Recombinant Expression and Purification of Latent Membrane Protein 1 of Epstein-Barr Virus: Potential Insights Into Oligomerization and Lipid Raft Association

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Abstract  
The presence of Epstein-Barr virus (EBV) and the expression of viral proteins in certain tumor cells suggest that the virus (EBV) contributes to the development of malignancies. The viral protein, latent membrane protein 1 (LMP1), functions as a tumor necrosis factor receptor analog, and is expressed in EBV associated cancers. Insufficient information is known about LMP1’s oligomerization and lipid raft association to induce cellular signaling. Understanding the functioning of LMP1’s activation in tumor cells is imperative for designing an approach to interfere with LMP1 signaling. To further study this protein, recombinant LMP1 was expressed from baculovirus-infected Sf21 cells. Recombinant LMP1 fractionated in membrane fractions, suggesting that it is folded correctly, and was successfully purified using a poly-histidine tag. Upon large-scale purification, LMP1 will be crystallized and analyzed by x-ray diffraction to determine its structure relationships. LMP1 structural information can be used to determine the oligomerization of LMP1 and give insights into how LMP1 is associated with lipid rafts. Understanding LMP1 complex formation will aid in the discovery of novel therapeutic strategies to block EBV-associated cancer growth through targeting LMP1.

Introduction  
The Epstein-Barr virus (EBV) is a human gamma herpesvirus associated with tumor development. EBV is the cause of infectious mononucleosis, and has also been found to contribute to carcinomas and lymphomas related to immunodeficiencies. New investigations have also found EBV to increase the risk of developing other diseases such as multiple sclerosis (Levin et al., 2011). EBV contributes to tumor cell growth and survival through infection involving a group of latent proteins. The viral signaling of a particular latent protein, latent membrane protein 1 (LMP1), is required to establish viral infection and immortalize cells in some EBV-related cancers and diseases. The work of this research is to understand LMP1’s structural and functional roles in relation to the development of EBV associated cancers and diseases.

Figure 1: Epstein-Barr virus life cycle  
Epstein-Barr virus infects B-cells through bodily fluids in the primary infection or by virus released within the body. EBV enters B-cells by fusion with the membrane after endocytosis. Latently infected B-cells do not express lytic viral proteins until they reactivate. Lytic infection causes gene expression of proteins and viral infection to other cells. During life-long infection, the virus will cycle between latent and lytic depending on immune system pressures.

*This author wrote the paper as a senior project under the direction of Alexander Shingleton*
Infectious Mononucleosis

The symptoms of mononucleosis include fatigue, swelling of the lymph nodes, pharynx, and tonsils, and fever. Most patients infected with infectious mononucleosis usually improve within two to four weeks, though a small proportion will experience fatigue for six months or more (Longnecker et al., 2013).

Various techniques have been adapted to detect EBV infection. A mononuclear spot test is a form of the heterophile antibody test used to detect EBV infection. A heterophile antibody specific to EBV, EBV immunoglobulin M (IgM) antibody, is used because the IgM antibody is present during the first two to three months of the illness. Antibodies for the Epstein-Barr virus nuclear antigens (EBNAs) can also be useful because they are expressed three to six weeks after illness and remain present for life (Longnecker et al., 2013). Treatment of mononucleosis is merely supportive. Anti-inflammatory drugs are administered if swelling becomes uncomfortable or obstructs airways.

Burkitt’s Lymphoma

Burkitt’s lymphoma (BL) is relatively rare, but accounts for 30-50% of lymphomas in children in Africa (Harris & Horning, 2006). BL has been identified as an EBV-positive tumor that occurs in equatorial Africa, climates in which malaria is also an endemic (Burkitt, 1958; Epstein et al., 1964). B-cell proliferation associated with malaria is thought to increase EBV potency and increase the risk for BL development (Longnecker et al., 2013). BL results from a translocation of the myc oncogene. The three most common translocations involve a breakpoint in the long arm of chromosome 8 at the 8q24 locus, adjacent to the first coding exon of the myc gene (Allday, 2009). The most frequent translocation (occurring in 80% of Burkitt lymphoma cases) transposes the telomeric region of chromosome 8 and the immunoglobin heavy chain gene on chromosome 14. The remainder involves either the translocation of chromosome 8 and chromosome 2, or chromosome 8 and chromosome 22 (Klein, 1981).

As a result of the translocation, myc becomes an active oncogene. The effects of the translocation involving the immunoglobin chains in mature B-cells cause the myc gene to constitutively express myc proteins. The levels of the proteins are higher than seen in normal B-cells, which causes tumor proliferation (Allday, 2009). How the myc gene becomes activated after the translocation in relation to EBV infection is still an area of investigation. Changes in myc levels that activate and repress specific sets of direct target genes of myc-transformed tumor cells indicate that myc levels fluctuate when active (Repellin, Tsimbouri, Phibey, & Wilson, 2010). Treatment for BL involves high doses of radiation, chemotherapy, and surgery to remove the tumor if severe enough.

Hodgkin’s Lymphoma

Hodgkin’s lymphoma (HL) results from white blood cells in the lymphatic system growing abnormally large. These giant malignant cells, called Reed-Sternberg cells, form tumor masses in swollen glands. Individuals with a history of infectious mononucleosis have been linked to a greater risk of HL (Longnecker et al., 2013). HL affects a wide range of individuals with various backgrounds as notable cases include: Mario Lemieux (NHL hockey player), Paul Allen (co-founder of Microsoft), and Flip Saunders (NBA head coach). Most EBV-positive Hodgkin’s lymphomas occur later in life when viral infection can cause long-term consequences. EBV latent proteins are present in transformed Reed-Sternberg cells, but the contribution of EBV is still under investigation.

Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma (NPC) is predominately associated with South East Asia (Khalli & Jeang, 2009). NPC affects epithelial cells of the pharynx behind the nasal passages. It is believed that NPC can result from lifestyle factors as well as genetics. South Asian Cantonese cultures, in particular boat people, include large amounts of salted fish in their diet. The fish are pickled and develop high levels of N-nitrosamines, chemicals that are known carcinogens (Longnecker et al., 2013).

While in the latent phase, EBV has been found to produce small amounts of the virus to allow replication in the epithelial cells of the oral cavity that can be passed to the pharynx. Levels of latent membrane protein 1 (LMP1) maintain epithelial cells in an undifferentiated state, while
latent membrane protein 2 (LMP2) promote growth in NPC. The role of EBV in NPC development is still under investigation, but the presence of the Epstein-Barr virus RNAs (EBERs), Epstein-Barr virus nuclear antigen 1 (EBNA1), and latent membrane proteins LMP1 and LMP2s have been detected in NPC (Longnecker et al., 2013). Treatment for NPC involves surgery, chemotherapy, and radiation therapy. EBV latent proteins are being targeted to apply immunotherapeutic strategies (Cao et al., 2014; Khanna, Moss, & Gandhi, 2005).

Gastric Carcinoma
EBV-associated gastric carcinoma (GC) occurs in approximately 10% of all GCs. Infected stomach cells express EBNA1, EBERs, and LMP2A. GC was first associated with EBV in 1990 when EBER1 was detected using PCR (Iizasa et al., 2012). Epstein-Barr virus nuclear antigen 1 (EBNA1) expression was found to reduce apoptosis in epithelial stomach cells (Dambaugh, Hennessy, Chamnankit, & Kieff, 1984). How EBV infects epithelial cells remains unclear, but once infected, cells are mediated by cell-to-cell contact to shed and release viral particles (Longnecker et al., 2013).

Multiple Sclerosis
The lifetime risk of multiple sclerosis (MS) is increased with a history of infectious mononucleosis or primary infection of EBV. Viral antibodies of EBNA1 were found to increase before the onset of MS (Levin et al., 2011). While EBV DNA has not been found in the brain, Epstein-Barr virus RNAs (EBERs) and LMP1 have been detected in B-cells located in white brain matter. The role of EBV, like many other diseases and cancers, still remains unclear.

Other Diseases
The link between EBV and various other diseases are still under investigation, such as rheumatoid arthritis, chronic lymphocytic leukemia, systemic lupus erythematosus, and AIDS (Longnecker et al., 2013).

Epstein-Barr Virus Latent Gene Products
The genetic template of EBV determines the behavior of the virus and its ability to infect B-lymphocytes and epithelial cells. The viral transcripts expressed provide the fundamental ability to cause disease. EBV exploits normal B-cell maturation pathways to establish latent infection by a series of viral expression designs that modify host behavior. Understanding how the latent gene products act, such interfering with cell growth signaling, is important to comprehending the biology of EBV diseases. A summary of EBV-associated diseases and gene products can be found in Table 1.

EBNA1
Epstein-Barr virus nuclear antigen 1 (EBNA1) was the first EBNA discovered, and arguably the most important because it prevents the EBV genome from becoming lost by cell division in latently infected cells (Rowe et al., 1987). The protein binds to chromosomal DNA during mitosis to ensure that copies of replicated viral DNA are shared equally between dividing cells. The transcription of EBNA1 is also essential for transformation of B-cells. Antibodies against EBNA1 were used to first discover EBV association with Burkitt’s lymphoma (Longnecker et al., 2013).

EBNA2
Epstein-Barr virus nuclear antigen 2 (EBNA2) is needed for B-cell transformation, and helps activate the other nuclear antigens and latent membrane products (Dambaugh et al., 1984). EBNA2 has also been found to increase transcription of cellular genes such as CD21 and c-fgr (Longnecker et al., 2013).

EBNA3a, B, and C
The Epstein-Barr virus nuclear antigen 3’s (EBNA3a) play a role in regulating LMP1 and LMP2 transcription (Kashuba et al., 2000). EBNA3A, B, and C have similar roles in B-cell growth, and work together to control RBP-Jκ, which monitors transduction pathways to activate transcription (Longnecker et al., 2013).

Latent Membrane Proteins
LMP1
The viral onconeural LMP1 is required to establish EBV infection and contributes to the nature of most EBV diseases (Ahsan, Kande, Nagashima, & Takada, 2005). LMP1 is transcribed from two different promoters separated by 600 base pairs, and encoded in B-cells by a 2.8 kb mRNA that interacts with EBNA2 (Longnecker et al., 2013). The half-life of LMP1 is fairly short, but the protein accumulates at high levels to establish latency by acting as a CD40 tumor necrosis factor receptor mimic (Hatzi-cassilou et al., 1996; Kilger, Kieser, Baumann, & Hammerschmidt, 1998). LMP1 is constitutively active, while CD40 acts as a ligand-dependent receptor protein found on antigen presenting cells, and interacts with adaptor proteins called tumor necrosis factor receptor-associated factors (TRAFs). Tumor necrosis factors are cytokines that trigger apoptosis and lead processes associated with cell survival. CD40 binds with its trimeric ligand CD40L and forms homo-trimers that drive downstream proteins and kinases such as nuclear factor kappa B (NF-kB), c-Jun N-terminal kinase (JNK), mitogen-activated protein kinases (MAPK), and phosphatidylinositol 3-kinase signaling from TRAF interactions (Adriaenssens et al., 2004; Mitchell & Sugden, 1995). CD40L is thought to move towards lipid rafts to become membrane associated and interact with the TRAFs (Kaykas, Worringer, & Sugden, 2001). LMP1 mimics CD40 by binding TRAFs from two C-terminal activating regions (CTAR1 and CTAR2), located on its carboxy-terminal tail (Figure 3). The CTARs bind TRAF1, TRAF2, TRAF3, and TRAF5, which are essential in facilitating immune and inflammatory responses (Higuichi, Izumi, & Kieff, 2001; McWhirter et al., 1999; Schneider et al., 2008). The roles of the TRAFs are to couple cell surface receptors with other cellular proteins and kinases, such as NF-κB and JNK, and to activate signaling pathways. LMP1 thus mediates B-cell transformation through signal transduction (Kilger et al., 1998; Rastelli et al., 2008). While it is known that LMP1 affects downstream factors, how these factors are recruited to LMP1’s signaling complex is still a matter of investigation.

Table 1: Epstein-Barr virus associated diseases and gene products. EBV associated cancers and diseases with correlating latent genes involved. Latent gene products differ between diseases. EBERs and EBNA1 are present in all diseases. EBNA1 ensures the viral genome replicates correctly in cell division. Latent membrane proteins are thought to vary between diseases, but still play an important role in B-cell transformation and survival.

Epstein-Barr Virus RNAs
Epstein-Barr virus RNAs (EBERs) are non-coding RNAs that are abundantly expressed in latently infected cells (Longnecker et al., 2013). The EBERs promote cellular transformation in Burkitt’s lymphoma, causing the cells to become resistant to apoptosis (Iwakiri & Takada, 2010; Takada & Nanbo, 2001). The two small RNA molecules, EBER1 (166 nucleotides) and EBER2 (172 nucleotides) are expressed at different levels (Glickman, Howe, & Steltiz, 1987). The EBERs localize to the cell nucleus and have been found to inhibit apoptosis (Takada & Nanbo, 2001).

Epstein-Barr Virus Nuclear Antigens
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Figure 3: Latent membrane protein 1 model. Latent membrane protein 1 contains a short N-terminal tail, a six-fold transmembrane domain, and a large C-terminal tail with two C-terminus activating regions (CTARI & CTAR2).
Latent Membrane Protein 1 Function and Structure.
Latent membrane protein 1 is a 62-kD integral membrane protein expressed in EBV-associated tumors such as Hodgkin’s lymphoma and nasopharyngeal carcinoma (Longnecker et al., 2013). LMP1 constitutively mimics the signaling of B-cell CD40 tumor necrosis factor receptors. CD40 is an oligomeric raft-associated single spanning membrane protein with an extracellular ligand binding domain and a cytoplasmic signaling domain (Figure 4). LMP1 differs dramatically in its structure from CD40 as LMP1 has six membrane spanning domains as opposed to CD40’s one. LMP1 also differs from CD40 in its apparent independence of a ligand for signaling (Longnecker et al., 2013). CD40’s ligand membrane receptor facilitates B-cell interaction with helper T cells, which then bind and activate downstream factors such as NF-κB (Kaykas et al., 2001; Kilger et al., 1998).

LMP1 is 386 amino acids long and has three structural domains: the N-terminus (aa 1-24), the six-polytopic membrane-spanning domains (aa 25-186), and the C-terminus (aa 187-386). LMP1 is believed to consist of a six-fold transmembrane domain across the plasma membrane, but this claim has yet to be confirmed. The amino-terminal and carboxyl-terminal are on the cytoplasmic side of the plasma membrane. The N-terminal is short, but essential to tethering the protein to the cytoplasmic side of the plasma membrane (Longnecker et al., 2013). On the other hand, the C-terminus is long and contains terminal activating regions CTAR1 and CTAR2 (Figures 3 and 4).

The transmembrane domain is believed to play a crucial role in signal facilitation, oligomerization, and lipid raft association (Clausse, et al., 1997; Higuchi et al., 2001). Removal of the first transmembrane domain disrupted CTAR signaling, suggesting proper orientation and anchoring of LMP1 is mandatory for LMP1 functioning (Li & Chang, 2003). The first two transmembrane domains were found as a loop exposed to the extracellular space (Coffin, Geiger, & Martin, 2002; Martin & Sugden, 1991). Experiments have also interchanged the C-termini of LMP1 and CD40 without signal disruption (Fioettmann & Rowe, 1997; Hatzivassiliou et al., 1998). LMP1’s ability to modify survival signaling is why it is considered a viral oncogene. LMP1 is therefore an attractive target to design therapeutic treatments against EBV diseases that express LMP1.

Latent Membrane Protein 1 Lipid Rafts and Oligomerization
Lipid Rafts
Plasma membranes create a barrier that separates the material within the cell from the extracellular environment. These membranes do not create an impermeable barrier, however, materials can get in and out of the cell, allowing signals to be relayed between the outside of the cell and the inside. Such signals can interact with proteins bound to the membrane. One way for proteins to become bound to the membrane is by aggregation in lipid rafts (Harder, Scheiffele, Verkade, & Simons, 1998). Lipid rafts are specialized regions in the cell membrane that contain lipids and cholesterol to compartmentalize cellular signaling molecules such as protein receptors (Gajate & Mollinedo, 2015; Lingwood & Simons, 2010). Since the cell membrane is bilayered, the membrane fluidity can be adjusted by associating sphingolipids and cholesterol into the membrane (Lingwood & Simons, 2010). These regions form drifting microdomains in the membrane with specific lipid and protein compositions. Lipid rafts can organize receptor proteins and their downstream molecules that play a role in modulating the cell cycle and programmed cell death (Hryniewicz-Jankowska et al., 2014).

LMP1 constitutive signaling has been linked to its ability to localize in glycosphingolipid-rich lipid rafts (Ardita-Orsorio et al., 1999; Clausse et al., 1997), but the mechanism by which LMP1 localizes in lipid rafts is still a matter of investigation. Studies have indicated that LMP1’s first two transmembrane domains encode a lipid raft targeting signal and information (Coffin et al., 2003). The lipid raft signal is important because the transmembrane domain is believed to be integral for downstream NF-κB signaling (Higuchi et al., 2001; Kaykas, Worringer, & Sugden, 2002; Yasui et al., 2004). Recent results support the notion that raft-associated LMP1 in oligomeric complexes bring the C-terminal of adjacent LMP1 molecules together to allow crosslinking to occur (Wrobel et al., 2013). The first 25 amino acids of LMP1’s N-terminal region were also found to play a role in lipid raft association and the binding of other cellular components (Rothenberger et al., 2001). LMP1’s transmembrane domain raft association is unique in that it can have enhanced Effects when fused together with CD40. If the raft-associated LMP1 N-terminal signaling domain is joined with CD40’s C-terminus, an enhanced affinity for TRAFs is observed (Kaykas et al., 2001).

Disruption of lipid rafts disturbs the association between TRAF2 and TRAF3 with CD40, suggesting that lipid rafts play a critical role in initiation of CD40 signaling (Vidalain et al., 2000). Since LMP1 and CD40 act in similar fashions, lipid raft disruption of LMP1 could possibly alter signaling in tumor development. Membrane rafts serve as a place of aggregation for signaling proteins, therefore, disrupting lipid rafts may provide a way to alter signaling pathways. Understanding LMP1 lipid raft formation could provide insights into the structure assembly mechanisms involved in malignant transformation.

![Figure 4: Latent membrane protein 1 versus CD40 signaling.](image)

Oligomerization
The process of oligomerization causes two or more polypeptide chains to form high ordered structures. Proteins can either homo-oligomerize (contain the same monomers) or hetero-oligomerize (contain two or more different monomers) through covalent, electrostatic, and hydrophobic interactions or hydrogen bonds (Goodsell & Olson, 2000; Gotte & Libonati, 2014). Protein oligomerization can occur by naturally forming covalently linked species, from active covalent complexes starting from inactive monomeric precursors, or through free cysteines of two subunits to form intermolecular disulfides (Gotte & Libonati, 2014). Protein oligomerization is often important in functional control and activating physiological pathways.

Receptor oligomerization has been linked to the activation process of tyrosine kinase receptors (Tartaglia & Goeddel, 1991). Structural studies of CD40 and tumor necrosis factor receptor (TNFR) oligomerization have demonstrated that they assemble into homotrimeric structures (Chan et al., 2000). The TNFRs appear to function as a group rather than
as individual receptor, and allow ligand-induced receptor signaling to initiate. The TNFR group is therefore believed to oligomerize and assemble before binding to the lipid raft domains as ligand independent structures (Chan et al., 2000; Clancy et al., 2005).

Oligomerization is thought to increase TRAFs binding affinity, similar to how hemoglobin’s four-subunit structure allows it to increase its affinity towards O2. TNFR’s affinity for receptors has been found to increase from the monomeric form to the trimeric form (Haswell, Glennie, & Al-Shamkhani, 2001; Pullen, et al., 1999).

The greatest TNFR bioactivity is thus seen in trimers. In ligand-induced CD40 (CD40L), the trimeric form causes conformational changes in its CD40L complex to increase its affinity for the tumor necrosis receptor associated factors and lipid raft establishment (Kaykas et al., 2001; Pullen, et al., 1999). NF-κB activation was found to increase 20-fold when CD40 was trimerized (as opposed to its monomeric form) (Kaykas et al., 2001). Crystal structures of TRAF2 have demonstrated that the trimeric structure contributes to NF-κB signaling (McWhirter et al., 1999).

Due to the fact that LMP1 is constitutively active (unlike CD40, the protein it mimics), understanding the constitutive activation is important for designing strategies to interfere with LMP1 signaling. Oligomerization of LMP1 is believed to be essential for both its incorporation into plasma membrane lipid rafts and the activation of cellular signaling. Oligomerized TRAFs bind to trimerized TNFRs and, by analogy, LMP1 is thought to bind to TRAFs as a constitutive oligomer (McWhirter et al., 1999). LMP1 is presumed to homo-oligomerize with other LMP1 polypeptides and activate as an oligomer (Coffin et al., 2002; Higuchi, et al., 2001).

LMP1’s six transmembrane domains are thought to play many roles in the protein’s ability to oligomerize and localize in membrane micro-domains. Oligomerization can increase lipid raft affinity and modulate raft components (Harder et al., 1998). LMP1’s transmembrane domains have a high level of intrinsic stability; therefore, each domain can be studied as independent domains in structural analysis (Sammond et al., 2011). LMP1’s transmembrane domains 1 and 2 were determined to contain a lipid raft signal that determines proper orientation for lipid raft association (Coffin et al., 2002; Kaykas et al., 2001). The greatest TNFR bioactivity is thus seen in trimers. In ligand-induced CD40 (CD40L), the trimeric form causes conformational changes in its CD40L complex to increase its affinity for the tumor necrosis receptor associated factors and lipid raft establishment (Kaykas et al., 2001; Pullen, et al., 1999). NF-κB activation was found to increase 20-fold when CD40 was trimerized (as opposed to its monomeric form) (Kaykas et al., 2001). Crystal structures of TRAF2 have demonstrated that the trimeric structure contributes to NF-κB signaling (McWhirter et al., 1999).

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The expression system was therefore changed to the insect cell system. However, if weak expression had been detected, the final two corrected mutagenesis sites would have been replaced and cloned into LMP1 DNA.

**Baculovirus/Insect Cell Expression**

Preliminary experiments indicate that LMP1 protein was expressed from recombinant baculovirus in Sf21 cells. Histidine-tagged LMP1 purification was achieved from fractioned membranes using a metal affinity resin. The SDS-PAGE Western blot analysis of LMP1 indicated correct expression of LMP1. LMP1 fractions analyzed by SDS-PAGE clearly indicate that LMP1 can be purified, as shown in Figure 10. The overall LMP1 recovery decreased over each fractioned step, but large-scale expression should increase the amount of purified protein and allow sufficient amounts for crystallization.

**Discussion**

This study is still ongoing in an attempt to determine LMP1’s structure and function. Although LMP1 oligomerization and lipid raft association analysis has yet to be conducted, this study is the first of its kind, involving novel expression and purification of full-length LMP1.

LMP1 expression was attempted with E. coli through recombinant techniques and IPTG induction. Rosetta and RIPL strains are designed to enhance the expression of eukaryotic proteins, but LMP1 was found to exhibit poor expression efficiency. This poor expression was thought to result from the Homo sapiens-E. coli codon bias frequency or incorrect protein folding from post-ribosomal translation. Six of the eight codon corrected sections were replaced and cloned into the LMP1 DNA sequence. Each designed site-directed mutagenesis block corrected 6-7 codons. LMP1 expression was investigated after each section (Figure 8). Increased expression should have been detected, but failed expression was continuously observed; thus, subsequent studies switched to eukaryotic expression.

Many eukaryotic proteins do not fold properly in E. coli and form insoluble inclusion bodies. Protein folding in eukaryotic cells is a complex process, and molecular chaperone machinery is often needed to assist with folding (Hartl & Hayer-Hartl, 2002). It is possible to resolubilize proteins from inclusion bodies or improve solubility by expressing the protein at lower temperatures. Due to the fact that we failed to see any LMP1 expression, it was not necessary to continue expression experiments with various temperatures.

The detection of LMP1 from the insect cells demonstrated that the protein could express at high enough levels to be purified and crystallized. Recombinant LMP1 fractionated in membrane fractions, suggesting that it is in a stable form and folded correctly (Figure 10). The next steps in the process include large-scale purification, which will allow us to crystallize LMP1, and expression with mutant versions of LMP1. The mutant LMP1 versions are LMP1-A5 (a modified version of LMP1 containing 5 alanine residues at positions 204-208, which mutate CTAR1 and the TRAF-binding domain from PQQAT to AAAAA), LMP1-1:220 (amino acids 1 to 220, CTAR2 deleted), and LMP1-1:220A5 (CTAR1 mutated, CTAR2 deleted). X-ray diffraction will be used to understand the structure and function of LMP1 and LMP1 mutants. Such knowledge may be useful in attempting to inhibit cellular pathways that lead to EBV-associated cancers.
Conclusion and Future Studies

Upon large-scale purification, LMP1 and LMP1 mutants will be crystallized and analyzed by x-ray spectroscopy in order to determine their structural relationships. LMP1 structural information can be used to determine the oligomerization of LMP1 and give insights into how LMP1 is associated with lipid rafts. Understanding LMP1 complex formation will aid in the discovery of novel therapeutic strategies to block EBV-associated cancer growth through targeting LMP1.

Materials and Methods

Bacterial Expression

Plasmids

The pET-19B vector (Novagen) was used throughout the experiment (Figure 11). pET-19B-LMP1 site-directed mutagenesis was created using PCR primers that were codon optimized for bacterial expression.

Each codon-optimized block contained a restriction enzyme site used to verify that each block was correctly cloned into the vector. The LMP1 DNA was digested with the corresponding restriction enzyme, purified (Qiagen PCR Purification Kit), ligated, and transformed into competent E. coli. Double-stranded oligonucleotides encoding each codon-optimized block were obtained from Integrated DNA Technologies.

Original sequence and primers for site-directed mutagenesis

<table>
<thead>
<tr>
<th>Block</th>
<th>Original Sequence</th>
<th>For Primer</th>
<th>Rev Primer</th>
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<tr>
<td>PR</td>
<td>5’-cggccccctcgaggaccccccctctctccctaggtctctc-3’</td>
<td>PR-for: 5’-cgTccGcAcgTggCcggctctctctggccctctc-3’</td>
<td>PR Rev: 5’-gggtcctctgaggggccgtcgcggccccgggtggccctctcaggtcgtg-3’</td>
</tr>
<tr>
<td>GG</td>
<td>5’-gtgactggacGcAgGgctctctttgctctcatgcttataaatctaatGtctatccctggtgagag-3’</td>
<td>GG-for: 5’-gtgactggacGcAgGgctctctttgctctcatgcttataaatctaatGtctatccctggtgagag-3’</td>
<td>GG Rev: 5’-gagggccGaccGgcGctcatctcaaagagtacagcgccaaacag-3’</td>
</tr>
</tbody>
</table>
| RR    | 5’-gctatcacttggagctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctctgctctct
**Sequenceing**
The Genomics Core Facility at Northwestern University sequenced and purified pET-19B-LMP1 after each codon-optimized block was complete. A sample sequence of the GG block can be observed in Figure 8.

**Cells**
SCS 110 competent E. coli were used for transformation onto Luria-Bertani (LB) plates containing 34 mg/mL of chloramphenicol. Plates were incubated overnight at 37°C Rosetta (Novagen) E. coli cells were used for protein expression in 200 mL of LB containing 34 mg/mL chloramphenicol and 100 mg/mL ampicillin. Cells were grown at 37°C to an OD600 between 0.6-1.0 and induced with 0.4 mM IPTG.

**Western Blotting**
Cells were harvested once they reached an OD600 between 0.6-1.0 by centrifugation 12,000 x g for 10 minutes. Cell pellets were lysed on ice with radio immunoprecipitation assay buffer (10 mM Tris-HCl, pH 8/0, 140 mM NaCl, 1% Triton X-100, 0.1% sodium dodeyl sulfate (SDS), 1% deoxycholic acid, protease and phosphatase inhibitors (Pierce)). Cell lysates were clarified by centrifugation and quantitated by Bio-Rad DC protein assay system (Bio-Rad). Samples were boiled in SDS-PAGE sample buffer at 95°C and separated using SDS-polyacrylamide gel electrophoresis. The gel was then transferred to nitrocellulose membranes (LiCor) for Western blotting analysis. Primary antibodies used to detect LMP1 were a mixture of four monoclonal antibodies diluted to 1:500 (Cao 7E10, Cao BG3, LMP1 IG6, and Cao 7G8; Ascenion GmbH). LMP1 antibodies were detected with RATH00 biotinylated anti-rat immunoglobulin G (heavy plus light) (16-16-12; Kirkegaard & Perry Laboratories, Inc.) followed by reaction with IRDye 680-labeled streptavidin (Li-Cor). Other bound proteins were detected with IRDye-labeled secondary antibodies by scanning with a Li-Cor Odyssey imaging system. Bands were quantitated using the Li-Cor imaging software. For more information about Western blotting techniques, please see Everly et al. (2009).

**Baculovirus/Insect Cell Expression**

**Plasmids**
The plasmids involved in the study were BABE HA-LMP1, which is a pBABEpuino-based vector (addgene), and pFastGlu (generously provided by Dr. Jun-yong Choe), a pFastBac (Thermo Fisher Scientific) (Figure 13) baculovirus transfer vector modified with a glucose transporter sequence (used in a previous study), and a thrombin cleavage site to eliminate the polyhistidine-tag after purification. LMP1 DNA was obtained from previously purified BABE HA-LMP1 with a polyhistidine-tag for purification purposes, digested with NdeI and HindIII, and purified using the QiAquick spin purification kit (Qiagen). The pFastGlu DNA vector was also digested with NdeI and HindIII restriction endonucleases using the same technique. This technique was also used for mutated versions of LMP1 (BABE HA-LMP1AS, BABE HA-1:220, and BABE HA-1:220AS) and cloned into pFastGlu baculovirus transfer vectors (Figure 14) for future structural studies (Everly, Mainin, & Raab-Traub, 2009).

**Competent Cells**
Bacterial transformations were performed with DH10Bac (Invitrogen) competent cells on fresh LB agar plates containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL X-gal, and 40 µg/mL IPTG to select for DH10Bac transformants. Transformations were incubated at 37°C for 16 hours. Blue/white selection was used to confirm the phenotype (Figure 15A). In addition, colony PCR with primers specific to LMP1 were used to confirm the correct phenotype (Figure 15B).

**Primers**
LMP1-1am (forward): 5’-gcttgatccatcagacgagacgtgagaggggcac-cgg-3’
L187-3 (reverse): 5’-atcagagagatgtagttaatcagatgttaaatcccagcagagac-caggagggc-3’

Colony PCR verified colonies were then inoculated in a liquid LB culture containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, and 10 µg/mL tetracycline overnight. DH10Bac bacterial cells were harvested, and DNA was purified using the ZR BAC DNA Miniprep Kit (Zymo Research).

**Protein Purification and Western Blotting**
SF21 cells were grown in suspension culture to a concentration of 2x106 cells/mL. Cells were scaled to 2 L of supplemented HyClone SFX-Insect Cell Culture media, and were infected with full-length LMP1 baculovirus having a multiplicity of infection (MOI) of approximately 0.1 pfu/mL. Samples were taken at time points 24 hours apart until harvest (0hrs, 24hrs, 48hrs, 72hrs, and 96hrs).

Infected cells were harvested 96 hours post-infection, centrifuged at 2000 x g to separate used media from the cells, and stored at -80°C. The cell pellet was then put back into suspension buffer containing 50 mM sodium phosphate (NaPi) (pH 7.5), 5% glycerol, 200 mM NaCl, lysozyme, and protease inhibitor. The suspension was sonicated four times using 10 second intervals. Cellular debris was removed by centrifugation at 10,000 x g for 2 minutes. The membrane fraction was collected by ultracentrifugation at 200,000 x g for one hour. The fraction was solubilized with 0.5% n-dodecyl-β-D-maltopyranoside (DDM) at 4°C for three hours. The solubilized membrane was separated using centrifugation at 12 x g for 10 minutes. Then the supernatant was loaded into a Talon metal affinity resin (Clontech) and washed with buffer containing 50 mM NaCl, 5 mM imidazole (pH 7.4), 5% glycerol, and 0.05% DDM. Pure protein was collected from the flow-through and eluted with 20mM Tris (pH 7.5) and 0.02% DDM. For more information on this process, please see lancu et al. (2013).

Protein samples (50 ng/mL) were then boiled in SDS-PAGE buffer at 95°C. The protein was separated using SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (LiCor) for Western blotting analysis. Bovine serum albumin (BSA) 10% was used to block the membrane from background binding. Primary antibodies were used to detect LMP1 was a mixture of four monoclonal antibodies diluted to 1:500 (Cao 7E10, Cao 8G3, LMP1 IG6, and Cao 7G8; Ascenion GmbH). LMP1 antibodies were detected with RATH00 biotinylated anti-rat immunoglobulin G (heavy plus light) (16-16-12; Kirkegaard & Perry Laboratories, Inc.) followed by reaction with IRDye 680-labeled streptavidin (Li-Cor). Other bound proteins were detected with IRDye-labeled secondary antibodies by scanning with a Li-Cor Odyssey imaging system. Bands were quantitated using the Li-Cor imaging software. For more information about Western blotting techniques, please see Everly et al. (2009).

**Cell Culture, Transfection, Viral Stock**
Figure 11: pET-19b vector. The pET-19b (Novagen) cloning vector was used to insert LMP1. Site-directed mutagenesis with the pET-19b vector allowed codon optimization blocks to be verified by restriction digestion. The vector had ampicillin resistance to E. coli. The arrows indicate the direction of transcription.

Figure 12: Baculovirus overview. Flowchart describing the separate steps throughout the course of the project. Prokaryotic and eukaryotic methods were both attempted to express LMP1. Diagram of the Bac-to-Bac expression system. Recombinant baculovirus was generated using DH10Bac E. coli cells and transfected using Sf21 cells.

Figure 13: pFastBac1 vector. The pFastBac1 (Life Technologies) vector was modified with a glucose transporter used in previous studies. The vector conferred ampicillin, kanamycin, gentamicin, and tetracycline resistance in E. coli. Arrow indicates the direction of transcription.

Figure 14: GluBac-BABE HA-LMP1 vector-insert. The diagram represents the vector-insert of GluBac-LMP1, GluBac-LMP1A5, GluBac-LMP1 1:220, and GluBac-LMP1 1:220A5. The vector and all inserts were digested with NdeI and HindIII.
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