Specifically, activation of NF-κB by LMP-1 correlates tightly with its localization in detergent resistant membranes (DRMs). I asked whether dimeric LMP-1 selectively partitioned with DRMs in sucrose gradients in an attempt to link the formation of disulfide-linked dimers to LMP-1 signaling activity. 721 cells were treated with or without NEM for 60 minutes and then detergent lysates were analyzed by flotation in sucrose step gradients. Gradient fractions were analyzed by reducing and nonreducing SDS-PAGE and Western blot for LMP-1 (Figure 3-8). Consistent with its presence in lipid raft microdomains, LMP-1 is found in the DRM fractions. As previously observed, LMP-1 is also found in the detergent soluble fractions (H Ardila-Osorio, et al., 1999; Hector Ardila-Osorio, et al., 2005; Clausse, et al., 1997; Coffin, et al., 2003; Higuchi, et al., 2001; J. Lee & Sugden, 2007; Yasui, et al., 2004). The dimeric form of LMP-1 is found only in extracts from non-NEM treated cells and is restricted to the DRM fractions. In addition, LMP-1 in the DRM fraction is independent of NEM treatments, demonstrating that the ability of LMP-1 to fractionate with DRMs is not a result of post-lysis crosslinking. These results suggest strongly that raft-associated LMP-1 is in the form of oligomeric complexes that bring the carboxy-termini of adjacent LMP-1 molecules together to allow crosslinking to occur when extracts are oxidized following lysis.
Figure 3-8 Disulfide-crosslinked dimeric LMP-1 is restricted to detergent resistant membranes.

721 cells were pretreated with (B and D) or without (A and C) 20 mM NEM for 60 minutes and then homogenized in MNE/0.2% Triton X-100 and fractionated by flotation through sucrose step gradients. Fractions were taken from the top of the gradient (fraction 1, top of gradient) and mixed with nonreducing SDS-sample buffer and heated to 85°C, with (A and B) or without (C and D) 50 mM DTT, for 15 minutes. Samples were resolved on 10% SDS-polyacrylamide gels and analyzed by Western blot for LMP-1. Detergent-resistant membranes (DRMs) float to the top of the gradient whereas detergent soluble material is found in the lower fractions (soluble). Fractions containing DRMs were identified by Western blotting for Lyn kinase, which localizes exclusively to DRMs (not shown). The distinction between DRMs and soluble material is noted beneath each blot and gradient fraction numbers are noted above. The migration of protein standards (in kDa) is noted to the right of each blot, the arrows point to the position of monomeric LMP-1 and the arrowhead points to the position of dimeric LMP-1. The faint ~98 kDa band in B and D is a cross-reacting cellular protein recognized by the rabbit polyclonal anti-LMP-1 antibody.
Discussion

Constitutive signaling by LMP-1 depends on its homo-oligomerization and lipid raft association, both of which are mediated by the LMP-1 amino-terminus and TMD. These results demonstrate a homo-dimeric form of LMP-1, upon cell lysis that is stabilized via disulfide bond formation between cysteines within the LMP-1 cytoplasmic carboxy-terminus upon cell lysis (Figures 3-1 and 3-2). Consistent with the highly reducing environment of the cytoplasm, formation of this disulfide linkage does not occur in the intact cell, but rather as a result of the change in the red/ox environment experienced by LMP-1 upon cell lysis (Figure 3-4). Pretreatment of living cells with a cell permeable crosslinker show that the C-termini of at least two adjacent LMP-1 monomers are close enough proximity to crosslink in intact cells (Figure 3-5A).

These results are significant with regard to defining the LMP-1 oligomer for two reasons. First, disulfide bond formation between cysteine residues requires the β-carbons of participating cysteine residues to be localized within 3.4 to 4.6 angstroms of one another (Balaji, et al., 1989; Careaga & Falke, 1992). Second, disulfide bond formation between cysteines of adjacent LMP-1 molecules occurs in isolated membranes and in solubilized extracts and cysteine residues of adjacent LMP-1 monomers in intact cells can be crosslinked with sulfhydryl crosslinkers that can cross the membrane. Thus, the fact that cysteine 238 can be crosslinked via disulfide formation provides direct evidence that a minimum of two LMP-1 monomers interact, either as homodimers or as part of a larger homo-oligomeric complex, in the intact membrane environment. This assertion is supported by two findings. First, LMP-1 dimers and larger species are observed following crosslinking with the trifunctional sulfhydryl-specific crosslinker TMEA (Figure 3-5C). Second, LMP-1 variants that have been shown to homo-oligomerize via co-immunoprecipitation from solubilized cell extracts can form disulfide linked homo-dimers while
non-oligomerizing LMP-1 variants cannot (Figure 3-1, 3-5, 3-7). Nonreducing SDS-PAGE analysis of TRAF3 immunoprecipitates demonstrates that dimerized LMP-1, but not monomeric LMP-1, copurifies with TRAF3 (Figure 3-6). Importantly, the dimeric form of LMP-1 is restricted to DRMs (lipid rafts) in EBV-immortalized B cells (Figure 3-8). Based on these results, I believe that the crosslinked dimeric form of LMP-1 is a "marker" of functional LMP-1 oligomers.

Oligomerization in the membrane is a common mechanism by which cell surface receptors initiate signaling in response to ligand binding. Ligand activation of many immune receptor classes triggers their association with raft microdomains where they can interact with, or recruit, critical signaling molecules to initiate signal transduction (Bouillon, et al., 2003; Cheng, Brown, Song, & Pierce, 2001; Eren, et al., 2006; Grassme, Jendrossek, Bock, Riehle, & Gubins, 2002; A.-O. Hueber, et al., 2002; Kovacs, et al., 2005; Pierce, 2002; Sadra, Cinek, & Imboden, 2004; Vidalain, et al., 2000). LMP-1 is unusual in this regard as it is a ligand-independent "receptor" and signals as a constitutive raft-associated oligomer. LMP-1 resembles CD40 in its signaling in that both LMP-1 and CD40 interact with a subset of the same TRAF proteins via their carboxy-termini and elicit similar signaling outcomes (i.e. NF-κB and JNK activation). Activation of these pathways by LMP-1 and CD40 leads to similar changes in cell behavior including proliferation, survival and differentiation (Bishop, et al., 2007; Brodeur, et al., 1997; O. Devergne, et al., 1996; Kulwichit, et al., 1998; Raab-Traub, et al., 2009; M. Sandberg, et al., 1997; Uchida, et al., 1999; Xie, et al., 2008). Both LMP-1 and CD40 are believed to signal as oligomers from lipid rafts.

The structure and dynamics of the ligand-activated oligomerization of members of the TNFR superfamily have been extensively studied. TNFRs have been shown to self-assemble
into trimers in a ligand-independent fashion via their pre-ligand-binding assembly domains (PLADs) (Chan, et al., 2000; Clancy, et al., 2005; Papoff, et al., 1999; Siegel, Frederiksen, et al., 2000). The affinity of TRAFs for monomeric receptors is low and increases upon receptor trimerization (Pullen, et al., 1999). CD40's membrane-associated (A Kaykas, et al., 2001) or soluble (Werneburg, et al., 2001) trimeric cytoplasmic domain activates NF-κB signaling significantly more robustly than CD40's monomeric cytoplasmic domain. These results indicate that CD40's trimerized carboxy-terminus is more efficient at activating signaling than its monomeric counterpart.

CD40 signaling activity has also been linked to formation of CD40 dimers (Girouard, Reyes-Moreno, Darveau, Akoum, & Mourad, 2005; Reyes-Moreno, et al., 2004; Reyes-Moreno, et al., 2007; Werneburg, et al., 2001). Preformed disulfide-linked CD40 homo-dimers are found at low levels in EBV-transformed B cells and their levels dramatically increase following coculture with CD40L-expressing fibroblasts or treatment with soluble trimeric CD40L. Formation of the disulfide-linked homo-dimer is required for CD40L activation of phosphatidylinositol 3-kinase and IL-8 secretion (Reyes-Moreno, et al., 2004; Reyes-Moreno, et al., 2007). As I have observed with disulfide-linked LMP-1 dimers (Figures 3-2 and 3-8), CD40L-induced disulfide-linked homo-dimers are restricted to detergent-resistant membrane microdomains and the target for disulfide oxidation is a cysteine residue in CD40's cytoplasmic signaling domain, just downstream of the PXQXT TRAF binding motif (Reyes-Moreno, et al., 2007). Ligand-activated trimeric TNFRs form higher order multimeric complexes as well. The degree of TNFR multimerization can dictate the strength and specificity of TRAF binding and thereby affect downstream signaling outcome (Baccam & Bishop, 1999; Fanslow, et al., 1994; Pullen, et al., 1999; Liu, et al., 2002).
Less is known about the oligomeric structure of LMP-1. LMP-1 patching in the membrane, cytoskeletal association, rapid turnover and functional similarity to the TNFR superfamily (NF-κB activation and TRAF binding) suggested early on that LMP-1 might function as a constitutively active, oligomeric receptor (Baichwal & Sugden, 1987; Hennessy, et al., 1984; Liebowitz, et al., 1986; Mann, Staunton, & Thorley-Lawson, 1985; Martin & Sugden, 1991a, 1991b; O. Devergne, et al., 1996; Huen, et al., 1995; Laherty, Hu, Opipari, Wang, & Dixit, 1992; Mitchell & Sugden, 1995; Mosialos, et al., 1995; M. Sandberg, et al., 1997). This hypothesis is supported by several observations. A fusion of the LMP-1 amino-terminus and transmembrane domain to CD40's cytoplasmic domain activates NF-κB in the absence of CD40L (Gires, et al., 1997; Hatzivassiliou, et al., 1998) and inducible aggregation of the LMP-1 carboxy-terminal signaling domain correlates with enhanced activation of signaling (Floettmann & Rowe, 1997; Gires, et al., 1997). In addition, LMP-1 co-immunoprecipitates with epitope-tagged LMP-1 variants in an LMP-1 transmembrane domain-dependent manner and NF-κB activation requires both oligomerization and membrane localization of the LMP-1 carboxy-terminal signaling domain (Gires, et al., 1997). NF-κB signaling from a CD2/LMP-1 chimera (CD2 extracellular and transmembrane domain fused to the LMP-1 cytoplasmic C-terminus) is dependent on crosslinking with anti-CD2 antibody NF-κB (Floettmann, et al., 1998). Targeting the LMP-1 monomeric cytoplasmic signaling domain to lipid rafts via modification with myristic and palmitic acid is sufficient to robustly activate NF-κB signaling and forced trimerization of the raft-targeted cytoplasmic domain has little additional effect on the magnitude of observed NF-κB activation (A Kaykas, et al., 2001). This observation is seemingly at odds with the hypothesis that LMP-1 activation depends upon its oligomerization. However, diffusion is limited in the highly ordered raft environment. It is plausible that the raft-targeted monomeric
LMP-1 cytoplasmic domains are packed tightly such that they are effectively oligomerized. Such enforced interactions have been observed by FRET analysis of co-expressed myristoylated/palmitoylated YFP and myristoylated/palmitoylated CFP (Zacharias, Violin, Newton, & Tsien, 2002) and by crosslinking analysis of tightly packed GPI-anchored proteins in live cells (Friedrichson & Kurzchalia, 1998). The stoichiometry of LMP-1 functional units (i.e. dimer, trimer etc.) has not been defined and the possibility that these functional units organize into superclusters multimerized oligomers has not been explored. Further work characterizing the formation of LMP-1 signaling complexes including stoichiometry of LMP-1 subunits and signaling components of these large platforms needs to be explored.

**Materials and Methods**

**Cells.**

721 is an *in vitro* transformed EBV-positive lymphoblastoid cell line (Kavathas, Bach, & DeMars, 1980). DG75 cells are an EBV-negative Burkitt-like lymphoma cell line (Ben-Bassat, et al., 1977). Both cell lines were grown in RPMI supplemented with 10% bovine calf serum (R10C). HEK 293T is a T antigen-positive human embryonic kidney carcinoma cell line and was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (D10C). All cell lines were maintained at 37°C, in high humidity, with 5% CO₂.

**Antibodies and reagents.**

Anti-LMP-1 antiserum is an affinity-purified polyclonal rabbit serum raised against the LMP-1 C-terminus (residues 188-352) fused to glutathione-S-transferase. CS1-4 is a pool of
monoclonal antibodies recognizing epitopes in the LMP-1 C-terminus (Dako). The rabbit LMP-1 antibody was used for blotting unless specifically noted. Mouse monoclonal myc (9E10) antibody was from Santa Cruz Biotechnology, Inc. Anti-HA (HA-11) is a mouse monoclonal antibody from Berkeley Antibody Company (BabCo). Anti-TRAF3 (H20) is a goat polyclonal antibody (sc-948-G) from Santa Cruz Biotechnology, Inc. Anti-LMP-1 antibodies and horseradish peroxidase-conjugated secondary antibodies (Promega) were used at a dilution of 1:2500 for Western blotting. Antibodies (anti-TRAF3, anti-myc and anti-HA) were used at a concentration of 1 µg/ml for immunoprecipitation studies. Horseradish peroxidase conjugated secondary antibodies were from Promega. BMH (Bismaleimidohexane), and TMEA (Tris-[2-maleimidoethyl]amine) were from Pierce. 1,10-Phenanthroline was from Sigma-Aldrich.

**Plasmids.**

pCMV-LMP-1 and pCMV-LMP-1/TMD5,6 are pCDNA3-based expression vectors encoding LMP-1 and the N-terminally truncated form of LMP-1 expressed during EBV's lytic cycle (the LMP-1/TMD5,6 (lyLMP-1) open reading frame encodes LMP-1 residues 129-386 which include the 5th and 6th transmembrane domains and cytoplasmic C-terminus (Erickson & Martin, 2000)). pCMV-LMP-1\textit{myc} and pCMV-LMP-1/TMD5,6\textit{myc} encode C-terminal myc epitope tags. The following cysteine substitution mutants are all constructed in the pCMV-LMP-1 background: pCMV-LMP-1/\textit{Csub}A1 has an alanine codon in place of cysteine 78 (C78C); pCMV-LMP-1/\textit{Csub}A1,2 has alanine codons in place of cysteines 78 and 84 (C78A;C84A); pCMV-LMP-1/\textit{Csub}A3 has an alanine in place of cysteine 116 (A116C); pCMV-LMP-1/\textit{Csub}A1-3 has alanine codons in place of cysteines 78, 84, and 116 (C78A; C84A; C116A); pCMV-LMP-1/\textit{Csub}A4 has an alanine codon in place of cysteine 238 (C238A); pCMV-LMP-1/\textit{Csub}A4-4 has alanine codons in place of cysteines 78, 84, 116, and 238 (C78A; C84A; C116A; C238A).
1/CsubA1-4 has alanine codons in place of cysteines 78, 84, 116, and 238 (C78CA; C84A; C116A; C238A). Cysteine substitution mutants were generated by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis XL-II, Stratagene) and confirmed by sequencing. pRSV-LMP-1 encodes the LMP-1 gene in pRC-RSV and has been described previously (Coffin, et al., 2003). The cysteine substitution mutant CsubA4 was subcloned into pRC-RSV to generate pRSV-LMP-1/Csub4. p1242 is a luciferase reporter in which the luciferase gene is driven by the minimal fos reporter with 3 upstream kB binding sites from the MHC class I gene (Mitchell & Sugden, 1995). pRSV-LacZ encodes the lacZ gene in the pRC-RSV vector.

Transfections.

DG75 cells were electroporated in 0.4 cm gapped cuvettes using a Bio-Rad gene pulser (0.25 kV, 960 μF, 5x10^6 cells/0.35 ml R10C). HEK 293T cells were transfected using Mirus TransIT-293 transfection reagent according to manufacturer’s instructions. Transfected cells were assayed two days posttransfection.

Membrane isolation.

Cells were resuspended in hypotonic lysis buffer (10 mM HEPES-KOH, pH 7.9, 0.5 mM KCL, 0.5 mM MgCl2, 0.1 mM EGTA, 0.5 mM DTT), incubated on ice for 30 minutes and triturated 10 times through a 26.5 gauge needle. The lysate was centrifuged at 13,000xg for 10 minutes (low speed spin) and the resulting supernatant was centrifuged at 105,000xg for 60 minutes (high speed spin) and the pellet was triturated and centrifuged at 10,000xg for 10 minutes. The pellet from the high-speed spin was combined with the low speed pellet in low salt buffer (LSB)(50
mM HEPES-KOH, pH 7.4, 100 mM β-glycerolphosphate, 25 mM NaF, 1 mM MgCl₂, 1 mM EGTA, 5% glycerol, 1 mM PMSF). This membrane preparation was used as the source of material for nonreducing SDS-PAGE and CuP crosslinking (Figures 3-1, 3-2, 3-5B). For preparation of solubilized membranes, an equal volume of LSB/10% Triton X-100 was added to the membrane pellet and the solution was incubated on ice for 30 minutes before centrifugation at 13,000xG for 15 minutes. The supernatant from this spin was centrifuged at 100,000xG for 60 minutes and the resulting supernatant was used for experiments shown in Figures 3-5C, 3-6, and 3-7).

**BMH Crosslinking.**

721 cells (2x10⁷ cells/µl) were resuspended in R10C containing 1 mM BMH and incubated at 37°C at 5% CO₂ for 1 hour. The reaction was quenched in 180 mM DTT for 10 minutes 37°C at 5% CO₂. Cells were then washed 2 times with 1xPBS and lysed 1:1 in 4x reducing SDS sample buffer, boiled at 85°C for 15 minutes.

**TMEA and EGS Crosslinking.**

721 cells (2x10⁴ cells/µl) were lysed in Syn7 lysis buffer (50 mM NaPO₄, pH 7.4, 150 mM NaCl, 25 mM sucrose, 5% glycerol, 1% Triton X-100). Insoluble material was removed by centrifugation and the supernatant was used for crosslinking reactions. EGS crosslinking: EGS was added to the soluble material at final concentrations of 0.5 mM, 1 mM and 1.5 mM (in DMSO; volume of EGS/DMSO was 5% of total volume of lysate) and incubated for 30 minutes at room temperature with rocking. The crosslinking reaction was quenched by the addition of
1M Tris, pH 7.5 to a final concentration of 20 mM followed by 15 minutes of incubation.

TMEA crosslinking: TMEA was added to the soluble material after adjusting the pH to 7.0 (final TMEA concentrations of 125 µM, 250 µM and 375 µM (in DMSO; volume of TMEA/DMSO was 5% of total volume of lysate) and incubated at room temperature for 30 minutes. 4X SDS-sample buffer containing 50 mM DTT was added to crosslinked lysates (1:1 dilution) and samples were heated to 85°C for 15 minutes and sonicated.

**CuP crosslinking.**

Membranes were incubated with 0.2 mM Cu(II) (1,10-phenanthroline)₃ and ambient O₂ (approximately 200 µM) for 20 minutes at 37°C. Oxidation was terminated by addition of 0.1 mM sodium persulfate (Careaga & Falke, 1992; Chervitz, Lin, & Falke, 1995). Membranes were then solubilized in 4X nonreducing SDS-sample buffer and heated to 85°C for 15 minutes, with or without addition of 50 mM dithiothreitol (DTT).

**NEM treatment.**

721 cells were pretreated with or without the membrane permeable, irreversible alkylating agent N-ethylmaleimide (NEM; 20 mM)(Pierce) at 10⁶ cells/ml in R10C for 60 minutes at 37°C. Cells were washed in ice cold phosphate buffered saline (PBS) and dounce homogenized (20 strokes) on ice in NEM lysis buffer (NLB; 50 mM NaH₂PO₄, pH 7.0, 300 mM NaCl, 20 mM NEM, 1% Triton X-100, protease inhibitors). This extract was diluted 1:1 with 4X nonreducing SDS-sample buffer, heated to 85°C for 15 minutes in the presence or absence of 50 mM DTT. For the experiment shown in Figure 3-4B, 721 cells pretreated with and without NEM were lysed in
NLB lacking NEM, incubated at 4°C for the indicated times and diluted 1:1 with 4X nonreducing SDS-sample buffer containing NEM. Samples were heated to 85°C for 15 minutes and analyzed by SDS-PAGE and Western blot for LMP-1. For the experiment shown in Figure 3-8, 721 cells were treated with NEM as described above and fractionated through sucrose step gradients as described below.

**SDS-PAGE and Western blotting.**

Samples were solubilized in 4X nonreducing SDS-sample buffer, sonicated and heated to 85°C for 15 minutes in the presence or absence of 50 mM DTT. Samples were then resolved on either 10% or 7.5% gels and transferred to Immobilon-P membranes (Millipore). Proteins were visualized on blots using the ECL Plus Western Blotting Detection System (GE Healthcare) following incubation with primary and secondary antibodies. Protein standards used were BenchMark Pre-Stained, SeeBlue Plus2 Pre-Stained Standard (Invitrogen) or Dual Color Precision Plus Recombinant Protein Standards (Bio-Rad).

**NF-κB reporter assay.**

HEK 293T cells were co-transfected with LMP-1 expression vectors, an NF-κB luciferase reporter, and the b-galactosidase reporter pRSV-lacZ. Each transfection was performed in duplicate. Cells were harvested two days post-transfection and cell lysates were assayed in triplicate by luciferase and b-galactosidase activity using the Tropix Dual Light assay system (Applied Biosystems). Luciferase relative light units are corrected for transfection efficiency by normalizing to b-galactosidase relative light units.
**Immunoprecipitation.**

Solubilized membranes from 721 cells were used as starting material for immunoprecipitation analysis. Antibody was added to the precleared starting material, incubated for 30 minutes on ice, and immune complexes were recovered with Protein-G agarose (Roche Diagnostics). Beads were washed five times in LSB/0.035% Triton X-100 and heated to 85°C for 15 minutes in 4X nonreducing SDS-sample buffer.

**Lipid raft assay.**

721 cells were pretreated with or without 20 mM NEM as described above and homogenized on ice with a Dounce homogenizer (10 strokes) in 1 ml of MNE (25 mM MES [morpholineethanesulfonic acid, pH 6.5], 150 mM NaCl, 5 mM EDTA) containing 0.2% Triton X-100. Extracts were diluted 1:1 in 80% sucrose/MNE, overlaid with 2 ml of 30% sucrose/MNE and 1 ml of 5% sucrose, and centrifuged at 4°C for 18 hours in an SW50.1 rotor at 200,000xg. Gradients were harvested from the top of the gradient in 400 ml fractions and mixed with 200 ml of 4X nonreducing SDS-sample buffer and heated to 85°C for 15 minutes in the presence or absence of 50 mM DTT.
CHAPTER 4 : LMP-1 FORMS MULTIPLE HIGH MOLECULAR WEIGHT COMPLEXES

Introduction

LMP-1 activates a variety of pathways (NF-κB/JNK) linked to B cell survival and proliferation like Tumor Necrosis Factor Receptors (TNFRs). Unlike the TNFRs, whose activity is ligand dependent LMP-1 signaling is ligand-independent and oncogenic. LMP-1 activates these pathways via binding of TRAFs to its C-terminal signaling domain.

LMP-1 is also coupled to non-TRAF associated signaling pathways via its hydrophobic transmembrane domain (TMD). Cells expressing LMP-1 have underphosphorylated Tyk2, a janus kinase, and are unresponsive to interferon alpha (Geiger & Martin, 2006). LMP-1 expression also activates cellular stress pathways leading to activation of the unfolded protein response (D. Y. Lee, Lee, & Sugden, 2009). The LMP-1 TMD is coupled to cytostatic signals when expressed at high levels (Coffin, et al., 2003; Floettmann, et al., 1996; A Kaykas & Sugden, 2000; M. L. Sandberg, et al., 2000). The LMP-1 hydrophobic TMD functions as the LMP-1 ‘activation’ domain by mediated homo-oligomerization and lipid raft association. Both of these activities are highly correlated with LMP-1 signaling activity and it is generally believed that LMP-1 signals as an oligomer from lipid rafts.

Work in this chapter aims to understand the complexes formed by LMP-1 to propagate its signaling. Understanding the mechanisms of LMP-1 signaling is challenging because it activates many different signaling outputs. To date, the study of LMP-1 signaling has been tackled using a structure/function approach. The signaling activities of LMP-1 variants are measured using reporter assays or biochemical assays and are compared to wild-type LMP-1. LMP-1 homooligomerization is assayed by co-immunoprecipitation of LMP-1 from detergent solubilized
extracts. However, the stoichiometry and mechanism by which LMP-1 homo-oligomerizes with itself is unknown. LMP-1 association with lipid rafts is assayed by measuring the fraction of cellular LMP-1 found in detergent resistant membranes following flotation of triton-solubilized extracts through sucrose gradients. The assembly of LMP-1 signaling complexes in membrane microdomains is likely required for active signaling, but nature of these complexes is unknown. Characterization of this signaling complex and the identification of its components would provide a toolkit to understand how these components act independently and the extent that they are dependent on each other.

Activation of signaling cascades with distinct and often opposing outcomes begs the question of whether LMP-1 forms one large signaling complex coupled to all outputs or multiple signaling complexes coupled to each output. This question was addressed using a combination of biochemical approaches aimed at characterizing the native LMP-1 signaling complex from EBV immortalized cells.

LMP-1 must form oligomers in order to signal from the membrane. The previous chapter demonstrated that LMP-1 forms dimers and these dimers are crosslinked upon oxidation of cell lysis (Chapter 3). I chose to address study LMP-1 oligomerization by characterizing native LMP-1 complexes using biochemical approaches and by characterizing complex heterogeneity. Specifically, the heterogeneity of LMP-1 containing complexes was examined to test the hypothesis that LMP-1 forms distinct high order complexes to signal to its downstream effectors. Because LMP-1 is a membrane protein I examined whole cell lysates from EBV immortalized B cells using Blue Native PAGE (BN-PAGE). BN-PAGE is well suited for resolving large native multi-protein complexes on the basis of shape and size. This method has been used successfully to characterize native mitochondria complexes from cellular (Nijtmans, Henderson, & Holt,
2002; Schägger, 2001). Recently BN-PAGE has been coupled with bimolecular complementation to find dimers and tetramers of Attachment Protein H from purified Measles virus (Brindley & Plemper, 2010).

**Results**

**Latent Membrane Protein-1 forms multiple high molecular weight complexes.**

Whole cell lysates from EBV immortalized B cells was used as the source of LMP-1 complexes. Immortalized cells were utilized to determine complexes LMP-1 forms when contributing to immortalization. Analyzing transient transfections would provide a mixed picture as transfected cells would express varying amounts of expressed proteins. 721 cells are an EBV positive lymphoblastoid cell line whose survival and proliferation are dependent on the activity of LMP-1. Cells were lysed in hypotonic lysis buffer and resolved by BN-PAGE. The lysis buffer contains Triton X-100 for solubilization of membrane components. At least two major bands ~440 kDa and ~669 kDa were resolved, and in addition very high molecular weight immunoreactive material of >669kDa was observed in 721 cell lysates (Figure 5-1). Previous work has shown that disulfide crosslinking between two adjacent LMP-1 proteins occurs in cell lysates (Chapter 3 and Wrobel et al. in submission). To verify that the high molecular weight complexes were not the result of post-lysis oxidation LMP-1, 721 were pretreated with N-ethyl-maleimide (NEM) to irreversibly block free sulphhydryls prior to lysis. NEM treatment dramatically increased the resolution of high molecular weight LMP-1 immunoreactive bands, suggesting that post lysis oxidation of sulphhydryls contributed to the relatively poor
Figure 4-1 LMP-1 forms multiple high molecular weight complexes.
4x10^5 721 cells treated or not treated with NEM for 30 minutes and were lysed and run on a 3-15% BN-PAGE and analyzed by Western blot for LMP-1 using rabbit polyclonal antibody for LMP-1 C-terminus.
resolution of complexes in lysates from non-NEM treated cells. In addition, these results suggest that the ladder of very high molecular weight bands (>669 kDa) observed in lysates from NEM treated cells does not result from post-lysis disulfide crosslinking.

**High molecular weight LMP-1 complexes immunoreactive contain monomeric LMP-1.**

To confirm that immunoreactive bands correspond to high molecular weight LMP-1 containing native complexes and are not the result of cross-reactivity, the migration of complexes from lysates pre-incubated with LMP-1 specific antibody was assessed (Figure 4-2). The addition of LMP-1 antibodies to lysates prior to electrophoresis shifted complexes to a higher molecular weight (Figure 4-2A). Lower molecular weight bands (i.e. 440 and 669 kDa) disappeared from lysates and new higher molecular weight bands appeared upon incubation with anti-LMP-1 antibody prior to BN-PAGE. Incubation with an affinity purified polyclonal serum specific for the LMP-1 C-terminus or a pool of monoclonal antibodies specific for epitopes in the LMP-1 C-terminus generated the same gel shift pattern (not shown). Importantly, pre-incubation with antibodies to GFP, a non-specific antibody, had no effect on the migration of LMP-1 complexes. LMP-1 immunoreactivity could be depleted from lysates by incubation with LMP-1 antibody followed by clearing with Protein-G agarose (Figure 4-2B). To further characterize the LMP-1 content of each high molecular weight band observed on BN-PAGE gels, samples were resolved by BN-PAGE one dimension and then resolved in a second dimension by SDS-PAGE. Should high molecular weight complexes contain LMP-1, immunoreactive dots will be detected in the second dimension Western blot corresponding in molecular weight to the molecular weight of monomeric LMP-1. High molecular weight complexes were resolved by BN-PAGE
Figure 4-2 Complexes resolved by BN-PAGE contain LMP-1
A) 721 lysates incubated with LMP-1 specific antibodies 1 hour prior to BN-PAGE analysis. Red arrows mark unshifted complexes that disappear upon addition of antibody, blue arrows indicate new bands that appear after addition of antibody. 721 cells were for BN-PAGE analysis lysed as in Figure 1. B) Lysates incubated with LMP-1 specific antibodies for 1 hour followed by addition of Protein-G agarose beads for 30 minutes, and then beads were removed by centrifugation, supernatant was resolved by BN-PAGE. Lane 1, no pretreatment with antibodies or beads; Lane 2 pretreated with LMP-1 antibodies only; Lane 3, pretreated with LMP-1 specific antibodies and then cleared with Protein-G agarose. C) 721 lysates incubated with LMP-1 specific or GFP specific antibodies were added 1 hour prior to BN-PAGE analysis. 721 cells were for BN-PAGE analysis lysed as in Figure 1 GFP or LMP-1 were added to lysates for 1 hour prior BN-PAGE analysis.
then subsequently resolved by SDS-PAGE (Figure 4-3). Detection of monomeric LMP-1 in the second dimension from the native complexes resolved in the first dimension shows that the higher molecular weight bands observed by BN-PAGE are composed wholly or in part of monomeric LMP-1 building blocks and are not the result of antibody cross-reaction with a cellular protein.

**Native LMP-1 complexes resolved by BN-PAGE resemble those resolved by size exclusion chromatography.**

Size exclusion chromatography was used to fractionate LMP-1 positive cellular lysates for further characterization by BN-PAGE. Triton solubilized lysates were resolved by size exclusion chromatography through a Sephacryl S-400 can resolve macromolecules from 20 to 8000 kDa. Column fractions were collected and analyzed by SDS-PAGE and Western blot for LMP-1. The amount of LMP-1 per fraction was quantified as shown in Figure 4-4A. LMP-1 eluted from the column in peaks ranging from >1MDa to ~50kDa. Column fractions marked with arrows in figure 4-4A were analyzed by BN-PAGE (Figure 4-4B). The molecular weight of LMP-1 containing complexes determined by size exclusion was confirmed by BN-PAGE analysis. High molecular weight complexes eluting within the first 40 fractions from the S400 column contained the very high molecular weight material (>669kDa) observed by BN-PAGE. Fractions eluted after fraction 40 contained the 669 and 440 kDa complexes, whereas complexes (or monomeric LMP-1) eluting after fraction 65 could not be resolved by BN-PAGE. The fact that complexes identified by these two different biochemical approaches are consistent in size and the fact that very high molecular weight complexes from size exclusion fractions enter and
Figure 4-3 High molecular weight complexes contain monomeric LMP-1.
721 cell lysates were analyzed by 2D PAGE and Western blot for LMP-1. A) 721 cell lysates were resolved by BN-PAGE and analyzed by Western blot for LMP-1 (1st Dimension). B) A lane from the BN-PAGE in “A” was cut off the gel, loaded across an SDS-PAGE gel for resolution in the 2nd dimension. The second dimension SDS gel was analyzed by Western blot for LMP-1.
Figure 4-4  LMP-1 complexes can be fractionated using size exclusion chromatography. Triton soluble cell extracts were fractionated by size exclusion sephacryl chromatography (S-400) and fractions collected for analysis by SDS-PAGE and BN-PAGE.  A) Quantification of LMP-1 in column fractions. Samples from fractions were analyzed by SDS-PAGE and Western blot for LMP-1. Blots were developed with ECL and analyzed with the Storm imager. The relative amount of LMP-1 per column fraction is plotted. Blots were normalized to each other using an LMP-1 marker. Dashed lines indicate elution of high molecular weight markers off the column (specifically, 2MDa eluted at fraction 20, 669kDa at 39, 440kDa at 58, and 232kDa at 78).  B) BN-PAGE analysis of selected column fractions eluates. Peak fractions from the S-400 column (marked with black arrows in part A) were resolved by BN-PAGE, and analyzed for LMP-1 by Western blot.
can be resolved by BN-PAGE argue strongly that these large complexes do not represent nonspecific LMP-1 aggregates.

**Native LMP-1 complexes are unequally distributed between detergent resistant membranes and soluble fractions.**

LMP-1 signaling activity correlates with its association with lipid rafts, which provide scaffolding and bring signaling components together to promote more efficient signaling (H Ardila-Osorio, et al., 1999; Hector Ardila-Osorio, et al., 2005; Coffin, et al., 2003; Higuchi, et al., 2001; J. Lee & Sugden, 2007; Yasui, et al., 2004). LMP-1 is constitutively active unlike the TNFR LMP-1 it mimics, CD40. CD40 requires ligand to bind and forms trimers in lipid rafts to activate signaling. LMP-1 is constitutively active and does not require ligand for signaling; therefore all LMP-1 in a cell could form signaling complexes. The requirements for LMP-1 signaling to date include homo-oligomerization and raft association. However, only a fraction of the total cellular LMP-1 is found membrane microdomains and the rest is found in bulk membranes.

To address the question of which native LMP-1 complexes resolved by BN-PAGE have active signaling potential, 721 cells lysates were fractionated into detergent resistant and detergent soluble fractions by sucrose gradient fractionation. Native LMP-1 complexes in gradient fractions where then resolved by BN-PAGE (Figure 4-5A) and SDS-PAGE (Figure 4-5B). As shown by SDS-PAGE, LMP-1 enriched in detergent resistant membranes (fractions 2 and 3 of the gradient) and also is found in the bottom of the gradient in the detergent soluble fractions.
Figure 4-5 HMW LMP-1 native complexes are enriched in Detergent Resistant Membranes.

Triton X-100 solubilized 721 cell lysates were fractionated through sucrose step gradients to obtain detergent resistant and detergent soluble fractions. Gradients were fractionated from the top (Fraction 1). Each fraction was analyzed by BN-PAGE and SDS-PAGE. A) BN-PAGE analysis. Samples from gradient fractions were resolved on a 3-15% acrylamide BN-PAGE and analyzed by Western blot for LMP-1. B) SDS-PAGE analysis. Samples from gradient fractions were mixed with 4 x Sample buffer and resolved by SDS-PAGE and analyzed by Western blot for LMP-1. The position in the gradient where detergent resistant membranes and detergent soluble fractions are numbered above the blot. Blots stained for Lyn kinase, a DRM marker was used to identify fractions containing DRMs (not shown). The migration of protein standards (in kDa) is noted to the left of each blot, the arrow points to the position of monomeric LMP-1.
Analysis of the same gradient fractions by BN-PAGE reveals that the highest molecular weight complexes (>669kDa) were enriched in detergent resistant membranes, whereas the smaller complexes (<669kDa) were enriched in detergent soluble fractions. Coomassie stain analysis of SDS-PAGE resolved samples for total protein throughout the sucrose gradient indicates less protein is found in the top of the gradient (detergent insoluble fractions) than in the bottom of the gradient (detergent soluble fractions)(data not shown). Thus, high molecular weight native LMP-1 complexes are highly enriched in detergent resistant membranes. LMP-1 can be found in the soluble and detergent resistant membrane fractions the complement of native complexes within these fractions differs. These results are consistent with the model that LMP-1 signals as an oligomer from raft microdomains and suggest that actively signaling LMP-1 complexes are large multi-protein complexes.

LMP-1 alone is not sufficient for formation of very high molecular weight native complexes.

The high molecular weight native LMP-1 complexes observed by BN-PAGE are likely to represent LMP-1 oligomers and associated cellular proteins. The migration of purified recombinant LMP-1 in BN-PAGE was compared to native LMP-1 from cell extracts. To ask whether any or all of the observed complexes are composed of “pure” LMP-1 homo-oligomers, a Flag and His-tagged LMP-1 cDNA was cloned into pFastBac1 and the resulting vector was transformed into DH10Bac E.coli cells for recombination into the parent bacmid. Recombinant LMP-1-bacmid was isolated from DH10 cells and transfected into Sf9 cells to generate Flag-LMP-1-6xHis expressing baculovirus. This virus stock was used for subsequent Sf9 cell infections and recombinant LMP-1 was purified by two-step affinity chromatography. LMP-1 was expressed in 293T cells and its migration in SDS-PAGE and BN-PAGE was compared to
Figure 4-6  Recombinant LMP-1 is not sufficient to form all the highest molecular weight complexes.

Analysis of purified recombinant LMP-1 by BN-PAGE and SDS-PAGE. Flag and 6xHis tagged recombinant LMP-1 was purified using a two step affinity column purification as described in methods. A) Analysis of 293T extracts transfected with LMP-1. 293T cells transfected with LMP-1 expression vector or purified recombinant LMP-1 was resolved by BN-PAGE and analyzed by Western blot for LMP-1. Molecular weight markers in kDa are noted to the left of the blot.  B) Analysis of recombinant purified LMP-1 by SDS-PAGE. The same starting material in B was mixed with 4x Sample buffer and resolved by 10% SDS-PAGE and visualized by Western blot for LMP-1. Markers on the left note migration of molecular weight markers in kDa.
the migration of purified recombinant Flag-LMP-1-6xHis (Figure 4-6A and 6B). LMP-1 expressed in 293T cells formed similarly sized high molecular weight complexes formed as observed in 721 cells (Figure 4-6; compare to Figures 4-1,4-2,4-4). The complexes were similar to both the very high molecular weight complexes (>1MDa) as well as the complexes falling into the 220 and 660kDa range (lower molecular weight). In contrast, recombinant LMP-1 was found exclusively in the lower molecular weight (669-440 kDa) complexes. This demonstrates that formation of the larger of the high molecular weight complexes requires interaction with cellular factors or cellular processes and that the complexes migrating at or below 669 kDa may in fact represent LMP-1 homo-oligomers. Analysis of 293T cell extracts and recombinant LMP-1 by SDS-PAGE and Western blot demonstrates that the recombinant purified LMP-1 analyzed by BN-PAGE (Figure 4-6A) was not degraded (Figure 4-6B) and in fact retained that ability to dimerized form crosslinked dimers (Chapter 3, Figure 3-4B). Furthermore, the fact that purified LMP-1 forms only the smaller complexes argues strongly that the very high molecular complexes found in detergent resistant membranes is a hetero-oligomer or requires cellular processing to form higher order homo-oligomers. Purified recombinant LMP-1 alone is not competent to form the highest molecular weight native complexes.

The LMP-1 TMD is required for formation of high molecular weight native complexes.

I have shown that LMP-1 is present in B cells and forms multiple high molecular weight complexes, but the functional relevance of these complexes is unclear. To dissect complex formation by actively signaling LMP-1, full length LMP-1 and functional vs. non-functional transmembrane domain deletion mutants of LMP-1 were analyzed by BN-PAGE (Figure 4-7A). 293T cells were transfected and harvested 48 hours post transfection and extracts resolved by BN-PAGE and analyzed by Western blot (Figure 4-7B). Mutants lacking any of the
Figure 4-7 LMP-1 complexes in transfected B cells are comparable to complexes identified in EBV+ Lymphoblastoid cell lines.
A) Schematics of LMP-1 mutants. For complete descriptions see Figure 2-4. B) Analysis of transfected cell extracts by BN-PAGE and Western blot LMP-1. C) Analysis of transfected cell extracts by SDS-PAGE and Western blot for LMP-1. Arrow on right indicates the migration of full length LMP-1. Molecular weight makers for both panels are shown on the left in kDa.
transmembrane domains of LMP-1 (LMP-1/TMD1,6; LMP-1/TMD1,2; LMP-1/TMD5,6) were unable to form the larger subset of higher molecular weight complexes. Uniformly, these mutants were found in and all of these mutants exhibit varying degrees of signaling deficiencies relative to full length LMP-1 (Chapter 2). Both LMP-1/TMD1,2 and LMP-1/TMD1,6 are compromised in their ability to activate NF-κB and inhibit IFNα signaling. LMP-1/TMD5,6 is completely unable to activate NF-κB signaling. These results suggest that the very high molecular weight complexes represent “active” LMP-1 signaling complexes. The fact that these very high molecular weight complexes are enriched in detergent resistant membranes (Figure 4-5), where LMP-1 is believed to signal is consistent with this conclusion. The smaller LMP-1 complexes (<669 kDa) may represent homo-oligomers of LMP-1 that are yet to form mature, active, signaling complexes.

**Discussion**

Homo-oligomerization of LMP-1 is crucial for LMP-1 ligand independent signaling activity (Floettmann, et al., 1998; Floettmann & Rowe, 1997; Gires, et al., 1997; Hatzivassiliou, et al., 1998). Full length LMP-1 activates NF-κB signaling, suppresses IFNα signaling, and alters cellular growth and proliferation. Here, I identified multiple high molecular weight native LMP-1 complexes (Figure 4-1). NEM independence reveals that LMP-1 complex formation is independent of post-lysis cysteine disulfide crosslinking (Figure 4-1). Higher molecular weight complexes are specifically shifted by the addition of LMP-1 antibodies to the cellular lysate and all these native complexes contain monomeric LMP-1 (Figure 4-2, 4-3). Similarly sized complexes can be resolved by BN-PAGE and size exclusion chromatography (Figure 4-4). The
fact that the same “collection” of native complexes can be resolved by two independent methods combined with the reproducibility of sizes of complexes observed by both methods strongly argues that the complexes are not random LMP-1 aggregates. Identification of similar higher molecular weight native complexes by different lysis and solubilization conditions are a testament to the stability and specificity (non-aggregate) of these high molecular weight complexes.

Crosslinking studies reveal that actively signaling LMP-1 in detergent resistant membranes is organized into dimers (Chapter 3). Consistent with the crosslinking results; BN-PAGE studies show that the largest subset of the high molecular weight complexes are enriched in the detergent resistant fractions with the smaller complexes are enriched in the soluble fractions (Figure 4-5). Importantly, non-functional LMP-1 mutants are found exclusively in the detergent soluble fractions and only form the smaller native complexes (Figure 2-14)(Coffin, et al., 2003). These results are consistent with the model that the higher molecular weight complexes contain (>669 kDa) complexes represent ‘active’ LMP-1 signaling complexes. Unlike LMP-1 from cell extracts, recombinant LMP-1 purified from Sf9 cells is not able to form the higher molecular weight complexes suggesting that the larger complexes are composed of cellular proteins and/or requires processing within the cell (Figure 4-6). TMD deletion mutants are not fully active and are unable to form the larger subset of complexes congruent with the idea that the larger native complexes are composed of active LMP-1 (Figure 4-7).

Oligomerization of LMP-1 is critical for its signaling activity. LMP-1 forms a disulfide crosslinked dimer is formed only in detergent resistant lipid rafts, suggesting that active LMP-1 is oligomerized in lipid rafts (Wrobel et al., submitted). LMP-1 signaling activity through NF-κB and JNK signaling pathways mimics signaling of endogenous CD40 in B cells. CD40, a
member of the Tumor necrosis factor receptor (TNFR) family, assembles as a trimer in response to activation by trimerized ligand. Our discovery of high molecular weight LMP-1 signaling complexes in detergent resistant membranes, and the correlation between active signaling LMP-1 and formation of large native complexes is consistent with a model in which LMP-1 must be organized in large higher order signaling clusters in order to activate LMP-1 signaling. Further experiments are needed to differentiate if these complexes have different signaling potential and interact differently with signaling interactors.

**Materials and Methods**

**Cells.**

721 is an *in vitro* transformed EBV-positive lymphoblastoid cell line (Kavathas). DG75 cells are an EBV-negative Burkitt-like lymphoma cell line (Ben-Bassat). Both cell lines were grown in RPMI supplemented with 10% bovine calf serum (R10C). All cell lines were maintained at 37°C, in high humidity, with 5% CO₂.

**Antibodies and reagents.**

Anti-LMP-1 antiserum is an affinity-purified polyclonal rabbit serum raised against the C-terminus (residues 188-352) of LMP-1 fused to glutathione-S-transferase. CS1-4 is a pool of monoclonal antibodies recognizing epitopes in the LMP-1 C-terminus (Dako). Anti-TRAF3 (H20) is a goat polyclonal antibody (sc-948-G) from Santa Cruz Biotechnology, Inc. Anti-LMP-1 antibodies were used at a dilution of 1:2500 (Rabbit) 1:1000 (mouse) for Western blotting,
anti-TRAF3 antibodies were used at a dilution of 1:500 for Western blotting, and at a concentration of 1\(\mu\)g/ml for immunoprecipitation, anti-LMP-1 antibodies were used at a dilution of 1\(\mu\)g/ml for immunoprecipitation. Horseradish peroxidase conjugated secondary antibodies were from Promega.

**Plasmids.**

pRSV-LMP-1 (LMP-1), pRSV-LMP-1/TMD5,6 (LMP-1 deletion lacking TMDs 1-4), pRSV-LMP-1/TMD1,2 (LMP-1 deletion lacking TMDs 3-6, and pRSV-LacZ are pRC-RSV (Empty Vector) based expression vectors (Invitrogen) and have been described previously (Coffin, et al., 2003; Geiger & Martin, 2006). pRSV-LMP-1/TMD1,6 (LMP-1 deletion lacking TMDs 2-5, with LMP-1 residue 51 (alanine) fused in frame to residue 166 (threonine);

**Transfections.**

DG75 cells were electroporated using a Bio-Rad gene pulser (0.25 kV, 960 \(\mu\)F, 5x10\(^6\) cells/0.35 ml R10C). Transfected cells were assayed two days post-transfection.

**NEM treatment.**

721 cells were pretreated with or without the membrane permeable, irreversible alkylating agent N-ethylmaleimide (NEM; 20 mM)(Pierce) at 10\(^6\) cells/ml in R10C for 30 minutes at 37°C prior to harvest.
**SDS-PAGE and Western blotting.**

Samples were solubilized in 4X nonreducing SDS-sample buffer (100mM TrisHCL pH6.8, 5%SDS, 10% Glycerol, 0.02g Bromophenol Blue), sonicated, and heated to 85°C for 30 minutes. Samples were then resolved on a 10% acrylamide gel at 80V for 10 minutes and 220V for 1 hour and transferred to Immobilon (Millipore) membranes at 100V for 75 minutes. Proteins were visualized on blots following staining with primary and anti-rabbit or anti-mouse HRP-conjugated secondary antibody using ECL Plus Western Blotting Detection System (GE Healthcare). Protein standards were BenchMark Pre-Stained (Invitrogen).

**Immunoprecipitation**- Column fractions from 721 cells were used as the starting material, precleared with protein-G agarose beads and antibody was added to the precleared supernatant for 30 minutes on ice. Immune complexes were recovered with Protein-G agarose (Roche). Beads were washed 5 times in column buffer and heated to 85°C for 30 minutes in nonreducing SDS-sample buffer.

**Blue Native PAGE.**

1x10^7 cells were harvested with or without NEM treatment (see NEM treatement section). Cells were lysed on ice for 30 minutes in 500ul of Native blue lysis buffer (20mM Bis-Tris, 500mM ε-aminocaproic acid, 20mM NaCl, 2mM EDTA, 10% glycerol, 0.2% Triton-X100 pH 7.0). Insoluble material was spun out at 13,000 RPM for 10 minutes in a microfuge. For antibody gel shift experiments antibody was added to lysates and nutated for 30 minutes at 4°C. 25ul of 5% Coomassie G-250 was added to the lysis solution and allowed to nutate for 30 minutes. Samples
were loaded onto a Native PAGE precast gradient gel 3%-18% (Invitrogen). Gels were run with Anode Buffer (500mM Bistris pH 7.0) and Cathode buffer (150mM BisTris, 500mM Tricine, 0.2% Coomassie G-250 pH7.0). Halfway through the run the cathode buffer was replaced with cathode buffer lacking Coomassie G-250. Gels were run until the dye front reached the bottom of the gel (~3 hours). After electrophoresis, gels were soaked in Gel Soak buffer (48 mM Tris base, 39mM Glycine, 0.25% SDS) for 30 minutes at 65°C, and transferred using Bio-Rad Semi Dry Blotter at 20V for 1 hour with Semi-Dry Transfer Buffer (48mM Tris, 39mM Glycine, 20%MeOH, 0.1% SDS). Cells were blocked and western blotted as previously described (Coffin , et al., 2001).

2D Gel Electrophoresis.

After resolution in the first dimension (by BN-PAGE) lanes were cut and soaked in 4xSDS sample buffer and allowed to equilibrate at room temperature for 20 minutes heated in the microwave for 20 seconds, and then allowed to incubate for another 10 minutes at room temperature. Each gel lane was then placed horizontally in the well of an SDS-PAGE gel and run as described in the SDS-PAGE section.

Sucrose Flotation assay (Lipid raft)- 721 cells were pretreated with or without 20 mM NEM as described above and homogenized on ice with a Dounce homogenizer (10 strokes) in 1.0 ml of MNE (25 mM MES [morpholineethanesulfonic acid, pH 6.5], 150 mM NaCl, 5 mM EDTA) containing 0.2% Triton X-100. Lyastes were then diluted 1:1 in 80% sucrose (40% final), overlaid with 2.0 ml of 30% sucrose and 1 ml of 5% sucrose, and centrifuged at 4°C for 18 hours in an SW50.1 rotor at 200,000xG. Gradients were harvested from the top of the gradient in 400ml fractions and mixed with 200ml of non-reducing 4xSDS-sample buffer and heated to
85°C for 30 minutes in the presence or absence of 50 mM DTT. Samples were analyzed by SDS-PAGE and Western analysis for LMP-1.

**Size Exclusion Chromatography.**

3x10^8 721 cells were treated with NEM and harvested by centrifugation, washed twice with 1xPBS, resuspended in 2 mLs of Syn 7 Buffer (50 mM NaPO₄, pH 7.4, 150 mM NaCl, 25 mM sucrose, 5% glycerol, 1% Triton X-100), and lysed by vortexing for 45 minutes at 4°C. Insoluble material was removed at 13,000 RPM in a microcentrifuge for 15 min. Lysates were passed through glass wool and loaded onto a HiPrep 16/60 Sephacryl S-400 HR FPLC column (120 mL column volume) and resolved using an ÄKTApurifier chromatography system and were run at a flow rate of 0.5mL/min. Fractions were 0.681mL.

**Baculovirus generation and Flag-LMP-1-6xHis purification.**

*Baculovirus vectors:* Flag-LMP-1-6xHis/pFastBac1 was generated by the addition of a 6xHis tag to the LMP-1 C-terminus in FLAG-LMP-1 by PCR and then the FLAG-LMP-1-6xHis cassette was cloned into pFastBac1 (Invitrogen). Flag-LMP-1-6xHis/pFastBac1 was transformed into DH10 Bac E. coli (Invitrogen) for recombination into its resident bacmid. FLAG-LMP-1-6xHis-bacmid was purified from DH10 Bac cells and transfected into Sf9 cells to generate Flag-LMP-1-6xHis expressing baculovirus. FLAG-LMP-1-6xhis baculovirus stocks were used to infect Sf9 cells and recombinant LMP-1 was purified as described below.

*Recombinant Flag-LMP-1-6xHis expression and purification:* 120 mLs of Sf9 cells at 2x10^6 cells/mL were infected with FLAG-LMP-1-6xHis baculovirus and incubated for 72 hours at 27°C with shaking at 130 revolutions/min. Harvested cells were washed twice with 1xPBS and...
lysed in 4 mL Syn7 lysis buffer (50 mM NaPO4, pH 7.4, 150 mM NaCl, 25 mM sucrose, 5% glycerol, 1% Triton X-100) with 1mM DTT and P8849 Protease Inhibitor Cocktail (Sigma) at 4°C by vortex for 45 min. Lysate was centrifuged in a refrigerated microfuge at 20,000xG for 15 min. Supernatant was filtered through a 0.45μm filter and filtered lysate was purified over a NiNTA column according to manufacturers directions (Qiagen). NiNTA column-purified eluate was then purified over a FLAG M2 affinity column according to manufacturer’s directions except the column buffers contained 0.65% Triton X-100 (Sigma). Column eluate (purified Flag-LMP-1-6xHis) was snap frozen in liquid nitrogen and stored at -80°C until use.

Purification of Flag-LMP-1-6xHis was verified by Silver stain and Western blot for LMP-1.
CHAPTER 5: SUMMARY AND FINAL DISCUSSION

EBV is a human gammaherpes virus that infects over 90% of the adult worldwide population. This virus is successful because of its ability to infect many people and remain latent after primary infection of the host. EBV is also associated with many cancers such as Hodkins disease, Nasophargeal carcinoma, and Burkitt’s lymphoma. EBV’s viral proteins mimic host cell signaling pathways to ensure its immortalization in the host’s memory B cell pool. Activation of cellular signaling pathways results in transformation of primary cells in vitro, enabling them to proliferate indefinitely. Detection of clonal EBV genomes in tumor biopsies is evidence of EBV’s presence at the time of tumorgenesis. Although EBV infection alone is not sufficient for cancer; co-factors such as diet, environmental factors, and immunosuppression are required for EBV associated cancers in vivo.

EBV’s immortalization of B cells in vitro requires expression of latent viral proteins, such as LMP-1. LMP-1 activates NF-κB and JNK signaling pathways from its C-terminus by binding to TNFR-associated factors (TRAFs). LMP-1 activity in this regard mimics that of the TNFR receptor CD40. Unlike CD40 however, LMP-1 is not ligand dependent and is constitutively active. Constitutive activity of LMP-1 is mediated by the LMP-1 TMD. LMP-1 TMD is required for LMP-1 to fractionate with DRMs (lipid rafts). The LMP-1 TMD also confers negative cell cycle regulation and is associated with IFNα suppression.

I approached my thesis with the goal of understanding signaling activities mediated by LMP-1 and the signaling complexes it formed. Previous research demonstrated an essential role for the LMP-1 TMD in suppression of IFNα signaling. My goal was to elucidate the mechanism by which expression of LMP-1 results in underphosphorylation of Tyk2 and suppression of
IFNα signaling. I found that suppression of IFNα signaling by EBV requires the first transmembrane domain (TM1) of LMP-1. Analyzing LMP-1 mutants in different cell lines revealed that although the first transmembrane helix is crucial in suppression of IFNα signaling, TM1 of LMP-1 is not sufficient for suppression in all cell types. My data suggests that NF-κB activation may also be required for this activity, although further experimentation is needed.

The LMP-1 TMD is critical for LMP-1 signaling; because this domain is embedded in the membrane, I believe its contribution to signaling lies in its ability to mediate complex formation (homo and hetero-oligomers). Therefore, I investigated the interaction of LMP-1 with itself and cellular proteins by analysis of LMP-1 complex formation in cellular lysates. I examined this by characterizing high molecular weight species in cellular lysates by non-reducing SDS-PAGE and native complexes by BN-PAGE analysis. An LMP-1 dimer is crosslinked upon oxidation post-lysis forming a high molecular weight LMP-1 dimer in unreduced cellular lysates. The dimer is crosslinked of LMP-1 monomers at cysteine 238. Adjacent LMP-1 monomers (within an LMP-1 oligomer) are crosslinked dimeric species is limited to the fraction of LMP-1 that co-migrates with detergent resistant membranes (DRMs), interacts with TRAF3, and requires the TMD. Therefore, I propose that the crosslinked dimeric form of LMP-1 is a “marker” for the active LMP-1 signaling complex. Specifically, I believe that the LMP-1 dimer is the result of crosslinking adjacent LMP-1 monomers within a higher order complex (for example, a multimer of trimers), whose C-terminal cysteine 238 residues are within crosslinkable distance (3-5 nanometers). Thus, only active, raft associated and oligomerized LMP-1 complexes will give rise to the crosslinked species. The TRAF binding and lipid raft associated form of LMP-1 is a dimer and formation of the LMP-1 dimer is dependent on the LMP-1 TMD.
Examination of lysates from EBV-immortalized cells by BN-PAGE demonstrated LMP-1 forms multiple high molecular weight native complexes. LMP-1 TMD mutants with reduced signaling activity are unable to form these native high molecular weight (HMW) complexes, demonstrating that high molecular weight complex formation by LMP-1 is dependent on LMP-1 signaling. These HMW native complexes are enriched in DRMs fractions, as is “active” LMP-1. This analysis of LMP-1 signaling brings us closer to understanding how the TMD of LMP-1 contribute to potent signaling activity. I will discuss how these findings add to our knowledge of LMP-1 signaling activity, and end with a model of LMP-1 complex formation and signaling activity.

**TM1 of LMP-1 and NF-κB activation are required for suppression of IFNα signaling**

EBV and other viruses activate the host’s immune system upon invasion. IFNα is a cytokine released by the body as part of the innate immune response. IFNα induces interferon-stimulated genes that are both anti-viral and anti-proliferative in nature. EBV’s ability to infect the host and coordinate a successful latent infection relies on the ability to overcome the host’s immune response. Robust IFNα signaling has high potential to alter the outcome of successful EBV infection, an outcome not in the best interest of the virus. Consistent with this notion is our finding that cells expressing LMP-1 respond poorly to IFNα. Suppression of IFNα signaling by LMP-1 was analyzed using a structure function approach. The first transmembrane domain of LMP-1 is required for IFNα suppression. Interestingly, all of the TM1 mutants (LMP-1/TMD1,2 and LMP-1/TMD1,6) retained some NF-κB signaling activity. Not all these mutants, however, retained the ability able to fractionate with lipid rafts, even though both NF-κB and lipid raft association have been shown to rely on TM1 of LMP-1 (Coffin, et al., 2003; J. Lee &
Sugden, 2007; Vishal Soni, et al., 2006; Yasui, et al., 2004). LMP-1 expressed in 293T cells suppressed IFNα signaling (Figure 2-9). However, LMP-1 lost the ability to suppress IFNα signaling without LMP-1 CTARs in 293T cells. Mutants lacking either CTAR1 or CTAR2 retain the ability to LMP-1 suppress of IFNα signaling, but loss of both CTARs results in loss of IFNα suppression (Figure 2-12). This contradicts our earlier findings in B cells that suppression of IFNα signaling does not require LMP-1 C-terminal activating regions (CTARs) (Geiger & Martin, 2006). Both CTAR1 and CTAR2 contribute individually to NF-κB activation via binding to TRAFs (Devergne, et al., 1996; Luftig, Cahir-McFarland, Mosialos, & Kieff, 2001; V Soni, Cahir-McFarland, & Kieff, 2007). These conflicting results suggest that NF-κB signaling does in fact contribute to IFNα suppression by LMP-1, and the difference in behavior of CTAR mutants in the two cell lines (293T vs DG75 B cells) reflects their different NF-κB signaling “environments”. LMP-1 activation of NF-κB is studied in 293T cells because of their very low background levels of NF-κB. The low NF-κB signaling background is desirable to measure stimulation of NF-κB by LMP-1 (signal to noise) and facilitates structure function analysis. However, the NF-κB signaling environment of 293T cells is very different than the signaling background of EBV’s host, the B cell. B cells have high basal levels of NF-κB signaling activity. Therefore in the cellular background of the B cell, the high basal levels of active NF-κB are sufficient and additional stimulation by LMP-1 CTARs is not be required for IFNα suppression. The results thus far suggest that stimulation of NF-κB may play a role of LMP-1 suppression of IFNα signaling. Understanding the role of NF-κB signaling in IFNα suppression requires further experimentation. It is not clear whether the mechanism of IFNα suppression is dependent on, or parallel to, the mechanism by which LMP-1 activates NF-κB. TM1 mutants of LMP-1 (LMP-1/TMD1,2, LMP-1/TMD1,6) retain intermediate abilities to suppress IFNα

122
signaling and activate NF-κB, while LMP-1/TMDN5,6 does not. This is strong evidence that NF-κB activation plays a role in IFNα suppression by LMP-1. While it is possible that high levels of NF-κB signaling are enough to suppress IFNα signaling, previous research demonstrates that NF-κB activation is a mechanism by which IFNα signaling is induced, not suppressed (i.e. Toll like receptor signaling, B-interferon stimulation, IRF-1 and NF-κB induce MHCI) (Drew, et al., 2009; Kawai & Akira, 2007; Lenardo, Fan, Maniatis, & Baltimore, 1989). In addition, decreased constitutive NF-κB activity by IFNα in immortalized B cells mediates anti-proliferative effects in these cells (Rath & Aggarwal, 2001). While increased activation of NF-κB by LMP-1 would limit effects of IFNα on B cells, it is unlikely that reduced NF-κB activity is sufficient to block Tyk2 phosphorylation and suppression of ISRE transcription.

Collectively, our data thus far supports a model in which the TM1 of LMP-1 is essential for suppression of IFNα signaling, in the background of actively signaling NF-κB. NF-κB regulates the expression of a very large number of genes. High NF-κB activity may be required for the transcription of genes or transcription factors required for LMP-1 to suppress IFNα signaling (Figure 5-1). This model relies on downstream activation of NF-κB signaling for LMP-1 to act in suppressing IFNα signaling. Since signaling from either CTAR domain ensures IFNα suppression in cells lacking basal NF-κB activity, it is unlikely that activation of either the non-canonical or canonical signaling pathway intermediates alone is sufficient for IFNα suppression, but rather, that upregulation of downstream κB induced gene products or a transcription factors provides the additional components needed to suppress IFNα signaling. In this regard, it does not necessarily have to be NF-κB activity driven by the expression of LMP-1,
Figure 5-1 Model of LMP-1 mediated IFNα signaling suppression.

LMP-1 activates NF-κB signaling via its cytoplasmic C-terminal TRAF binding domains (CTAR 1,2), and suppresses IFNα signaling. I postulate NF-κB activation is necessary but not sufficient for IFNα suppression. A) 293T cells. LMP-1 suppression is dependent on CTAR1,2 activation of NF-κB. 293T cells have very low basal NF-κB activation. LMP-1 activation of NF-κB contributes to suppression of IFNα signaling. B) B-cells. LMP-1 suppression of IFNα signaling is independent of CTAR1,2 activation of NF-κB activity which contributes to LMP-1 mediated suppression of IFNα signaling.
as B cells with a high signaling background of NF-κB will suppress IFNα signaling in the absence of LMP-1 mediated NF-κB signaling. Further experimentation is necessary to test this model and to fully the mechanism of LMP-1 suppression of IFNα signaling.

**LMP-1 forms a high molecular weight dimer**

Receptor oligomerization commonly initiates signaling in response to ligand binding. LMP-1 functions as a constitutively active receptor that is ligand independent. CD40 signaling mimicked by LMP-1 requires interaction with TRAF proteins following receptor trimerization and raft association. The C-termini of LMP-1 and CD40 interact with a subset of the same TRAF proteins to elicit NF-κB and JNK signaling. Activation of these pathways leads to B cell proliferation, survival, and differentiation (Bishop, et al., 2007; Brodeur, et al., 1997; O Devergne, et al., 1996; Kulwichit, et al., 1998; M. Sandberg, et al., 1997; Uchida, et al., 1999; Xie, et al., 2008). Both LMP-1 and CD40 are believed to signal as oligomers from lipid rafts.

Neighbors LMP-1 monomers, within an LMP-1 oligomer, are crosslinked at cysteine 238 in the C-terminus of LMP-1 upon oxidation of cellular lysates (Figure 3-1). Crosslinking can be blocked by pretreatment of the cells with N-ethyl-maleimide (NEM) before cell lysis (Figure 3-4) demonstrating that the disulfide crosslink was not pre-existing in the cell. Crosslinking experiments in which sulphydryl specific, membrane permeable reagents were added to live cells demonstrated that LMP-1 monomers are “dimerized” prior to lysis. LMP-1 deletion mutants lacking the first four TMDs (LMP-1/TMD5,6) cannot oligomerize, activate NF-κB signaling, or dimerize despite retaining cysteine 238 (Figure 3-7). LMP-1 fractionates to detergent resistant membranes when fractionated by a sucrose step gradient. LMP-1 dimers are
found only in the detergent resistant fraction of this gradient (Figure 3-8). LMP-1 recruits TRAFs to lipid rafts from where it is thought to signal. Co-immunoprecipitation analysis revealed that dimeric, not monomeric, LMP-1 associates with TRAF3 (Figure 3-6).

Furthermore, dimeric LMP-1 was restricted to detergent resistant membranes; indicating LMP-1 monomers within these membrane microdomains are in close proximity to crosslink. When oxidation induced crosslinking was blocked with pretreatment by NEM, LMP-1 was still able to migrate to DRMs, indicating that crosslinking upon oxidation is not sufficient to cause LMP-1 to migrate to the detergent resistant fractions. These results demonstrate that actively signaling LMP-1 complexes contain monomers with close enough proximity to form disulfide crosslinks. Constant with this idea is our finding that nonfunctional LMP-1 mutants do not form these high molecular weight dimers, and presumably do not form active signaling complexes.

The identification of preformed LMP-1 dimers shed light on the nature of the LMP-1 oligomer. Dimeric LMP-1 species are found only in DRMs, suggesting raft-associated LMP-1 is in an oligomeric form while detergent soluble LMP-1 is not. This is consistent with the hypothesis that “active” LMP-1 signaling complexes are oligomerized and raft-associated. Disulfide bond formation between LMP-1 molecules indicates cysteine 238 residues in the cytoplasmic carboxy-termini of two LMP-1 molecules are within angstroms of each other. Cysteine 238 is ~24 amino acid residues downstream from the TRAF binding consensus sequence in CTAR1 (P_{204-XQXT208})(O. Devergne, et al., 1996). The LMP-1 core TRAF3 binding peptide (P_{204-QQATDD210}) binds to the same binding crevice on TRAF3 as does CD40 by X-ray crystallography (Wu, et al., 2005), suggesting that LMP-1 and CD40 interact with TRAF trimers similarly. TRAF2 trimers bind CD40 in the interior of the receptors' trimerized carboxy-terminal TRAF binding domains, with each of CD40's P_{250-VQET254} motifs bound
symmetrically to the external face of a TRAF-C domain of the mushroom shaped TRAF2 trimer (McWhirter, et al., 1999). If trimerized TRAF3 binds to the CTAR1 motifs of trimerized LMP-1 in an analogous fashion, then it is unlikely that cysteine 238 of two adjacent LMP-1 C-termini are within the necessary 3-5 angstrom disulfide-crosslinkable distance of each other. Lack of predicted secondary structure of the cytoplasmic domains of LMP-1 and CD40 suggests these C-terminal domains are relatively "unfolded". The proximity of cysteine 238 to CTAR1, together with the unstructured nature of the cytoplasmic domain, suggests that cysteine 238 is unlikely to form intra-trimer disulfide links.

The findings are consistent with a model in which cysteine 238 of adjacent LMP-1 trimers in a TRAF-binding LMP-1 multimer, or "supercluster", are within disulfide-crosslinkable distance (Figure 5-2). This model assumes that LMP-1 binds TRAFs like CD40. This model supposes that TRAF binding to the interior of the trimer prevents cysteine 238 from crosslinking to cysteine 238 in other LMP-1 molecules within a functional trimer. Instead, cysteines from LMP-1 monomers of adjacent trimers are involved in disulfide crosslinks. This model leads to the possibility that the "core" LMP-1 trimer is localized in the "bulk" membrane and only associates with lipid rafts upon formation of multimeric complexes. Core LMP-1 trimers could organize into a highly ordered supercluster or form an unordered supercluster. The findings that dimeric LMP-1 is only found in the DRMs (Figure 3-8), that HMW native LMP-1 complexes are enriched in DRMs (Figure 4-5), and that a considerable amount of LMP-1 is also found in the detergent soluble membranes are consistent with this model (H Ardila-Osorio, et al., 1999; Hector Ardila-Osorio, et al., 2005; Clausse, et al., 1997; Coffin, et al., 2003; Higuchi, et al., 2001; J. Lee & Sugden, 2007; Yasui, et al., 2004).
Figure 5-2. Model illustrating the hypothetical relationship between LMP-1 trimerization, multimerization, and formation of LMP-1 disulfide-linked dimers.

A) A hypothetical LMP-1 dimer of trimers bound to a TRAF trimer, with the position of CTAR1 and cysteine 238 marked by arrows (based on the model of CD40 cytoplasmic domain bound to TRAF2 trimers (McWhirter, et al., 1999)). LMP-1 is hypothesized to be a trimer in this model based on analogy to CD40 and its interaction with TRAFs. Proximity of cysteine 238 to CTAR1 favors disulfide bond formation between cysteines in adjacent interacting trimers rather than between cysteines with a trimer. The cysteines within the box are predicted to be those crosslinked upon oxidation. B) Hypothetical relationship between LMP-1 monomers, "core" LMP-1 trimers and multimers of the core LMP-1 trimer (superclusters). Circles represent LMP-1 monomers and each trimer of the multimer is distinguished by shading. The black line marks the position of disulfide-crosslinks between cysteine 238 residues in adjacent trimers (in multimers of trimers). Cysteine 238 of adjacent trimers (in the multimer) can be crosslinked by oxidation or by addition of membrane permeable sulfhydryl crosslinkers, thus giving rise to covalently linked dimers upon solubilization in SDS-sample buffer under nonreducing conditions. High molecular weight LMP-1 species are formed upon cross-linking with the amine-reactive crosslinker EGS or the sulfhydryl reactive crosslinker TMEA (Figure 3-5C) or when LMP-1 complexes are resolved by BN-PAGE (Figure 4-1), the latter of which I propose represents the multimerized LMP-1 "supercluster". Core LMP-1 trimers could potentially aggregate in a variety of configurations that lead to multimerization in high molecular weight superclusters. Shown in the figure are two hypothetical configurations of multimerized core trimers, each representing an extreme of possible levels of organization: a less ordered aggregate of trimers (left) and a highly ordered supercluster built by trimeric interactions of core LMP-1 trimers (right). It is predicted that either of these configurations would localize in detergent resistant membrane fractions (lipid raft) microdomains because the LMP-1 crosslinked dimer is found only in the DRM fractions of sucrose gradients and the larger HMW complexes are enriched in those fractions (Figure 3-8; 4-5). Other arrangements with varying degrees of order, or combinations of different arrangements are equally possible.
A) CTAR1

Cysteine 238

B) Unordered LMP-1 Supercluster

Ordered LMP-1 Supercluster

- Cysteine 238 disulfide crosslink
- LMP-1 monomer
- LMP-1 trimer
- LMP-1 dimer of trimers
- LMP-1 trimer of trimers
This model raises many questions: To what extent does the hypothetical core LMP-1 trimer have signaling activity? What is the relationship of this signaling to LMP-1 signaling from raft-associated superclusters? What are the sequence requirements for formation of the core LMP-1 trimer? Are requirements for forming the trimer distinct from those resulting in the formation of the LMP-1 supercluster? Does LMP-1 actively promote clustering of microdomains to potentiate its own signaling? Answers to these questions will reveal the mechanism underlying LMP-1 constitutive signaling activity.

**High Molecular Weight Native LMP-1 Complexes**

The pleiotropic signaling activity of LMP-1, together with the crosslinking results (Chapter 3), prompted a characterization of native LMP-1 complexes in EBV-immortalized B cells. I found that solubilized extracts from 721 cells contain multiple high molecular weight complexes resolvable by BN-PAGE (Figure 4-1), and corresponding high molecular weight complexes are resolvable by size exclusion chromatography (Figure 4-4). Complexes visualized by BN-PAGE contain monomeric LMP-1 as shown by 2D-PAGE (BN-PAGE 1st dimension; SDS-PAGE 2nd dimension) and are specific for LMP-1 (Figure 4-3). Purified LMP-1 and nonfunctional TMD deletion mutants are unable to form the larger higher molecular weight complexes (Figure 4-6,4-7). The largest of the high molecular weight complexes (>669kDa) are enriched in DRMs, whereas the smaller complexes (<669kDa) are enriched in the detergent soluble membranes (Figure 4-5). The smaller complexes are similar to the complexes formed by TMD mutants of LMP-1 that are deficient in signaling activity (Table 5-1).

These findings support a model in which LMP-1 assembles to form large active signaling complexes upon association of all of its constituents (Figure 5-3). This model is formed based on observations from partially functional LMP-1 TMD mutants. The predominance of large high
Table 5-1 Summary of signaling activities of TMD mutants of LMP-1.
Table displays findings of LMP-1 TMD mutants presented in Chapters 2, 3, and 4 of Thesis.
Legend: -, no activity; +, low activity; ++, intermediate; +++, wildtype activity.

<table>
<thead>
<tr>
<th>Signaling Activity</th>
<th>LMP-1</th>
<th>LMP-1/ TMD1,6</th>
<th>LMP-1/ TMD1,2</th>
<th>LMP-1/ TMD5,6</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMW complex formation</td>
<td>++++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lipid Raft Association</td>
<td>++++</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Homo-oligomerizes</td>
<td>+++++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Binds TRAFs</td>
<td>++++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Suppresses IFNα Signaling</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Activates NF-κB</td>
<td>++++</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 5-3 Hypothetical relationships between LMP-1 complex formation and signaling complexes.

**Left:** *Category I:* Monomeric, non-raft associated LMP-1 typified by TM deletion mutants LMP-1/TMD1,6 and LMP-1/TMD1,2. This class demonstrates intermediate NF-κB and IFNα suppression activity. Is unable to oligomerize or localize to rafts. I envision monomeric LMP-1 in bulk membrane to have reduced NF-κB activating capacity and intermediate IFNα suppression activity (See Table 5-1).

**Right:** *Category II:* Higher order, LMP-1 signaling platforms associated with lipid rafts. These type II complexes are represented by fully functional, oligomerized, and raft associated LMP-1. I envision these complexes to be varied in size due to variability in number of core LMP-1 trimers and cellular components, and thus to vary in terms of signaling output.
molecular weight complexes are observed in cellular lysates and the migration of these large complexes to DRMs is in agreement with the formation large signaling complexes representing fully active LMP-1. This model is congruent with evidence that activation of IFNα signaling and dimerization of LMP-1 relies on the TMDs to create this ‘active’ LMP-1 signaling platform. This platform starts with the basic core LMP-1 unit that has been demonstrated to signal and builds to the fully active higher-order LMP-1 signaling complex based on characterized signaling activities at this point.

TMD deletion mutants retaining the first transmembrane domain of LMP-1 and C-terminal signaling domain (LMP-1/TMD1,2; LMP-1/TMD1,6) fall into category I (monomeric complexes). These mutants are deficient in NF-κB activation but still retain intermediate levels of activation. If these mutants are expressed at high enough levels they can activate NF-κB to wild type levels. Because these mutants do not oligomerize, bind TRAFs, and are compromised in raft association, I believe their NF-κB activity differs mechanistically from that of full length LMP-1. Importantly, this class of mutant retains IFNα suppressing activity. Activities of these mutants revealed that that TM1 per se is not sufficient for targeting LMP-1 to DRMs. Suggesting that TM1, in conjunction with TM2, is required for raft targeting. Importantly, the ability to migrate to lipid rafts is not required for the residual NF-κB activation and suppression of IFNα signaling of these mutants.

Category II represents fully functional, higher order LMP-1 complexes in lipid rafts. These complexes are comprised of “core” LMP-1 trimers that have gained the ability to migrate to lipid rafts and interact with varying combinations of cell proteins to form active platforms. I believe that the ability to crosslink LMP-1 dimers in intact cells actually reflects the association of core trimers within the lipid raft. The fact that the crosslinked species is a dimer and not a
trimer simply reflects that fact that LMP-1 monomers within a core trimer are not sulfhydryl
crosslinkable, whereas LMP-1 monomers in adjacent core trimers are. Hence, crosslinked
dimers reflect the association of two LMP-1 trimers. Data from BN-PAGE analysis mutants and
recombinant LMP-1 support this model.

Conclusion

LMP-1 contribution to B cell immortalization demonstrates its importance and role in
EBV-associated tumorgenesis. EBV associated malignancies are a great threat to
immunosuppressed populations such as those infected with HIV/AIDS and malaria. LMP-1 is
oncogenic and a potent activator of signaling. In order to design approaches to disrupt LMP-1
signaling it is essential to understand how LMP-1 activation differs mechanistically from the
receptor it mimics, CD40. My research focuses on understanding how LMP-1 activation results
in potent and varied signaling outputs. The striking finding is the absolute requirement for the
TMDs in all LMP-1 activities. The TMDs of LMP-1 are largely uncharacterized with respect to
their role in LMP-1 signaling activation (Figure 5-4). The goal of my thesis was to characterize
native LMP-1 signaling complexes and determine how specific complexes mediate downstream
outputs. I found LMP-1 forms very large HMW signaling complexes that correlate with
signaling activity. While I couldn’t associate particular signaling outcomes to particular
signaling complexes, I did find that these large signaling complexes are dependent on the LMP-1
TMD for their formation. Signaling competent LMP-1 can form these large complexes whereas
signaling “incompetent” LMP-1 cannot. Further research elucidating mechanisms of LMP-1
activation could generate powerful LMP-1 drug targets. My results demonstrated that the LMP-1
Figure 5-4 LMP-1 TMD mediates LMP-1 complex formation leading to greater signaling potential.
The results presented describe a model of hierarchical signaling potential by LMP-1, with NF-κB and IFNα signaling at the base of the pyramid, easily activated or inhibited by LMP-1. Increased signaling potential by LMP-1 require uncharacterized activity from its TMD. As activity of LMP-1 mutants reaches that of wildtype LMP-1, their signaling potential increases, ultimately cumulating in robust NF-κB signaling and HMW complex formation. The robustness of LMP-1 signaling activity is associated with the TMDs as transmembrane deletion mutants are not active and are very low in signaling potential. Disruption of LMP-1 TMD could be an exciting potential target to disrupt LMP-1 signaling activity potential. Reduced signaling potential could weaken the oncogenicity of LMP-1, reducing the frequency of EBV-associated malignancy.
TMD are essential for both suppression of IFNα signaling and LMP-1 ability to form high molecular weight complexes. Understanding how LMP-1 complexes form, how multiple high molecular weight LMP-1 complexes differ from each other, and identifying the signaling outputs of each complex will contribute greatly to our understanding of how LMP-1 functions as a potent and oncogenic activator of signaling.
Works Cited


