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The LMP2A signalosome – a therapeutic target for Epstein-barr virus latency and associated disease

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1. ABSTRACT

Most adults are infected with Epstein-Barr virus (EBV), a virus that establishes a lifelong latent infection in B lymphocytes and is associated with a variety of cancers. In normal individuals, latent infection with EBV typically poses no health risk, but upon immunosuppression, either following organ transplantation or HIV infection, malignancies and lymphoproliferative diseases can result. We have utilized both transgenic mice and EBV transformed lymphoblastoid cell lines (LCLs) as models of EBV latent infection to explore the function of latent membrane protein 2A (LMP2A) of EBV. This has allowed us to identify important functional domains of LMP2A, essential host proteins necessary for LMP2A function, and the effect of LMP2A on normal B cell function. These studies have provided a more complete understanding of the role of LMP2A in EBV latency and tumorigenesis and may allow for the identification of novel therapeutics for the treatment or eradication of EBV latent infections and associated proliferative disorders.

2. INTRODUCTION - EPSTEIN-BARR VIRUS AND HUMAN DISEASE

Epstein-Barr virus (EBV), one of eight human herpesviruses, routinely establishes latent infections in human hosts following initial infection and is associated with a variety of cancers (1,2). The eight human herpesviruses are organized into three families, alpha, beta, and gamma, depending upon biological characteristics and evolutionary relatedness. The three human alpha-herpesviruses, herpes simplex virus 1 (HSV-1), HSV-2, and Varicella Zoster virus (VZV), are characterized by their rapid reproductive cycle and capacity to establish latent infections in sensory ganglia. The three human beta-herpesviruses, human cytomegalovirus (HCMV), human herpesvirus 6 (HHV6), and HHV7, typically have a longer lytic reproductive cycle in tissue culture. The two human gamma-herpesviruses, Epstein-Barr virus (EBV) and HHV8, are distinguished by their latent infection of transformed lymphocyte cell lines in culture and their link with human proliferative disorders (2-4). Each family of herpesviruses likely requires distinct host and viral factors to establish and maintain latent infections within the human

host. These host and viral factors may serve as therapeutic targets to treat latent herpesvirus infections.

Considerable interest has focused on EBV since its discovery and its link with Burkitt's lymphoma in the early 1960s (5-7). Along with HHV8, EBV is the only herpesvirus with a known role in human malignancies. Infection with EBV usually occurs early in childhood resulting in an asymptomatic infection. Disease syndromes in humans caused by EBV reflect the cell types that EBV infects, being primarily of lymphoid or epithelial origin (2,8). The most notable lymphoid disease, infectious mononucleosis, is a self-limiting lymphoproliferative disease that occurs in normal adolescents upon primary infection (2). Children are normally able to resolve the primary EBV infection with few or no symptoms. By the age of 25 most individuals are EBV seropositive and harbor a lifelong EBV latent infection. Carriers of EBV latent infection develop cellular immunity against a variety of both lytic and latency associated proteins (2,3,9). Periodically, virus is shed from latently infected individuals by the induction of lytic replication in B-lymphocytes. The true site of latent infection has not been determined, but the virus likely resides in B-lymphocytes. Potential sites of EBV latency include bone marrow, lymph nodes, or other lymphoid organs. Recent studies have shown that EBV can be detected in circulating peripheral blood lymphocytes in carriers of EBV latent infections by PCR for both viral DNA and viral mRNA (10-15). The virus can also be isolated by culturing peripheral lymphocytes (16). This latent infection can be demonstrated by the presence of EBV infected lymphocytes at a frequency of about 1 in 10^5 to 10^6 B lymphocytes, which is stable over time (10,13,17-20). Lytic replication is presumed to occur when EBV infected B-lymphocytes traffic through oral epithelium. The resulting infectious virus provides a source for infection of other individuals. There is considerable disagreement as to whether lytic replication occurs in epithelial cells and if this replication is important for transmission. Recent studies using samples from patients with acute infectious mononucleosis indicate no detectable lytic replication in oral epithelial cells despite abundant lytic replication in lymphocytes that have trafficked to the epithelium (21). Studies from AIDS patients with unusual epithelial hyperplasia of the tongue indicate that the virus can gain access to epithelial cells and undergo lytic replication (2,3,9,22,23). Whether this is a pathological consequence of the underlying immune suppression found in AIDS patients will need to be resolved.

EBV is associated with variety of hematopoietic cancers such as African Burkitt's lymphoma (BL), Hodgkin's lymphoma (HL) and adult T-cell leukemia. This is based upon detection of viral DNA or gene expression in a fraction of these malignancies (2,8). EBV associated lymphoproliferative disease occurs in individuals with congenital or acquired cellular immune deficiencies (2,8). EBV associated lymphoproliferative disease also occurs in 1% to 10% of transplant recipients and in AIDS patients (24-27). The two notable epithelial diseases associated with EBV infection are nasopharyngeal cancer, a malignancy endemic to southern China, and oral hairy leukoplakia, an epithelial hyperplasia of the lingual squamous epithelium in AIDS patients (2,8). Designing effective therapies against EBV latency and EBV-associated cancers is of high priority and could be beneficial for the treatment of EBV-associated disease.

3. EBV *IN VITRO* LATENT INFECTION

B-lymphocytes infected with EBV and grown *in vitro* are immortalized and are termed lymphoblastoid cell lines (LCLs). These EBV transformed LCLs contain EBV episomes and nine virus encoded proteins. Six are nuclear proteins, EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNALP (3,28). Three are the integral membrane proteins, LMP1, LMP2A, and LMP2B. These nine proteins mediate latent virus infection and B lymphocyte proliferation. EBNA2, EBNA3A, EBNA3C, and LMP1 are essential for EBV transformation of B-lymphocytes (3,28). EBNA1 also has an important role in lymphocyte growth transformation since it is required for EBV episome maintenance (29). EBNA3B, LMP2A, and LMP2B are dispensable for lymphocyte transformation (3,28). Of the viral proteins expressed in latent infection, EBNA1, LMP1, and LMP2A are the proteins consistently detected in NPC tumor biopsies, EBV related malignancies, and lymphoproliferative diseases in AIDS patients (2,8).

4. EBV *IN VIVO* LATENT INFECTION

Studies analyzing gene expression in normal individuals harboring EBV latent infections have been informative in suggesting which viral proteins may be important for EBV latency and persistence in the human host. In these studies, B cells infected with EBV *in vivo* demonstrate three distinct patterns of latent gene expression, depending on the differentiation stage of the cell. In the peripheral blood, EBV is restricted to memory B cells that are resting and express very few viral genes (14,15,30-33). Most well documented is LMP2A, but some studies have indicated that the EBERs and BARTs are expressed (14,15,30-33). Naive B cells in tonsils express a repertoire of viral genes similar to what is observed in EBV-infected LCLs grown in tissue culture (30,34-36), whereas germinal center centroblasts and centrocytes as well as tonsillar memory B cells express a more restricted pattern of latent genes, including EBNA1, LMP1, and LMP2A (30,34-38). This pattern of expression is similar to that observed in EBV positive tumors such as NPC and HL as indicated above. Thus, LMP2A must play an important role *in vivo* in viral replication, persistence, and EBV related diseases and could serve as a target for therapies directed against EBV.

5. THE *LMP2* GENES

The *LMP2* gene, the subject of this review, is simultaneously transcribed under the control of two promoters separated by three kilobases (39-42). The two LMP2 proteins are encoded by two mRNAs that have different 5' exons followed by eight common exons (39-

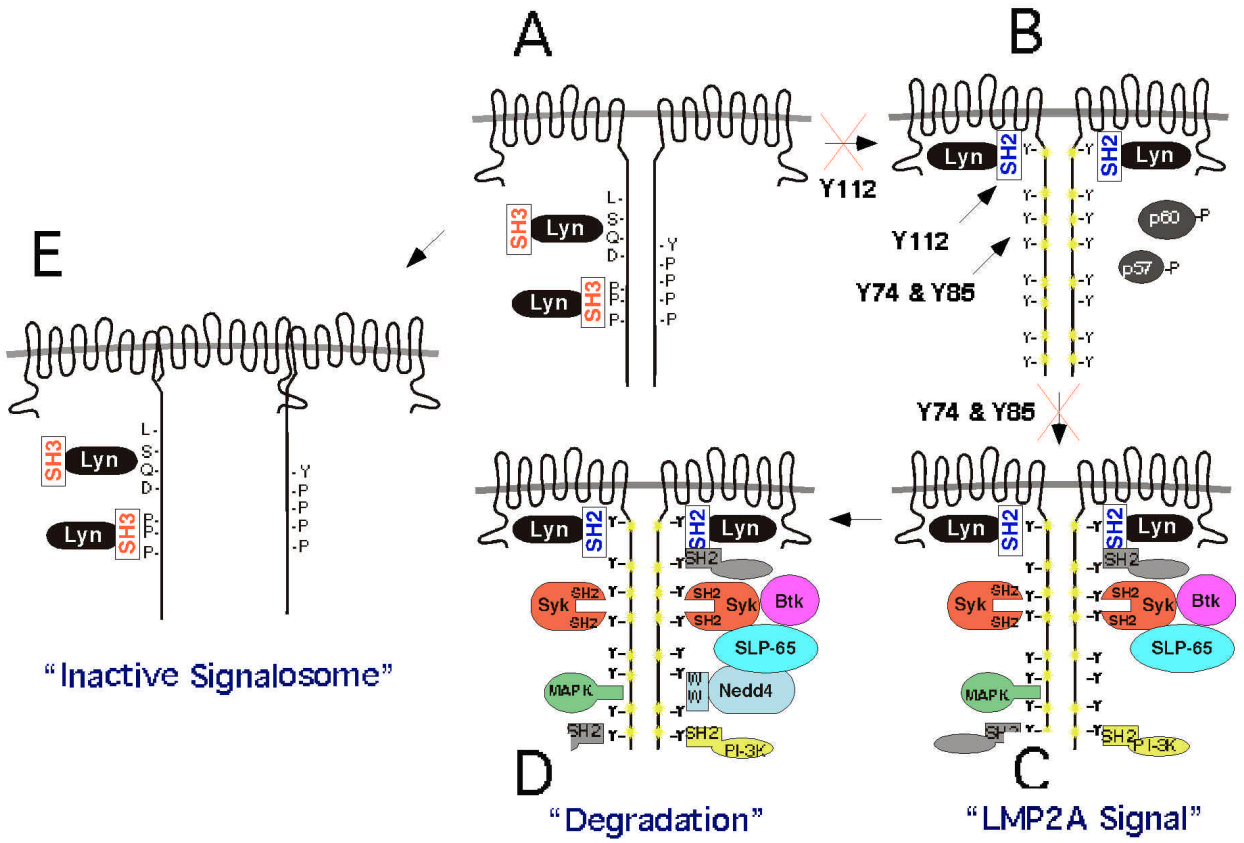

Figure 1. Model for the formation of the LMP2A signalosome. A. The Src family PTK Lyn is recruited to LMP2A, possibly by interaction of the Lyn SH3 domain with an LMP2A proline-rich region or interaction of the Lyn unique region with the LMP2A DQSL sequence. B. LMP2A is phosphorylated at Y112 by the Lyn PTK. Two unknown host proteins are also phosphorylated. Once Y112 is phosphorylated, the Lyn SH2 domain binds and the remaining LMP2A tyrosines are phosphorylated, including the LMP2A ITAM (Y74 and Y85). Binding of Lyn to Y112 and the subsequent phosphorylation of LMP2A are blocked in the Y112F mutant. C. The Syk PTK and other SH2 containing proteins bind to phosphorylated LMP2A. Once bound to LMP2A their activities are altered and they are no longer able to participate in BCR signal transduction. Binding of Syk is blocked in LMP2A ITAM (Y74F and Y85F) mutants, but LMP2A phosphorylation is not inhibited. Following complex formation, LMP2A provides a surrogate BCR signal and blocks normal BCR signal transduction. D. Nedd4 ubiquitin ligases bind to the LMP2A PY motifs and mediate internalization and degradation of LMP2A and LMP2A-associated proteins. E. LMP2B, lacking the LMP2A amino-terminal domain, may negatively regulate LMP2A activity.

42). The LMP2A primary amino acid sequence includes a 119 amino acid receptor tail-like domain at the amino terminus, twelve hydrophobic domains of at least 16 amino acids, each of which traverses the plasma membrane, and a 27 amino acid carboxyl terminal domain. Both the amino and carboxyl termini are in the cytoplasm. The LMP2A 119 amino acid amino-terminal domain includes eight tyrosine residues, some of which are phosphorylated by host tyrosine kinases (43). Each of these phosphorylated tyrosine residues provides a potential site for binding cellular proteins containing Src homology 2 (SH2) domains. This recruitment is likely important for the formation of the LMP2A signalosome, which will be described in detail in figure 1. SH2 domains are non-catalytic domains conserved among cytoplasmic signaling proteins which bind tyrosine phosphorylated proteins (44). Comparing the eight LMP2A tyrosine residues and their surrounding motifs reveals homology to several motifs that predict optimal binding to identified proteins involved in signal transduction, such as PI3Kinase, Syk, and the Src family protein tyrosine kinase Lyn. Additional evidence for the putative importance of the LMP2A tyrosines has been obtained from the comparison of LMP2A sequences from clinical EBV isolates. Of the eight tyrosines, only Y23 and Y64 were not found in all of the EBV isolates that were analyzed (45,46). In addition, seven of the eight LMP2A tyrosine residues are homologous and conserved between LMP2A and the amino acid sequence of LMP2A homologues in non-human gamma-herpesviruses that infect primates (47,48). Y23 is the only tyrosine not found in the non-human LMP2A-like protein sequence (47,48).

In latently infected B-lymphocytes, LMP2A localizes to numerous small patches contained within raft domains in the plasma membrane and other cellular membranes (43,49,50). Most of the phosphotyrosine reactivity in latently infected B-lymphocytes is associated with these LMP2A patches (43). LMP2B initiates at a

methionine 120 amino acids into LMP2A and lacks the entire amino terminal receptor tail-like domain (39,40,42). Lacking this domain, LMP2B may function as a dominant-negative regulator of LMP2A activity by interacting with LMP2A (figure 1E).

6. LMP2A, B CELL SIGNAL TRANSDUCTION, AND REGULATION OF EBV LATENCY

EBV transformed B lymphocytes resemble antigen and cytokine activated B-lymphocytes in their expression of a set of B lymphocyte activation markers and adhesion molecules and by initial proliferation. The B cell receptor (BCR) consists of membrane immunoglobulin (IgM) and the associated proteins Ig-alpha and Ig-beta (51-53). Antigen induced B lymphocyte proliferation and differentiation involves protein tyrosine phosphorylation of these components of the BCR by associated protein tyrosine kinases such as the Src family protein tyrosine kinases (PTKs) Lyn, Fyn, and Blk. Once phosphorylated, the B-cell specific tyrosine kinase Syk associates with the BCR via an interaction of its two SH2 domains with the immunoreceptor tyrosine-based activation motif (ITAM) contained in Ig-alpha and Ig-beta (51-53). Additional proteins such as SLP-65, Vav, Btk, and PI3Kinase are recruited to these complexes, also termed a signalosome, resulting in activation of gene transcription in the nucleus (54,55). Expression of LMP2A completely blocks normal BCR signal transduction (41). We have identified many features of LMP2A that are required for this function. We have demonstrated that the Src family protein tyrosine kinases and the Syk protein tyrosine kinase are associated with LMP2A (56-59), and that LMP2A induces the phosphorylation of Syk and Lyn (57-59). In LMP2A expressing B cells, BCR activation fails to activate Lyn, Syk, PI3Kinase, phospholipase Cgamma2 (PLCgamma2), Vav, Shc, and MAPKinase. Syk, PI3Kinase, PLCgamma2, and Vav are constitutively tyrosine phosphorylated, and their tyrosine phosphorylation does not change following BCR activation (59). In contrast, activation of the BCR on cells transformed by LMP2A null EBV recombinants triggers the same protein tyrosine kinase cascade as in non-infected B lymphocytes (60). In wild-type LCLs, activation of lytic viral infection is blocked by LMP2A, whereas in LCLs null for LMP2A, lytic replication is efficiently induced following BCR activation (60).

7. MODEL OF LMP2A FUNCTION

LMP2A is anchored in the plasma membrane by 12 hydrophobic membrane spanning regions and assembles into large aggregates within glycolipid-enriched microdomains (GEMs), or so-called lipid-rafts (figure 1A) (49). By forming constitutive membrane complexes, LMP2A is able to mimic an activated BCR receptor or BCR signalosome. Initially, the Src family PTKs, such as Lyn, are recruited to LMP2A complexes, possibly through the interaction of the Lyn SH3 domain with a LMP2A proline-rich region (figure 1A). Alternatively, Lyn may be recruited to the DQSL sequence that is similar to the DCSM sequence important for recruitment of Lyn to the BCR associated protein Ig alpha (61). Upon recruitment of Lyn to LMP2A, a 60 kDa protein and 57 kDa protein become phosphorylated (figure 1B). This is followed by the phosphorylation of LMP2A and binding of Lyn via its SH2 domain to tyrosine 112 of LMP2A (58). Once bound, Lyn phosphorylates the remaining LMP2A tyrosines. When tyrosine 112 is mutated to phenylalanine, the initial phosphorylation of LMP2A at Y112 is blocked, thereby preventing interaction of the Lyn SH2 domain with Y112 and subsequent phosphorylation of LMP2A (58). Following phosphorylation of LMP2A, other SH2 containing proteins are recruited to LMP2A complexes. Specifically, the Syk PTK binds to the phosphorylated LMP2A ITAM (figure 1C) (57). Binding of Syk to LMP2A is blocked when either of the tyrosines within the LMP2A ITAM is mutated, although LMP2A is tyrosine phosphorylated in both ITAM mutants (57). Other unidentified SH2 domain-containing proteins may also be recruited to LMP2A phosphotyrosines or the LMP2A signalosome, such as PI3Kinase, MAPK, SLP-65, or Btk. At this point, LMP2A can deliver a positive signal and by its recruitment of Lyn and Syk prevent normal signal transduction through the BCR (figure 1C, "LMP2A Signal"). Finally, the WW domain-containing Nedd4 ubiquitin ligases are recruited to the LMP2A signalosome by interaction of the WW domains contained within the Nedd4 ubiquitin ligases with the two LMP2A PY motifs (figure 1D) (62,63). This interaction is important for the specific internalization and degradation of LMP2A and LMP2A-associated proteins in an ubiquitin-dependent fashion (62,63). This internalization and degradation is inhibited or significantly reduced in cells expressing the double LMP2A PY motif mutant (62,63). The LMP2A complex renders B cells largely unresponsive to BCR activation in that they fail to induce protein tyrosine kinase substrate phosphorylation and subsequent mobilization of calcium (59,60,64). This signaling block prevents BCR-induced lytic production of EBV particles and thereby contributes to a successful escape of the virus from immune recognition.

8. LMP2A AND *IN VIVO* LATENT INFECTION

Our studies suggest that LMP2A may play multiple roles in EBV latent infection. These are delineated in figure 2. LMP2A may provide the necessary signals allowing the virus to establish and maintain a latent infection (figures 2A and 2B). Our *in vitro* studies indicate that LMP2A may prevent activation of lytic EBV replication by cell surface mediated signal transduction. By keeping latently infected cells in an "inactivated" state, LMP2A may allow the virus-infected cells to evade immune surveillance by cytotoxic T cells (figure 2C). This function of LMP2A would be important in preventing lytic replication in latently infected B lymphocytes as they circulate in the peripheral blood, bone marrow, or lymphatic tissues, where they might encounter antigens, super antigens, or other ligands which could engage B cell receptors and activate EBV lytic replication. An important prediction from this model is that certain stimuli will activate lytic replication when latently infected lymphocytes traffic near epithelial surfaces and that this would result in release of virus for the spread of EBV to

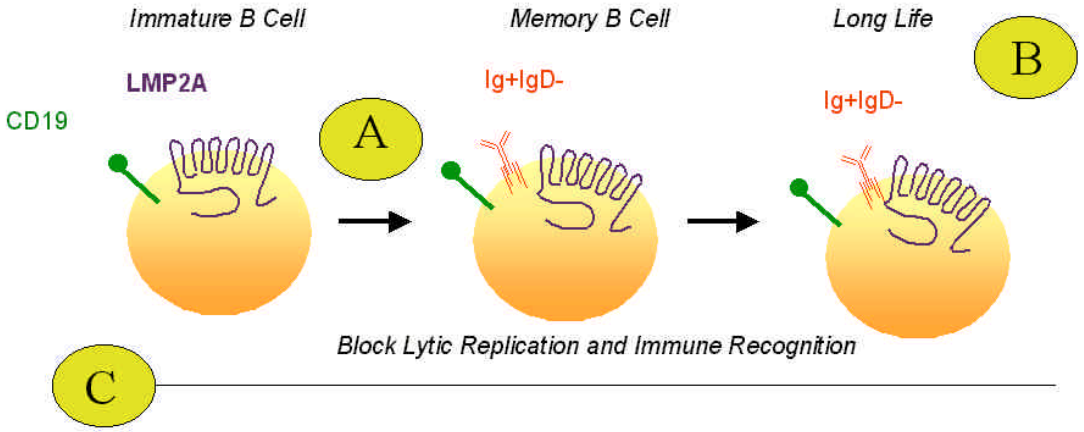

Figure 2. Model of LMP2A function in latent EBV infections. (A). LMP2A may be important in providing a required signal that allows EBV to establish a latent infection in a memory B cell. (B). LMP2A may provide an important cell survival signal allowing EBV to persist in a memory B cell. This survival signal may be important in EBV-mediated pathogenesis. (C). LMP2A may be important in blocking activation of EBV-infected B cells, thus preventing lytic infection, and may prevent immune recognition by maintaining the latently infected B cell in an inactivated state.

uninfected hosts. This activation could be due to interactions of infected cells with epithelial cell produced cytokines, interactions with epithelial surfaces, or down-regulation of LMP2A activity by the expression of LMP2B.

Other roles for LMP2A in EBV latent infections are suggested by our *in vivo* work utilizing transgenic mice. Mice transgenic for the LMP2A gene of EBV were generated by inserting a chimeric LMP2A gene downstream of the immunoglobulin heavy chain promoter and enhancer (65,66). Five independent transgenic lines, TgB, TgC, TgE, Tg6 and Tg7, were analyzed. All lines exhibited no gross developmental defects and demonstrated similar growth and survival rates in a barrier facility when compared to littermate controls. LMP2A expression was verified by immunoblot analysis and quantitative PCR (65-67). The transgenic lines have been maintained and propagated in the C57BL/6 background. This is the standard mouse strain utilized for the analysis of B cell function and is the parental line for many of the knockout and transgenic murine lines we have mated or plan to mate with our LMP2A transgenic mice.

Of the five lines we established, lines TgE and Tg7 display the most dramatic phenotype. In these lines, LMP2A provides both a developmental and survival signal that allows BCR negative cells to exit the bone marrow and colonize peripheral lymphoid organs. Normally these cells would rapidly die by apoptosis (68-73). Further analysis has indicated that LMP2A expression in precursor B cells alters normal B cell development by transmitting signals normally attributed to the pre-BCR. Specifically, appearance of CD43- bone marrow cells that lack appropriate immunoglobulin rearrangements suggests that signals from LMP2A may shut off immunogobulin recombination in a premature enforcement of allelic exclusion, while concomitantly allowing cells to progress to a CD43- stage, exit the bone marrow, and colonize the periphery.

To determine whether the CD43- cells present in transgenic bone marrow could proliferate in response to IL-7, *in vitro* bone marrow cultures were established for each LMP2A genotype in wild-type and recombinase-activating gene (RAG)-/- murine backgrounds. Bone marrow B cells are responsive to the growth and differentiation inducing properties of IL-7 only after rearranging immunoglobulin heavy chain genes, expressing a functional pre-BCR, and transiting from a CD43+ pro B cell to a CD43- pre-B cell (74,75). Within one week of growth in IL-7-containing methylcellulose media, individual wild-type bone marrow B cells form microscopically detectable foci of proliferating cells. All LMP2A transgenic bone marrow B cells were able to proliferate and form colonies when cultured in IL-7-stimulated culture. There were no significant differences in the number of total colonies identified in either wild-type or LMP2A cultures. By comparison, the growth properties of RAG-/-,LMP2A cells were significantly different from those of RAG null animals. RAG null cells do not develop to a stage responsive to IL-7 and therefore do not survive or proliferate under these culture conditions. In contrast, the RAG-/-,LMP2A cells grew in response to IL-7 stimulation, resulting in the appearance of BCR negative cells in the IL-7 culture conditions. Taken together, the *in vivo* and *in vitro* characteristics of LMP2A transgenic bone marrow cells indicate that LMP2A can provide developmental signals that mimic those initiating from an immunoglobulin heavy chain.

A quantitative analysis of LMP2A transcription in the bone marrow of each of the LMP2A transgenic lines was performed utilizing a quantitative RT-PCR assay. Absolute levels of LMP2A mRNA were determined for

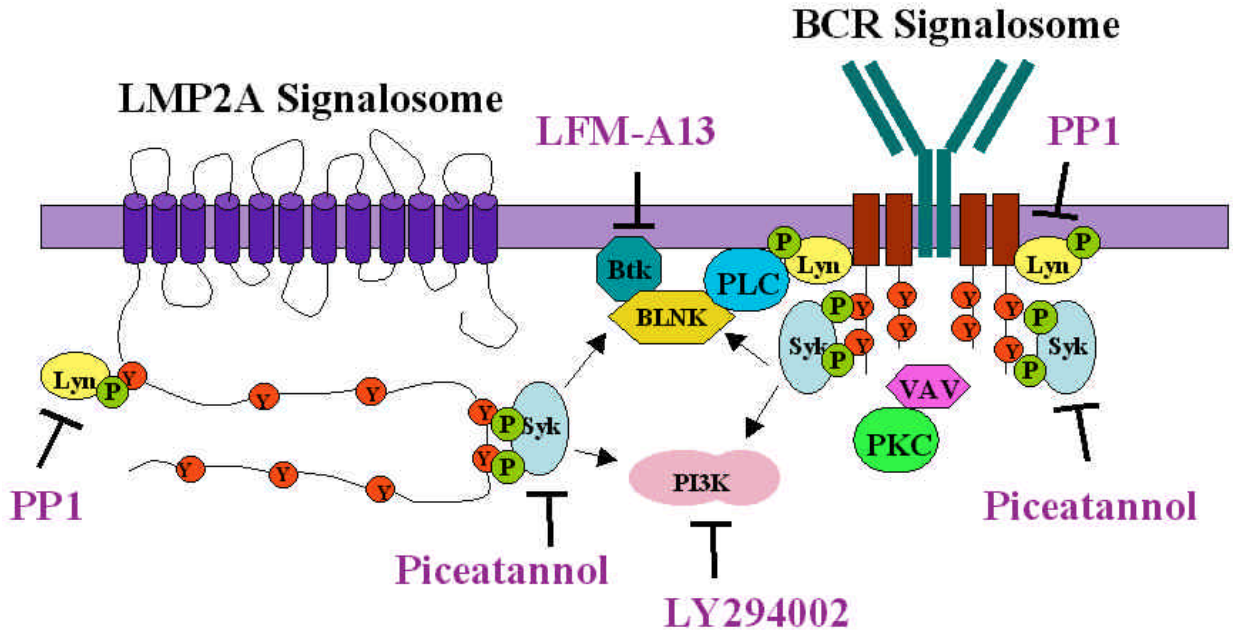

Figure 3. Host cell proteins we have shown to be important for LMP2A *in vitro* or *in vivo* function. Potential inhibitors which block the relevant host proteins are indicated.

each of the LMP2A transgenic lines (66). Relative levels of LMP2A expression were then determined by dividing the LMP2A RNA transcript copy number by the absolute number of GAPDH RNA copies per sample and normalizing for the proportion of B cells in the primary bone marrow sample. As expected, no LMP2A transcripts were detected in cells from wild-type littermate control animals, whereas LMP2A mRNA was readily detected in all transgenic bone marrow samples examined (66). As a group, LMP2A TgB, Tg6, and TgC mice transcribe almost 50% less LMP2A mRNA than LMP2A Tg7 and TgE animals. In a wild-type murine background, the TgB, Tg6, and TgC lines do not display a dramatic phenotype, rather a more subtle phenotype characterized by slightly reduced numbers of B lymphocytes is observed. The more dramatic LMP2A phenotype is only observed when these lines are mated to RAG knockout animals. In summary, our transgenic lines, which display distinct phenotypes, have offered us a unique opportunity to explore the role of LMP2A in EBV latent infection.

9. HOST CELL PROTEINS REQUIRED FOR LMP2A-MEDIATED DEVELOPMENTAL AND SURVIVAL SIGNALS

We have begun to examine the importance of specific host proteins for LMP2A activity in B cells. In addition, mutation of specific LMP2A functional domains has been useful in identifying host cell protein interactions critical for LMP2A function *in vivo*. These studies have allowed us to determine that the interaction of Syk with LMP2A is essential for LMP2A function (67). The various host proteins we have investigated or plan to investigate are shown schematically in figure 3. Identification of host proteins targeted by LMP2A may allow for utilization of specific inhibitors of the altered host protein activities to abrogate LMP2A activity.

10. THE ROLE OF KINASES IN LMP2A FUNCTION

10.1. Syk and LMP2A ITAM Mutant Transgenic Mice

The LMP2A Y(74/85)F mutant ITAM transgene was constructed in a similar manner to our LMP2A transgenic lines except for the specific mutation of tyrosines 74 and 85 to phenylalanines within the LMP2A ITAM (67). Our previous *in vitro* studies had shown that the LMP2A ITAM bound the Syk PTK and that this interaction was essential for LMP2A to efficiently block BCR signal transduction in EBV transformed B cells grown in tissue culture. Isolation and characterization of LMP2A ITAM mutant transgenic mice has allowed us to directly test the importance of this motif for LMP2A-mediated B cell developmental and survival signaling. Two transgenic lines were identified and designated Tg-ITAM1 and Tg-ITAM2. Both Tg-ITAM1 and Tg-ITAM2 expressed LMP2A levels similar to the previously described LMP2A transgenic mice. Spleen and bone marrow samples from Tg-ITAM1 and Tg-ITAM2 were analyzed by flow cytometry to determine whether LMP2A ITAM mutant mice display a B cell developmental phenotype similar to that observed in the previously described LMP2A transgenic lines TgE and Tg6. Spleen and bone marrow samples from each transgenic line were compared to cells from wild-type littermate controls. LMP2A ITAM mutant mice could not support B cell development or survival in the RAG-/- background, nor growth in methylcellulose containing IL-7. Finally, the LMP2A ITAM transgenics could not provide B cells with the developmental or survival signals observed in our other LMP2A transgenic lines, indicating the absolute requirement for the LMP2A

ITAM and the interaction of Syk with this motif for LMP2A function (67).

10.2. Btk and LMP2A Function

Activation of the BCR results in activation of the Src family kinase Syk and Tec family kinase Btk (51-53). Since LMP2A has been shown to interact with both Syk and Lyn, we have investigated the role of Btk in LMP2A signaling by mating our LMP2A transgenic mice to Btk knockout mice (76). Btk is a member of the Tec family of tyrosine kinases, consisting of an amino-terminal pleckstrin homology (PH) domain, a Src homology 2 (SH2) domain, a SH3 domain, and a catalytic domain (77). Btk is a 77 kDa protein critical for normal B cell development and BCR signaling. Loss of Btk function in human B cells results in X-linked agammaglobulinemia (XLA), a severe form of immunodeficiency resulting in the near absence of mature B cells. In mice, loss of Btk results in a less severe, but multifaceted phenotype known as X-linked immunodeficiency (*xid*). The *xid* phenotype in $Btk^{-/-}$ mice is characterized by the loss of peritoneal B-1 lineage B cells, a slight reduction in conventional B-2 lineage B cell numbers, an increase in the percentage of B cells displaying an immature cell surface marker phenotype, and the inability of B cells to respond to a number of B cell mitogens (78). To investigate whether LMP2A developmental and survival signals are dependent upon Btk, LMP2A transgenic mice were crossed into the $Btk^{-/-}$ background. Spleen and bone marrow cells from TgE,$Btk^{-/-}$ animals exhibited a complete loss of the LMP2A phenotype and instead displayed a much stronger *xid* phenotype compared to $Btk^{-/-}$ littermates. In the bone marrow of TgE,$Btk^{-/-}$ mice, there was a reduction in the percentage of B cells bearing a CD19+IgM+ phenotype when compared to $Btk^{-/-}$ littermates. In addition, a modest increase in CD19 expression, which we routinely observe in our LMP2A transgenic lines, was not observed in TgE,$Btk^{-/-}$ B cells, indicating that Btk is necessary for this aspect of the LMP2A phenotype (76). The percentage of CD19+IgM- B cells in the bone marrow of TgE,$Btk^{-/-}$ mice was slightly elevated compared to wild-type mice, but reduced compared to TgE animals. The slight increase may indicate that LMP2A alters the balance of B cell development in the $Btk^{-/-}$ background. A reduction in the number of CD19+IgM+ B cells was also observed in the spleens of TgE,$Btk^{-/-}$ mice. These results indicate that Btk is critical for the generation of two key aspects of the LMP2A *in vivo* phenotype: the bypass of Ig heavy chain rearrangement and the upregulation of CD19. Western blots confirmed expression of LMP2A in B lymphocytes isolated from the bone marrow, indicating that the alteration of the LMP2A phenotype was not due to loss of LMP2A expression (76). In EBV transformed LCLs grown in tissue culture, LMP2A expression was shown to induce the phosphorylation of Btk. In particular, phosphorylation of Y223 and Y551, tyrosines normally phosphorylated upon Btk activation, was observed using phosphopeptide specific antibodies kindly provided by Dr. Witte and Dr. Wahl (79).

10.3. Lyn and other Src Kinases and LMP2A Function

Our previous *in vitro* experiments have indicated that the interaction of the Src family protein tyrosine kinase Lyn with tyrosine 112 of LMP2A is essential for the ability of LMP2A to block BCR signal transduction (58). To test the importance of Lyn in LMP2A signaling *in vivo*, we have mated our LMP2A transgenic TgE line to Lyn knockout animals. Lyn-deficient mice contain reduced numbers of peripheral B cells with a greater proportion of immature cells and a higher than normal turnover rate (80,81). Splenic B cells from Lyn knockout mice initiate early BCR signaling events that are delayed compared to littermate controls (80-82). In addition, Lyn knockout B cells exhibit enhanced MAP kinase activation and an increased proliferative response to BCR engagement (80). These studies indicate that Lyn is important for both positive and negative regulation of BCR signal transduction. The first mating of Lyn knockout mice with the TgE LMP2A transgenic line has been performed and we are currently screening mice to establish breeding pairs for generation of mice heterozygous for the LMP2A transgene and homozygous for the Lyn knockout. Once the appropriate mice have been generated, they will be characterized to identify any alterations in phenotype when compared to the parental TgE transgenic line, Lyn knockouts, and wild-type littermate controls. Depending upon the results of these experiments, we may mate our other LMP2A transgenic lines to the Lyn knockout line. Along with Lyn, two other Src family protein tyrosine kinases, Fyn and Blk, are abundantly expressed in B lymphocytes. Knockouts of any one gene do not dramatically alter B cell development (52). Studies have demonstrated that these protein tyrosine kinases serve largely redundant or overlapping functions. Our previous studies have suggested that Lyn is the Src protein tyrosine kinase that LMP2A preferentially binds (56,58). However, if other Src family protein tyrosine kinases can substitute for Lyn, an altered phenotype may not be observed when LMP2A transgenic mice are mated with the Lyn knockouts. In this case, we will make a tyrosine to phenylalanine mutation similar to the LMP2A Y112 mutation we have tested in EBV transformed LCLs grown in tissue culture. As indicated above, this mutant is non-functional in EBV transformed B-lymphocytes in culture and is unable to bind to any of the Src family PTKs. These experiments should establish the role of the Src family protein tyrosine kinases in LMP2A *in vivo* function and will provide an indication of the potential specificity of LMP2A for particular Src family protein tyrosine kinases.

10.4. SLP-65 and LMP2A Function

The SH2 Domain-containing Leukocyte Adaptor Protein SLP-65 (83), (also called BLNK (84) or BASH (85)) is an early substrate of the Syk PTK and couples Syk and Btk phosphorylation and hence activation of phospholipase Cgamma2 (PLCgamma2) (86-90). This triggers the mobilization of intracellular calcium, which is a hallmark of the BCR activation signal (86-90). SLP-65 has been shown to be a critical component of the BCR signalosome since SLP-65 knockout mice show a drastic reduction in the number of mature B cells due to a block at the transition from B220+CD43+ progenitor B (proB) to

B220+CD43- precursor B (preB) cells (83,84). To investigate the role of SLP-65 in LMP2A *in vivo* function, SLP-65-deficient animals were bred to TgE and Tg6 LMP2A transgenic mice. Spleen and bone marrow samples were analyzed by flow cytometry and indicated that all aspects of the LMP2A phenotype required SLP-65 (91). Further studies analyzing LMP2A function in EBV transformed cells grown in tissue culture demonstrated that LMP2A expression results in the constitutive phosphorylation of one of the two SLP-65 isoforms and complex formation between SLP-65 and the proto-oncoprotein CrkL (CT10 regulator of kinase like) (91). CrkL is an intracellular adaptor protein consisting of one SH2 domain and two SH3 domains and has been shown to be important in lymphocyte proliferation (92-95). SLP-65/CrkL complex formation leads to antigen receptor-independent phosphorylation of Cbl and C3G. In contrast, PLCgamma2 activation is completely blocked. These results establish that LMP2A not only sequesters signaling elements from the B cell antigen receptor, but also selectively activates or represses certain SLP-65 related signaling events. In summary, this set of experiments revealed that LMP2A expression affects not only the phosphorylation status of the p70 SLP-65 isoform but also abolishes the requirement of BCR activation for a phosphorylation-dependent complex formation with CrkL. The CrkL-Cbl-C3G signaling module is constitutively active in EBV transformed LCLs.

11. MICROARRAY ANALYSIS OF LMP2A TRANSGENICS

The developmental and survival phenotypes observed in LMP2A transgenic mice suggest that LMP2A is capable of mimicking signals stemming from the BCR (65,66). We have begun DNA microarray analysis to identify changes in gene expression in LMP2A expressing B-lymphocytes. DNA microarrays provide a powerful tool to explore complex changes in gene expression (96-98). Microarray analysis of B-lymphocytes from LMP2A transgenic mice affords a unique opportunity to understand the molecular mechanism of how LMP2A alters normal B cells and the potential importance this may have in affecting B lymphocyte function. Specific cellular pathways may be activated by LMP2A, resulting in the up-regulation of certain transcription factors and specific changes in gene expression. This may allow for the identification of additional cellular targets for the development of therapies directed against EBV latent infection and EBV associated cancers.

12. POTENTIAL THERAPEUTICS TO TARGET EBV INFECTION

The studies described above elucidating cellular factors important for LMP2A function *in vivo* provide the impetus for our most recent experiments in regard to LMP2A function. In these experiments, we have begun to investigate the potential of utilizing specific inhibitors of normal cellular proteins whose activity or function is required for LMP2A mediated developmental and survival signals. The rationale behind performing such an analysis relies on the hope that LMP2A may dramatically alter the activity of a normal cell protein that is necessary for mediating LMP2A developmental and survival signals. By inhibiting the activity of this protein, the ability of LMP2A to establish or mediate EBV persistence in the human host may be prevented. Treatment with a specific inhibitor, possibly for only a short time period, may have dramatic effects on the number of B-lymphocytes harboring EBV in the human host. They may simply apoptose because they lack the LMP2A surrogate BCR signal. By reducing the number of cells that harbor the virus, malignancies associated with EBV may be prevented or reduced. In particular, patients recently diagnosed with HIV infection or patients who will be undergoing transplantation may be treated before immunosuppression occurs, thus reducing the number of infected B cells that may contribute to the development of EBV associated immune proliferative disorders.

The specific targets we have begun to investigate are based upon our work using EBV transformed LCLs grown in tissue culture as well as experiments using LMP2A transgenic mice. We have previously demonstrated that Src family PTKs specifically bind to tyrosine 112 of LMP2A. This interaction is required for LMP2A to block BCR signal transduction *in vitro* and for the phosphorylation of LMP2A and subsequent recruitment of Syk to the LMP2A signalosome (58). Thus, inhibition of Src family PTKs may be useful in blocking LMP2A function. Studies proposed above using Lyn knockout mice may clarify the use of Lyn specific inhibitors as a method to block LMP2A function. Both our *in vitro* and *in vivo* studies with the LMP2A ITAM have shown that the interaction of LMP2A with the Syk PTK is absolutely required for LMP2A function, thus making Syk a potential target (57,67). Our recent studies utilizing Btk knockout mice suggest that blocking Btk kinase activity may also block LMP2A function (76). In Btk knockout mice, LMP2A was unable to promote B cell development and survival. One caveat with these results is that Btk appears to be less critical for B cell development and function in mice compared to humans. Mutation of Btk in humans leads to the severe immunodeficiency X-linked agammaglobulinemia (XLA), whereas mutation of Btk in mice leads to the milder X-linked immunodeficiency (77). Studies suggesting PI3Kinase as a potential target are based upon our analysis of the activation of Akt in B-lymphocytes (99). In the presence of inhibitors of PI3Kinase, the induced phosphorylation of Akt by LMP2A was completely abrogated in LMP2A expressing B cells (99). Experiments in epithelial cells also suggest an important role for LMP2A and its activation of Akt by PI3Kinase in epithelial cell survival (100). Finally, our studies utilizing SLP-65 knockout mice in conjunction with our LMP2A mice suggest that c-Abl may be a target of LMP2A (91). The Crk family of adaptor proteins has been shown to transactivate the c-Abl PTK (101) and we have demonstrated the activation of CrkL in LMP2A expressing cells. Table 1 is a listing of proteins whose activity is altered in LMP2A expressing cells and for which inhibitors are commercially available. We plan to obtain inhibitors for each of these proteins and measure colony formation of

Table 1. Potential Targets and Inhibitors

Cell Protein	Reference[1]
Lyn	58
Syk	57,67
Btk	76
PI3K	99,100
Abl	91

[1]Reference for paper suggesting target.

bone marrow B cells in IL-7 methycellulose cultures and apoptosis in splenic B cells from LMP2A transgenic and wild type mice. Finally, our transgenic mice provide an *in vivo* system in which to test specific inhibitors if potential candidates are identified and depending on the toxicity and bioavailability of the compounds in experimental animals.

13. DISCUSSION

Studies to identify the site of EBV latency in immune competent human hosts have demonstrated that in peripheral blood EBV resides in memory B lymphocytes (13-15,33,102,103). Other potential sites of EBV latency may include bone marrow, lymph nodes, or other lymphoid organs as EBV can be isolated from virtually any lymphoid tissue. Our recent experiments with a transgenic mouse model of EBV latency (65) and the observation that bone marrow cells can harbor EBV (104,105) have led us to speculate that bone marrow may serve as the site of EBV latency. In this model of EBV latency, progenitor B cells may become infected by EBV when circulating B cells containing the virus traffic to bone marrow. Progeny of latently infected bone marrow cells could generate the relatively stable number of EBV infected lymphocytes observed in the peripheral blood of latently infected individuals (13,16,17,19,103,106). Theoretically, once latently infected bone marrow cells enter the periphery, constitutive signaling from LMP2A could provide a survival signal that would maintain these cells in the absence of a competent BCR or the requirement for BCR signal transduction. Other peripheral organs may also be important for the generation of EBV latently infected cells. In combination with LMP1, LMP2A may provide essential signals that allow EBV to reside in memory B lymphocytes in a largely latent state (20).

Our *in vitro* and *in vivo* studies have identified important functional domains of LMP2A and cellular proteins that are essential for LMP2A activity. These studies have been important in elucidating the mechanisms underlying the persistence of EBV in latently infected humans. The development of a transgenic mouse model system to study LMP2A function in non-proliferating B lymphocytes in the context of the whole animal has provided important new information in regard to how EBV alters the phenotype of latently infected B lymphocytes. The ability of EBV to remain latent in the human host is important for the development of EBV related cancers. The discoveries outlined in this review regarding LMP2A function provide insight into how LMP2A alters the normal B cell phenotype, its importance in maintaining EBV latency, and its role in the development of EBV-related malignancies. For example, LMP2A may be important in the development of Hodgkin's disease (HD). Reed-Sternberg cells in HD can contain somatic mutations resulting in the absence of BCR expression, indicating that these cells do not require BCR signaling for survival (107,108). By providing a survival signal, LMP2A may allow these cells to be maintained in the absence of a competent BCR. This may be an important first step in the development of HD. By understanding LMP2A function in EBV latency and tumorigenesis, novel therapeutics may be derived for the treatment or eradication of EBV latent infections and associated cancers.

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