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Original Research Article

Type 1 and type 2 epstein-barr virus transform B cells differently due to BS69 interaction

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Abstract

Two main strains of Epstein-Barr virus (type 1 and type 2) can be defined largely based on the sequence of their EBV nuclear antigen 2 (EBNA2) gene. Type 1 EBV is more efficient in transforming B-cells than type 2, but the mechanism underlying this difference in transformation efficiency is unclear. Evidence indicates that type 2 EBNA2 may activate some cellular genes less efficiently than type 1 EBNA2. In this report, we summarized on the hypothesis that whether the differences between type 1 and type 2 EBNA2 in activating cellular genes correlates with their ability to bind a host cell tumour suppressor and transcription repressor protein, BS69. Previous studies identified two PXLXP peptide motifs in conserved regions of type 1 EBNA2, which bind BS69. These motifs are also present in the sequence of type 2 EBNA2. A single amino acid mutation (S442D) increases the transformation efficiency of type 2 EBNA2, indicating that this amino acid is the defining factor in the difference in the transformation efficiency of type 1 and type 2 EBV. This residue is present near the second BS69 binding site with BS69. In conclusion, we hypothesize that it is the stronger association between BS69 and type 2 EBNA2 that could contribute in the reduction of type 2 EBV transformation efficiency.

Keywords: Epstein Barr-Virus, Burkitt's Lymphoma, EBNA2, BS69 protein, MYND, protein-protein interaction, transcription regulation

Introduction

Epstein-Barr Virus (EBV) is a human herpesvirus that belongs to the Gammaherpesvirinae subfamily and the genera Lymphocryptovirus (Goedert, 2000) [18]. EBV is also known as Human Herpesvirus 4. It was first discovered in 1964 by Epstein, Achong and Barr in a cell line derived from a Burkitt's lymphoma (BL) biopsy sample and was the first virus to be directly linked to human cancer (Epstein *et al*., 1964) [16]. EBV is widespread and is carried by more than 90% of the worldwide population. EBV is transferred through the exchange of the saliva and genital secretions (Neiderman *et al*., 1976) [37]. Primary infection is usually asymptotic and occurs in childhood but the virus can cause the benign lymphoproliferation, infectious mononucleosis (glandular fever) if primary infection is delayed to adolescence or early childhood (reviewed in Kieff and Rickinson, 2007) [27]. EBV is associated with B cell

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cancers such as Burkitt's Lymphoma, Post-transplant lymphoproliferative disease (PTLD), AIDS associated B-lymphoma; Hodgkin's disease; epithelial tumors, such as undifferentiated nasopharyngeal carcinoma (NPC); and gastric cancer (Crawford, 2001) [14].

EBV infection

The EBV genome is a double stranded linear DNA (reviewed in Young and Murray, 2003) [50], enclosed within a viral nucleocapsid (Cohen, 2000) [12]. The nucleocapsid is surrounded by a viral envelope. Initial infection of EBV is thought to begin in the epithelial cells of the oral cavity. There, the virus undergoes lytic replication to produce progeny viruses and spread throughout the lymphoid tissue. The virus attaches to naïve B cells by binding its viral envelope glycoprotein gp350 to compliment receptor 2 (CD2/CD21) on B-cells. Then EBV gp42 interacts with B-cell HLA II (Human leukocyte antigen class II) and triggers fusion with the host membrane (reviewed in Odumade *et al*., 2011) [38]. Upon infection, the EBV genome undergoes circularisation and is maintained as an episome, which is transcribed by cellular transcription machinery to encode the EBV proteins needed to transform the host cells (Hurley and Thorley- Lawson, 1988) [21]. EBV proteins activate the B-cell growth program (also called Latency III), leading to the proliferation of the blasting B cells (Figure A). In latency III, the latent genes expressed by EBV include six nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C and EBNA-LP), three membrane proteins (LMP1, LMP2A and LMP2B), and various non-coding small RNAs (EBERs and miRNAs). Epstein-Barr virus nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3C) and LMP-1 are essential for generation of predominantly proliferating cell lines. (Young *et al*., 2004; Kieff *et al*., 2007) [50, 27], while, promoting the G_0–G_1 phase transition of the cell cycle (Sinclair *et al*., 1994) [45]. EBNA-LP and LMP-2 contribute to the optimal efficiency of transformation (Lee *et al*., 1999; Longnecker *et al*., 1993) [29, 33]. Latency III, a growth latency program involved the transformation of infected B cells into proliferating blasts and is detected in AIDS patients or in post-transplant patients (Figure A). Usually, these blasting B cells are destroyed by cytotoxic T cells (reviewed in Odumade *et al*., 2011) [38]. The remaining cells enter the pool of memory B cells where the virus switches to the default program (also called latency II) and express EBNA1; and LMP 1 and 2. Latency II is the state where expression pattern of the latent genes is restricted by the virus in the diseases like differential nasopharyngeal carcinoma, Hodgkin's disease, and T-cell lymphoma. (reviewed in Young and Murray, 2003) [46]. In latency 1 only EBNA-1 expression takes place which allows the EBV genome to replicate when the memory B cell divides (reviewed in Odumade *et al*., 2011) [38]. The memory cells exit the cell-cycle and enter the peripheral circulation detected in Burkitt's lymphoma group I and AIDS- related DLBCL (Diffuse Large B-Cell Lymphoma). The memory B cells stay as resting B cells in latency 0, where no EBV latent proteins are expressed. On reactivation, in response to some unrelated infection, memory B cells differentiate into plasma cells, which can switch on the lytic genes leading to the replication of the virus (reviewed in Thorley-Lawson and Gross, 2004; Odumade *et al*., 2011; Epstein *et al*., 1964) [46, 38, 16].

Types of EBV

Differences predominantly in the EBNA2 gene sequence classifies EBV into two types: type 1 and type 2 (also known as type A and B) (Kieff *et al*., 2001) [20]. The sequence identity between the two types of EBNA2 is 50% (Adldinger *et al*., 1985; McCann *et al*., 2001) [2, 35]. Some EBV associated diseases have specific geographic distributions. For example, NPC is prevalent in parts of southern China (Epstein *et al*., 1964) [16] and Burkitt's lymphoma in Sub-Sahara Africa (Burkitt and Conor, 1961) [5]. Natural variation in EBV may contribute to the differences in disease incidence in different parts of the world. The main component of variation is type 1/type 2 classification (Palser *et al*., 2015) [40]. Type 1 strains of EBV are found worldwide, whereas type 2 strains are equally abundant in some areas of Central Africa

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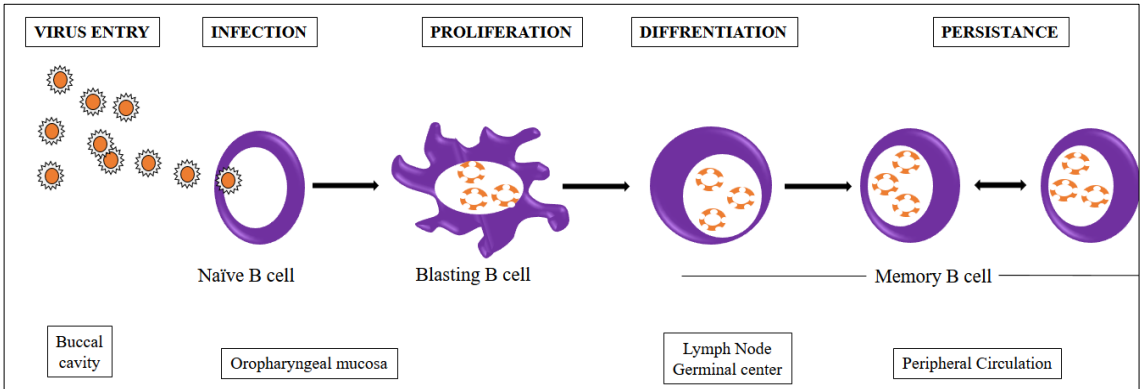
and other malaria prevalent parts, but much less frequent in Europe and the United States (Young *et al*., 1987; Rickinson *et al*., 1987) [50, 43]. However, recent studies indicated the presence of coinfection in the samples with higher EBV load and a small percentage of white Caucasian people carrying type 2 EBV almost exclusively (Correia *et al*., 2017) [13].



Latency Type	Type 3	Type 2	Type 1	Type 0
Description	Growth	Default	EBNA1	Latency
Viral Proteins	EBNAs1, 2, 3a, 3B, 3C, LP, LMP1, 2	EBNA1, LMP1, 2	EBNA1	
Normal B Cell	Naïve B	Germinal Center B	Dividing Memory B	Resting Memory B
Malignant Counterpart	PTLD	HD, NPC	BL, Gastric Cancer	

Fig A: *EBV infection cycle and proteins expressed in different latencies. EBV infection is associated with the initial expression of latency III proteins such as EBV nuclear antigens (EBNAs) 1, 2, 3A, 3B, 3C, Leader Protein (LP), Latent Membrane Protein (LMP) 1 and 2 which transform the B cells into proliferating blasts. These blasts then enter the pool of memory B cells where virus switches on its default program (latency II). The memory B cells exit the cell cycle and enter the peripheral circulation in latency I. Then memory B cells enter a persistence phase (latency 0) and on reactivation, Memory B cells differentiate into plasma cells, which can switch on the lytic cycle. PTLD: Post-transplant lymphoproliferative disease, HD: Hodgkin's Disease, NPC: Nasopharyngeal Carcinoma and BL: Burkitt's Lymphoma.*

Type 1 EBV is much more effective in transforming B cells and producing immortalized cell lines compared to the type 2 strain. EBNA2 alone is responsible for this difference in the efficiency of transformation between the EBV types. By replacing the EBNA2 gene in type 2 EBV with type 1 EBNA2, the transformation efficiency of type 2 EBV is increased to type 1 levels (Cohen *et al*., 1989) [11].

The EBNA2 Protein

On comparing the EBNA2 allele sequences from type 1 and type 2 EBV and different primate lymphocryptoviruses, nine evolutionarily conserved regions (CR1-CR9) were identified (Figure B), which define some key functional domains (Rickinson *et al*., 1987) [43]. The N-terminus of EBNA2 contains CRs 1 to 4 and the C-terminus contains CRs 5 to 9, these two regions are separated by a diversity region. EBNA2 sequence homology between type 1 and type 2 viruses at the N-terminus, diversity region and C-terminal is 62%, 30% and 48%, respectively (Cancian *et al*., 2011) [6]. Two N-terminal domains, Dim1 (1-58 a.a.) and Dim2/SAD (Self Association Domain) (97-121 a.a.), separated by a polyproline region mediate the self-association of two EBNA2 proteins. Two transactivation domains at N and C terminal (N-TAD and C-TAD) have been mapped by tethering EBNA2 fragments fused to the yeast GAL4DBD (DNA binding domain) to GAL4 dependent reporter genes. When GAL4 DNA binding domain fusion proteins of the N- or C-TAD are compared directly, they score equally well in transient transactivation assays (Peng *et al*., 2004) [41]. C-TAD domain can recruit components of the basic transcriptional machinery; and chromatin modifiers; and

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might directly bind to the EBNA-LP (Peng *et al*., 2004) [41]. N-terminus transactivation domain (N-TAD) coincides with Dim1 and appears to mediate multiple molecular functions including self-association, transactivation, and functional cooperation with EBNA-LP (Peng *et al*., 2004; Chabot *et al*., 2014) [41, 8]. So far is it unknown if the N-terminus of EBNA2 directly provides all these functions or if these activities merely depend on the dimerization involving the N- terminal domain. CR5 and CR6 facilitate the binding of EBNA2 with the host DNA-binding protein, RBP-Jk (Harada *et al*., 2001; Zhou *et al*., 2000) [20, 53]. CR7, CR1 and CR2 are required for co-activation with EBNA-LP (Peng *et al*., 2004) [41]. CR8 is an acidic transactivation domain (TAD) (Ling *et al*., 1994) [31], whereas CR9 contains nuclear localization signal (NLS).

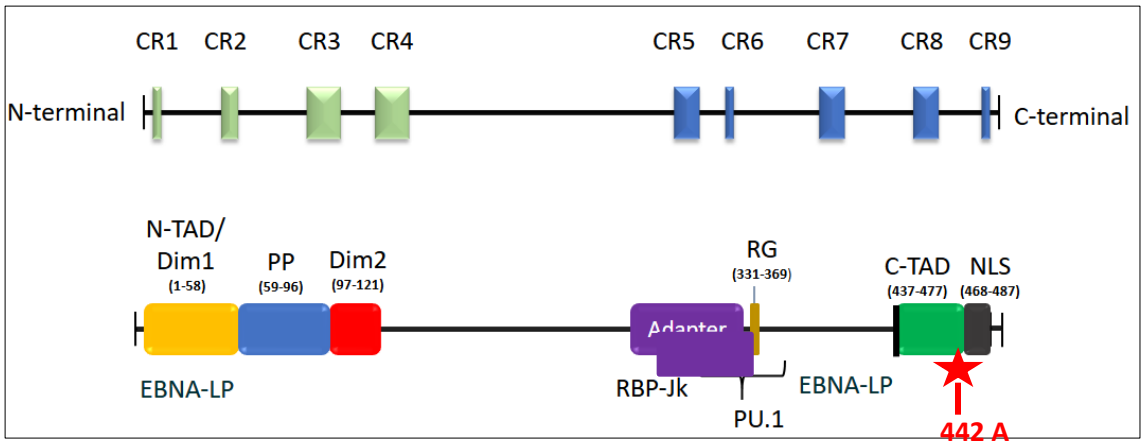

Fig B: *Schematic representation of important features of the EBNA2 protein sequence of EBVThe two dimerization regions (Dim1 and Dim2/SAD (Self-association domain)) are present in the N-terminus. CR1-9 (Conserved Region) indicates the conserved domains identified in EBNA2 sequence. CR1, CR2 and CR7 regions of EBNA2 bind with EBNA-LP (EBV Nuclear Antigen - Leader Protein). Repetitive primary sequence motifs are also present like poly-proline (PP) and poly arginine-glycine (RG) stretch. CR5 and CR6 bind with DNA-binding proteins, RBP-Jk and PU.1 respectively. CR1 and CR8 are N and C-terminal TAD (Transactivation Domain). Dim1 coincides with N-TAD region. An Aspartate at 442 position in the C-TAD region is highlighted with a red star (see section 1.5). CR9 contains NLS (Nuclear Localization Signal).*

EBNA2 acts as a transcription factor for the expression of viral genes and numerous host cell genes. EBNA2 activates transcription from the viral Cp (C promoter), which generates a 120kb transcript encoding all six of the EBNAs proteins. In addition to this, EBNA2 activates promoters of the three viral LMP genes. In the case of B cell genes, EBNA2 regulates hundreds of B cells genes including the B cell Complement Receptors: *CD23, CD21(CD2);* Chemokine receptor*: CXCR7* (C-X-C Chemokine Receptor type 7*);* Proto-oncogene*: MYC;* and Transcription Factor*: RUNX3* (Cohen *et al*., 2000) [12]. EBNA2 does not bind to DNA directly. EBNA2 binds DNA via other sequence specific DNA binding proteins such as RBP-Jk (Recombination signal Binding Protein 1 for Jk) (CBF1), AP-2 and PU-1. EBNA2 binds to RBPJ via a RAM (RBP-J associated molecule) motif (WWP). According to the ChIP sequencing studies, 72% of EBNA2 human genome binding sites overlap with RBPJ sites (Zhao *et al*., 2011) [52]. With its acidic activation domain (TAD) EBNA2 interacts with several transcription factors and co-activators including TFIIB (transcription factor II B), TAF40 [(TATA-box-binding protein)-associated factor 40], HATs (Histone Acetyltransferases): p300/CBP [CREB (cAMP- response-element- binding protein)-binding protein]. EBNA2 also recruits chromatin remodelers, such as the SWI/SNF remodeling complex to modify chromatin structure, which further help in the regulation of the viral and host cell genes.

Type 2 EBNA2 S442D Mutagenesis

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Interestingly, the difference in the transformation efficiency of type 1 and type 2 EBV has been mapped to a single residue. Aspartate is in the TAD region of type 1 EBNA2 at 442 (amino acid) position (Figure B) and being an acidic amino acid confers a stronger transactivation function and increased binding to some sites of the key genes including the viral oncogene, LMP-1 and the host cell gene, CXCR7 (which are both required for proliferation of EBV-infected lymphocytes). Since, EBNA2 is a transcription factor, increased binding of TAD region of EBNA2 results into stronger and more rapid induction of viral and host cell genes, consequently, it may direct higher B cell transformation and proliferation, which also lead to the superior growth of the cells containing type 1 EBNA2. On the other hand, type 2 EBNA2 contains a serine residue at 442 position. Changing serine to aspartate (S442D) in type 2 EBNA2 allows type 2 EBNA2 to activate the two key genes (LMP-1 and CXCR7) more efficiently.

An EREB2.5 cell growth assay was also carried out to investigate the contribution of changing serine with aspartate at 442 position in type 2 EBNA2 protein. EREB 2.5 is a human B-cell line containing EBV (ΔEBNA2) in which the endogenous EBNA2 is replaced with an estrogen (β-estradiol)-inducible EBNA2-estrogen receptor (ER) fusion protein. The results showed that changing a single amino acid serine with aspartate in type 2 EBNA2 conferred a type 1 growth phenotype in LCL growth maintenance assay. A reciprocal amino acid change, D442S was also made in type 1, which abolished the activity of type 1 EBNA2 in this assay. So, the result confirmed that aspartate at 442 position is essential for type 1 EBNA2 function in the cell growth assay and S442D point mutation is sufficient to convert type 2 EBNA2 to be as effective as type 1 EBNA2 in this assay. (Tzellos *et al*., 2014) [47].

Type 1 and 2 EBNA2 Transcription Regulation

To understand the mechanism of difference in the transformation efficiency and growth maintenance in type 1 and type 2 EBV. The host cell genes induced by type 1 and type 2 EBNA2 were compared with a microarray analysis of EBNA2 target genes. CXCR7; LMP-1; MARCKS; IL-1β and ADAMDEC were identified to be more strongly induced by type 1 EBNA2 (Lucchesi *et al*., 2008) [34]. CXCR7 (Fredriksson *et al*., 2003; Joost *et al*., 2002) [17, 25] and LMP-1 were the most differentially regulated genes and both required for LCL (Lymphoblastoid cell lines) proliferation (Cancian *et al*., 2011) [6]. In a EREB2.5 growth assay (explained in section 1.5), expression of an EBV oncoprotein, LMP1 was assessed. Western blot analysis revealed that estrogen starved cells expressing type 1 EBNA2 showed LMP-1 protein expression at the same level as detected in EREB2.5 cells normally grown in the presence of estrogen. In contrast, LMP-1 expression was lost in type 2 EBNA2. The qRT-PCR analysis for the mRNA of cell gene CXCR7, showed an increased level, following a 14-fold increase in expression of EBNA2 type 1 than type 2, compared to the non-transfected cells. In the cells expressing type 2 EBNA2, loss of LMP-1 and CXCR7 is rapidly followed by growth arrest of the cells. Whereas in the cells expressing type 1 EBNA2, a maintained expression of LMP1 and CXCR7 is followed with the sustained proliferation of the cells. Therefore, higher induction of LMP-1 and CXCR7 accounts for the superior ability of type 1 EBNA2 to promote B cell growth, compared to the type 2 (Cancian *et al*., 2011) [6]. MARCKS activates the PKC (Protein Kinase C) (Aderem *et al*., 1992) [1] and regulates ruffling of the membrane and size of the host cell (Myat *et al*., 1997) [36]. IL-1β is a cytokine involved in lymphocyte proliferation. It is not yet known how MARKS and PKC affect the transformation efficiency of EBV.

BS69/ ZMYND11 Protein

The motifs in the CR7 and CR8 regions of EBNA2 have been shown to interact with the B-cell protein, BS69. BS69, also known as ZMYND11 (Zinc finger MYND-type containing 11) (Hateboer *et al*., 1995) [23], BRAM1 and MRD30, is a tumour suppressor protein and a

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transcriptional co-repressor that is frequently deleted in cancers e.g. renal-cell carcinoma and early T-cell precursor acute lymphoblastic leukemia. BS69 was originally identified as a nuclear protein that binds to adenoviral E1A oncoprotein and inhibits its transactivation activity (Hateboer *et al*., 1995) [23]. Its reduced expression is associated with poor prognosis in breast cancer and its overexpression suppresses growth of cancer cells *in vitro* as well as *in vivo* (Wen, 2014) [49]. BS69 binds to the EBNA2 protein with its C-terminal cysteine-rich domain, known as MYND (Myeloid translocation protein 8, Nervy and DEAF-1, residues 521–562) (Figure C) (Hateboer *et al*, 1995) [23], and suppresses the transactivation activity of EBNA2 (Ansieau and Leutz, 2002) [3]. The MYND domain of BS69 recognises a common PxLxP (x denotes any amino acid) sequence motif present in E1A and EBNA2 proteins (Ansieau and Leutz, 2002) [3]. The N-terminus of BS69 protein contains a PHD (Plant Homo Domain) zinc finger. Recent studies also identified the interaction of BS69/ZMYND11 and the histone H3 variant, H3.3 (H3.3K36me3, histone H3.3 trimethylated at lysine 36). The histone variant, H3.3 is associated with childhood and young-adult tumors, such chondroblastoma and giant-cell tumors of the bone. Importantly, some H3.3 cancer mutations are predicted to abrogate the H3.3K36me3/BS69 interaction, suggesting that this interaction may play an important role in tumor suppression (reviewed in Lan and Shi, 2015) [30]. The tandem Bromo-PWWP domains of BS69 specifically recognize histone H3.3 H3.3K36me3, thereby linking BS69 to transcriptional elongation, tumor suppression and pre-mRNA splicing (reviewed in Lan and Shi, 2015) [30].

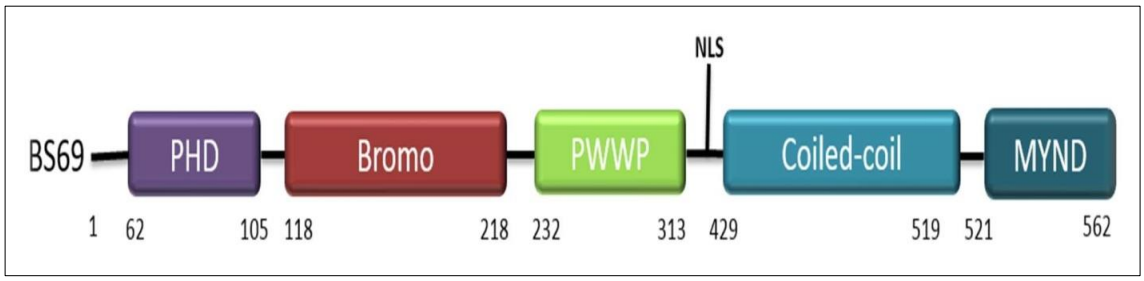

Fig C: *Domain architecture of BS69. Coiled-coil (residues 429-520) and MYND (Myeloid translocation protein 8, Nervy and DEAF-1, residues 521–562) domains are present at the C-terminal of the protein. BS69 N-terminus contains a PHD (Plant Homo Domain), a Bromo domain and a PWWP domain. PWWP and coiled-coil domain contain a region in between for nuclear localization signal (NLS).*

The EBNA2 and BS69 interaction

Two pentamer peptide motifs, PxLxP were found in the EBNA2 CR7 and CR8 regions of type 1 EBV (Ansieau and Leutz, 2002) [3] (Figure D). The interaction of these two motifs (EBNA2 type 1 381-389 aa and 435-445 aa) is thought to occur with two different MYND domains (521-562 aa) of a BS69 homodimer (Figure D). The conserved residues of the PxLxP motif (P383, L385 and P387) bind with $BS69_{CC\text{-}MYND\ (521\text{-}562)}$ (residues Y532, C534, Y540, Q546, W550, C558 and R560) through hydrogen bonds and van der Waals contacts (Harter *et al*., 2016) [22]. The proline residues in PxLxP mainly provide structural rigidity while the central leucine residue pointing inward makes hydrophobic contacts. The BS69 structure revealed the presence of a coiled-coil domain (residues 429–520), preceding the MYND domain. The coiled-coil domain mediates dimerization of two $BS69_{CC\text{-}MYND}$ molecules to form a homodimer and brings two MYND domains of the molecules in proximity (Figure E). The W536 residue of one MYND domain is found to engage with the neighboring MYND domain in the $BS69_{cc\text{-}MYND}$-$EBNA2_{(381\text{-}389)}$ complex, suggesting that the coiled-coil domain in BS69 has a role in enhancing the interaction between $BS69_{MYND}$ domain and EBNA2 (Harter *et al*., 2016) [22]. In vitro interaction studies showed that mutation of Q546 and W550 residues in $BS69_{MYND}$ abolishes the binding with EBNA2 and assays in B cells showed that these mutations prevent BS69 from inhibiting EBNA2-mediated

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transactivation and LCL cell growth (Harter *et al*., 2016) [22].

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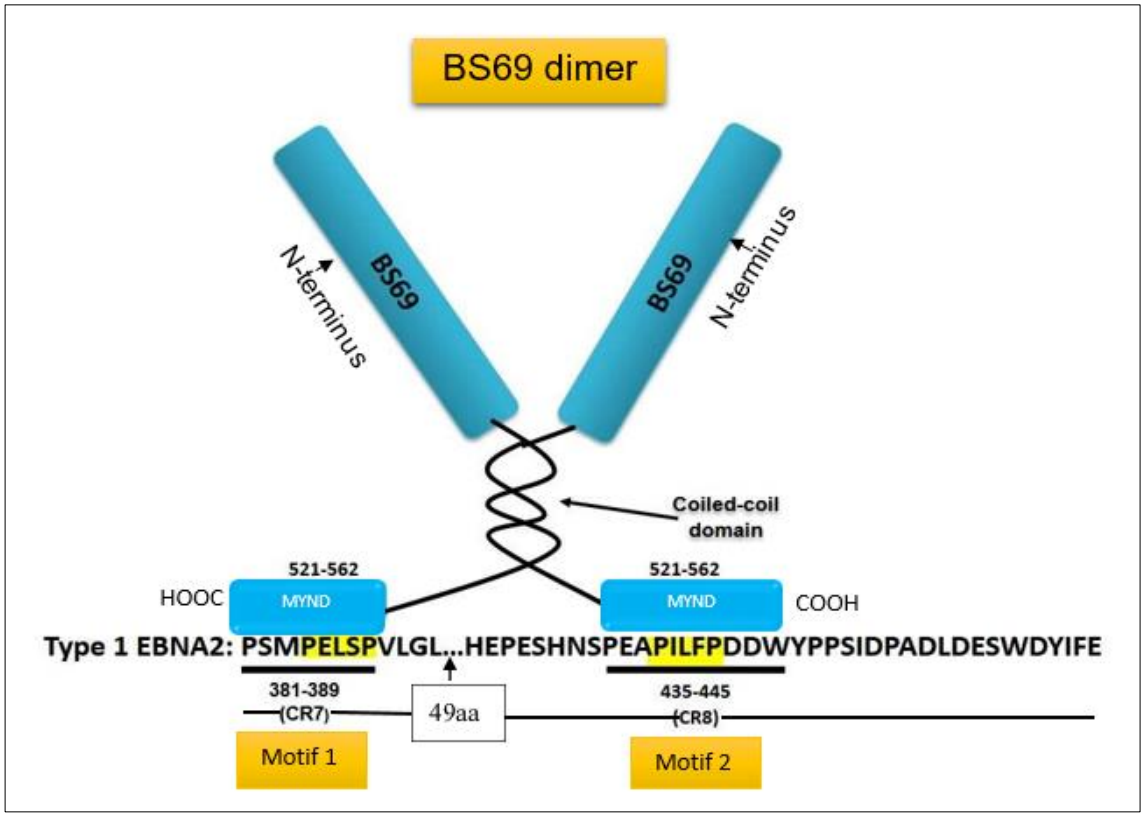

Fig D: *Interaction between BS69 MYND$_{521-562}$ domain and the PXLXP motifs of EBNA2. Two BS69 MYND domains bind to the two PXLXP pentamer motifs (EBNA2 type 1 motif 1 (381-389 aa) and motif 2 (435-445 aa) in the CR7 and CR8 regions.*

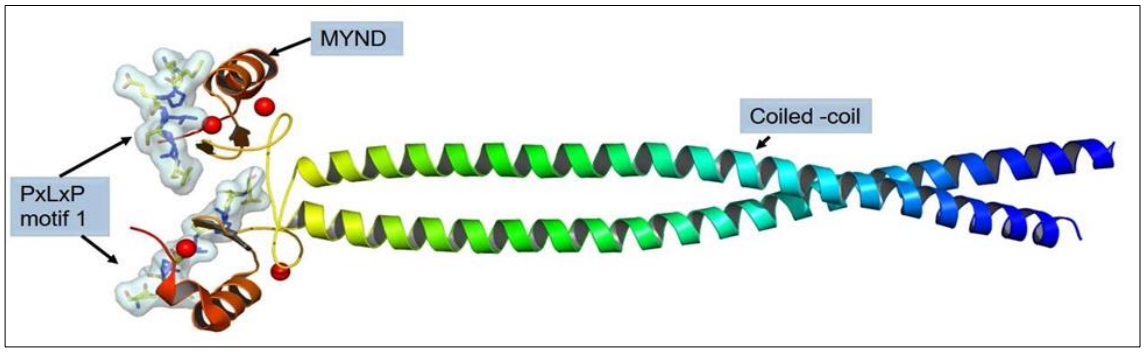

Fig E: *Structure of the BS69$_{CC-MYND}$-EBNA2$_{381–389}$ motif 1 complex. Ribbon representation of the complex of BS69$_{CC-MYND}$ domains (coiled-coil: multi-color; MYND: brown) and two EBNA2$_{(381–389)}$ peptide motif 1 (P and L residues: blue, X residues: yellow) showing intermolecular interaction between both MYND domains individually. The zinc ions are shown in red spheres.*

Using Isothermal Titration Calorimetry (ITC), the BS69$_{CC-MYND}$ and BS69$_{MYND}$ were shown to bind an EBNA2$_{(381–389)}$ motif 1 peptide from the CR7 region with dissociation constants (K_D) of 7.4 μM and 25.6 μM, respectively. The binding of EBNA2$_{(435–445)}$ peptide in the CR8 region had a K_D of 34.9 μM and 93.4 μM, respectively. Although there is no indication of direct binding of EBNA2 individual motifs with the coiled-coil domain, the inclusion of the coiled coil domain with the MYND domain increases the binding affinity of the PXLXP motifs. It is likely that the coiled-coil region increases the overall stability of the MYND domain and indirectly contributes to the binding affinity of EBNA2 peptides. When the BS69$_{CC-MYND}$ binds to a large region of EBNA2 containing the two PXLXP motifs (residue 381–445) the Kd is 0.24 μM, indicating that the binding avidity is 30-150-fold stronger compared to the binding of EBNA2$_{381–389}$ or EBNA2$_{435–445}$ peptides alone. Thus, it appears

that the $BS69_{CC\text{-}MYND}$ homodimer binds to the $EBNA2_{381–445}$ motifs in a synergetic manner which further aids in the enhancement of the BS69-EBNA2 interaction (Harter *et al*., 2016) [22].

Conclusion

EBV has the unique ability to transform resting B cells into permanently proliferating cell lines. EBV Nuclear Antigen 2 (EBNA2) is a key regulator of viral and cellular gene expression for this transformation process. EBNA2 gene sequence classifies EBV into type 1 and type 2. Type 1 EBNA2 TAD region contains an aspartate residue adjacent to the BS69 binding motif 2 at 442 position, which has reported for the superior growth maintenance in type 1 EBV. In type 2 EBNA2 protein, a serine residue is present at the equivalent position. Changing serine to aspartate (S442D) conferred type 1 growth phenotype in LCL.
No interaction study is reported whether the difference in amino acid at 442 position makes any change in the binding of type 1 and type 2 EBNA2 protein with BS69. Earlier it is hypothesised that the type 2 EBNA2 and BS69 might have higher binding affinity with second PxLxP motif than type 1, which might be the reason for EBNA 2 type 2 transformation efficiency is repressed more than type 1. Interaction studies showed that Type $1_{(435\text{-}445)}$ EBAN2 motif 2, containing aspartate at 442 position binds with the transcriptional repressor protein, $BS69_{cc\text{-}MYND\ (480\text{-}602)}$ more strongly than type $2_{(402\text{-}412)}$ EBNA2 which has a serine at the equivalent position. We know that aspartate in type 1 EBNA2 confers a stronger transactivation function and increases binding to some sites of the key genes like LMP-1 and CXCR7. Possibly, aspartate, being an acidic amino acid, adjacent to the motif 2 also interfere with the binding of type 1 EBNA 2 motif 2 with BS69 as compared to the type 2 motif 2. So, on the basis of motif 2 peptide interaction studies with BS69, it is proved that the difference in a single amino acid residue at 442 position did not show any increase in the binding affinity of EBNA2 type 2 as compared to the type 1.

Either this difference in the amino acids at 442 position is not the defining factor with respect to BS69 mediated higher repression of the transformation efficiency of type 2 EBNA 2 than type 1 or this would be too early to predict based on interaction study of a single EBNA 2 motif 2 peptides with BS69. By changing the serine with aspartate residue at 442 position in type 2 EBNA2 (containing all the three motifs) and then carrying out an interaction study with $BS69_{cc\text{-}MYND\ (480\text{-}602)}$ might better reveal the impact. If the binding affinity, stoichiometry, and other thermodynamic parameters of the mutated type 2 EBNA2-BS69 will appear as EBNA2 type 1-BS69 then it might confirm that this single amino acid, aspartate, is the defining factor which makes the difference in EBNA2 type 1 and type 2 transformation efficiency in EBV.

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