

EBV genes and B cell proliferation

Evelyne Manet *, Pierre-Yves Bourillot, Lucas Waltzer, Alain Sergeant

U412 INSERM, Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, 69364 Lyon Cedex 07, France

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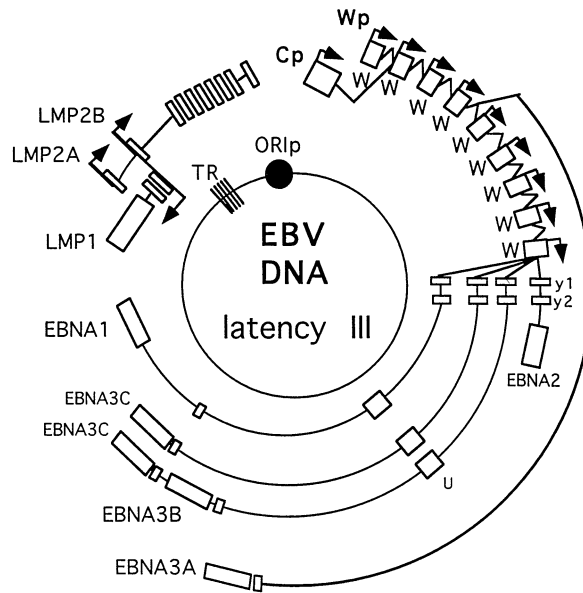
1. Introduction

The Epstein–Barr virus (EBV) has been the first virus isolated from a human tumour biopsy [1] the Burkitt's Lymphoma [2]. Since then, numerous reports had accumulated demonstrating that EBV, a B lymphotropic γ -herpesvirus, is widespread in the human population and is the causal agent of infectious mononucleosis [3]. It is also associated with several human cancers, including undifferentiated nasopharyngeal carcinoma (NPC) [4–6], Hodgkin's disease [6–8], rare nasal T cell lymphomas [7], gastric carcinoma

[9,10], breast carcinoma [11] and B and T cell lymphomas in immunocompromised individuals [6,7,12–14]. One early recognised effect of EBV on in vitro infected cells, was the induction of the indefinite proliferation of quiescent B lymphocytes (or immortalisation). Subsequently, it was also demonstrated that only a limited set of viral genes products was expressed in such cells defining a latency of type III: six nuclear proteins (EBNA1, 2, 3A (or EBNA3), 3B (or EBNA4), 3C (or EBNA6) and EBNA-LP (or EBNA5), three membrane proteins (LMP1, LMP2A (or TP1) and LMP2B (or TP2) and two small nonpolyadenylated nuclear RNAs (EBER1 and EBER2) (Fig. 1A) [15]. These genes products were expressed from mRNAs generated from a giant precursor RNA initiated at the

* Corresponding author. Tel.: +33 4 72728176; fax: +33 4 72728686.

A



B

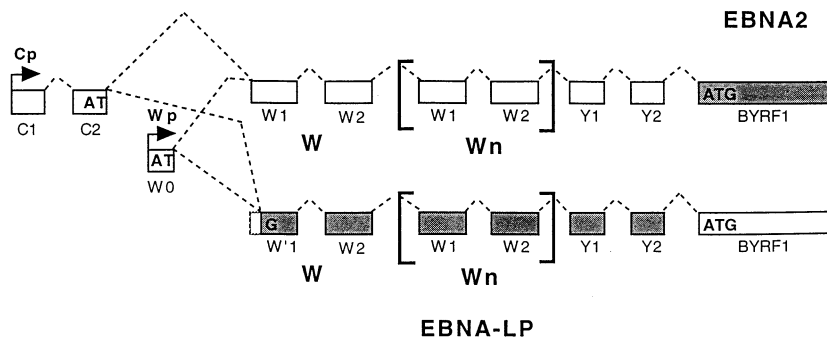


Fig. 1. Schematic representation of the EBV mRNAs expressed in B lymphocytes during latency III *in vitro*. (A) Several mRNA precursors are initiated at three different promoters: Cp, LMP1/LMP2B and LMP2A (the initiation sites and the sense of transcription are depicted by arrows). From the precursor RNAs initiated at promoter Cp, the mRNAs for EBNA2, EBNA-LP, EBNA3A, EBNA3B, EBNA3C, EBNA1 are generated by facultative splicing. From precursor RNAs initiated at the bidirectional LMP1/LMP2B promoter and at the LMP2A promoter, LMP1, LMP2B and LMP2A mRNAs are generated (open boxes represent exons and lines represent intron sequences). The representation of the mRNA encoding EBNA3B is only hypothetical since no cDNA containing the EBNA3B ORF has been isolated. (B) Detailed representation of how EBNA2 and EBNA-LP mRNAs are generated from a unique RNA precursor by facultative splicing. Boxes represent exon sequences and broken lines represent intron sequences. The exons coding for EBNA2 or EBNA-LP respectively, are indicated by the grey boxes. The EBNA-LP protein AUG is created following the use of an alternative acceptor splice site, five bases into the W1 exon. This alternate splice provides the G of the EBNA-LP initiation codon. In latency III, only the Cp promoter is active.

Cp promoter or alternatively at a downstream promoter called Wp (Fig. 1A). It became also clear that some or all of these genes products might act on the cell cycle regulators to induce the permanent proliferation of B cells. The first major contributions that allowed the EBV researchers to evaluate the function of these genes in the proliferation of B cells *in vitro* came from the demonstration that the EBNA2/EBNA-LP-deficient EBV strain P3HR1 could be trans-complemented with

or recombined with the EBNA2 gene [16–18]. Such princeps experiments demonstrated unambiguously, that EBNA2 was required for immortalisation of B cells but not EBNA-LP, and were followed by similarly designed experiments which demonstrated that among the EBV genes expressed in latency III, only EBNA2, EBNA3A, EBNA3C and LMP1 were required for the *in vitro* immortalisation of B lymphocytes by EBV [19,20]. Another major contribution consisted in the

generation of mini EBV plasmids containing non-contiguous EBV DNA sequences from which all the latency III EBV genes products were expressed [21,22]. When introduced in primary human B lymphocytes, these mini EBV genomes were sufficient both to initiate and to maintain the proliferation of B cells. Altogether, the results described above demonstrated that a limited set of EBV genes was required for B lymphocyte immortalisation *in vitro*.

What will be described and discussed in this review is the latest information obtained on the molecular mechanisms by which some of these EBV genes products affect the growth of B cells *in vitro*.

2. EBV genes required for B cell proliferation *in vitro*

2.1. EBNA2 and EBNA-LP

2.1.1. Co-operation in cell cycle progression

EBNA2 and EBNA-LP pre-mRNAs are initiated either at the Cp or Wp promoters, and EBNA-LP is encoded by the leader of each EBNA mRNA, but is expressed only from those mRNAs in which exon W' is included, creating the EBNA-LP ATG (Fig. 1B) [23]. Both EBNA2 and EBNA-LP are nuclear proteins, but only some of the mechanisms of action of EBNA2 have been now established. It is a transcription factor recruited to EBNA2-responsive elements by the cellular specific DNA-binding factor RBP-J κ /CBF1/KBF2 (Fig. 2) [24–29]. It activates transcription initiated at the Cp, LMP1 and LMP2A promoters and thereof of all the EBV genes expressed in latency III (see Fig. 1). Accordingly, EBNA2 is essential for B cell immortalisation, and this has been unambiguously shown by using a mini EBV expressing an EBNA2 chimeric fusion protein with the hormone binding domain of the oestrogen receptor (Fig. 3A) [30]. Immortalisation of B cells by this recombinant mini EBV was oestrogen-dependent. However, upon withdrawal of estrogen, the expression of EBNA1, EBNA2, EBNA-LP, EBNA-3A, -3B, -3C, LMP1 and LMP2 decreased dramatically, and the B cells stop proliferating in G1 and in G2/M (Fig. 3A). Upon re-addition of oestrogens, only the cells arrested in G1 re-entered the cell cycle. Although these experiments did not evaluate the direct contribution of EBNA2 in the regulation of the cell cycle, they clearly demonstrated that EBNA2 was a key activator of EBV genes expression, was essential for both initiation and maintenance of B cell permanent proliferation, and that among the EBV EBNA2-activated genes some might be acting on the regulation of the cell cycle.

In one report, however, a direct contribution of EBNA2 in the deregulation of the cell cycle has been evaluated. It was shown that by exposing resting human B-lymphocytes to purified EBV glycoprotein gp340,

which interacts with the EBV receptor, CD21, it was possible to efficiently express transfected DNA in primary B cells without causing the cells to enter the cell cycle [31]. Thus it has been possible to assess the direct effect of EBNA2 expression on primary B cells. Interestingly, an induction of cyclin D2 expression was observed, suggesting that EBNA2 induces resting B cells to progress through the G1 phase, but this was observed only when EBNA2 was acting in concert with EBNA-LP [31] (Fig. 3B). It appeared that EBNA2 in co-operation with EBNA-LP does participate in the initial disruption of the cell-cycle control in a transient expression assay, and might therefore be essential for the initiation of the immortalisation of resting B lymphocytes by EBV. These results are not completely in agreement with early reports suggesting that EBNA-LP was not required for B cell immortalisation *in vitro*, although EBNA-LP might potentiate the growth of *in vitro* immortalised B cells. This EBNA-LP function was attributed to the two last coding exons Y1 and Y2. Indeed, EBV recombinant viruses lacking the EBNA-LP Y1 and Y2 exons showed a reduced although still detectable immortalizing effect [32]. However, and as suggested by the experiments of Sinclair et al. [31], two recent reports clearly suggest that EBNA-LP might be an essential gene for B cell immortalisation by EBV [33,34].

2.1.2. Co-operation in transcriptional activation

It has been shown independently by two groups that EBNA2 and EBNA-LP co-operate in activating LMP1 expression, although these two proteins do not seem to co-operate in activating transcription initiated at the Cp promoter [33,34]. Two independent experimental approaches have been used, one essentially based on transient expression assays in EBV negative B cells [33], the other based on transient expression assays in latency I Burkitt's lymphoma cells [34]. From these reports, one learned that EBNA2 and EBNA-LP co-operate in activating the expression of LMP1, probably by increasing transcription initiated at the LMP1 promoter. This up-regulation of LMP1 is dependent on RBP-J κ binding sites, and the two allelic forms of EBNA2 were as efficient in co-operating with EBNA-LP. Interestingly, an EBNA-LP with four copies of W1W2 repeat but lacking the Y1Y2 domain was almost as active as the wild type EBNA-LP protein in co-operative activation of transcription at the LMP1 promoter. Therefore recombinant EBV viruses carrying a deleted Y1Y2 domain might have retained a partial wild type activity in co-operative activation of LMP1 with EBNA2 [32], leading to the conclusion that EBNA-LP was only accessory to the immortalizing process of B cells by EBV. It is therefore important to examine the effect of EBNA-LP W1W2 mutations to assess the function of EBNA-LP in immortalisation. Moreover, since EBNA2 and EBNA-LP co-operate to induce cyclin

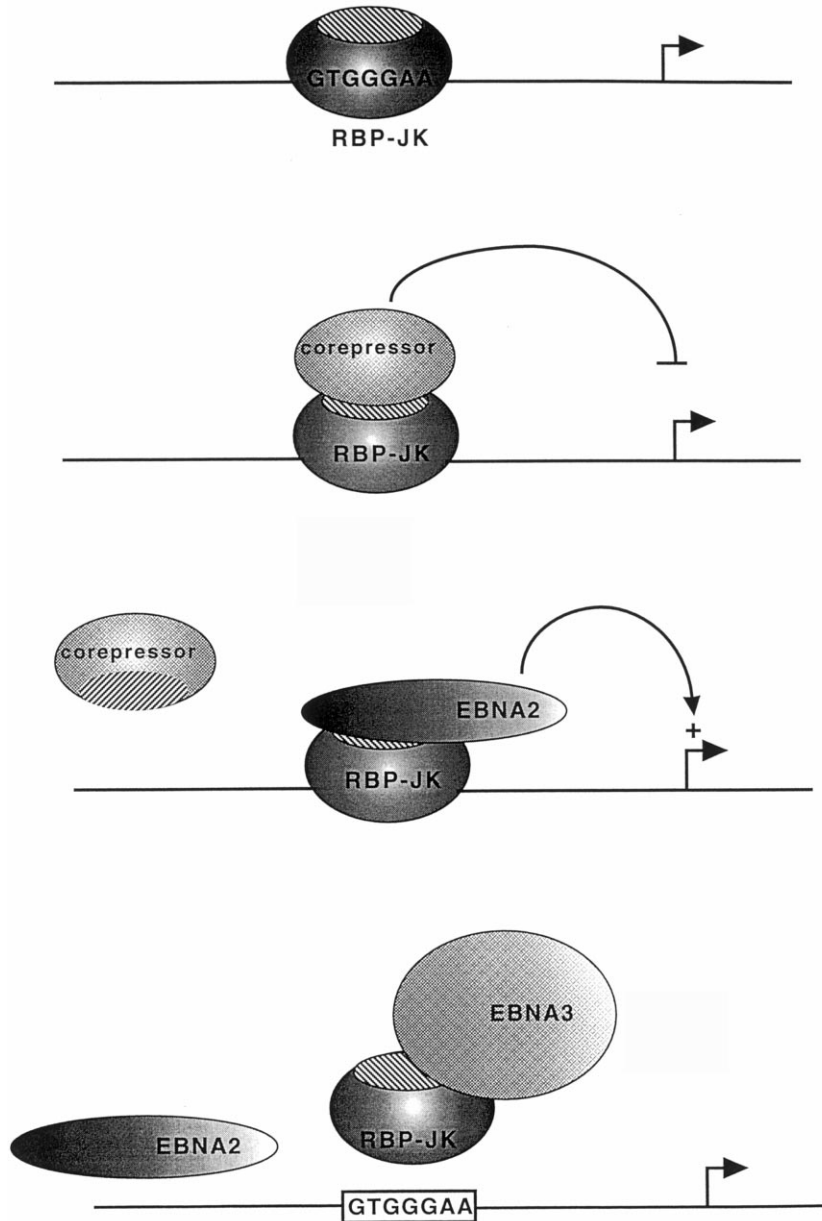


Fig. 2. RBP-Jk/CBF1 is a key regulator of EBV genes expressed during latency III. RBP-Jk binds to EBNA2 responsive elements (GTGGGAA) localised in promoters Cp, LMLP1/LMP2B and LMP2A (see Fig. 1A), and recruits EBNA2 to EBNA2-responsive genes. This recruitment has two effects, it masks the repressing activity of RBP-Jk probably by interfering with the function of a co-repressor bound to RBP-Jk, and it provides a transcription activation domain located at the C-terminal of EBNA2. The EBNA3s repress EBNA2-activated transcription by interacting with RBP-Jk and probably by destabilising the binding of RBP-Jk to DNA.

D2 expression [31], it would also be interesting to see whether they do so directly or indirectly. Since it has been shown that EBNA2 activates cellular genes such as CD23, CD21 and cfgr [35–38], it would be important to see if more genes could be characterised that are activated by co-expressing EBNA2 and EBNA-LP. Last but not least, it is also urgent to identify the mechanism(s) by which EBNA2 and EBNA-LP co-operate in transcriptional activation at the LMP1 promoter.

2.2. EBNA3A and EBNA3C

By means of EBV or mini EBV recombinant viruses, it has been established that among the three EBNA3 genes products, only EBNA3A and EBNA3C are essential for B cell immortalisation [20]. How these nuclear proteins participate in the immortalisation process is still unknown, but some proposals based on experimental data have been made.

2.2.1. EBNA3A, EBNA3B and EBNA3C represses EBNA2-mediated transcriptional activation

Investigating the effect of the EBNA3s on the EBV TP1 (LMP2a) promoter, Le Roux et al. [39] observed that the EBNA3s reduced strongly the EBNA2 activated transcription initiated at this promoter. Since then, it has been demonstrated that both EBNA3A and EBNA3C repress EBNA2-activated transcription by directly contacting RBP-J κ and by destabilising the binding of RBP-J κ to DNA (Fig. 2) [40,41]. This is also

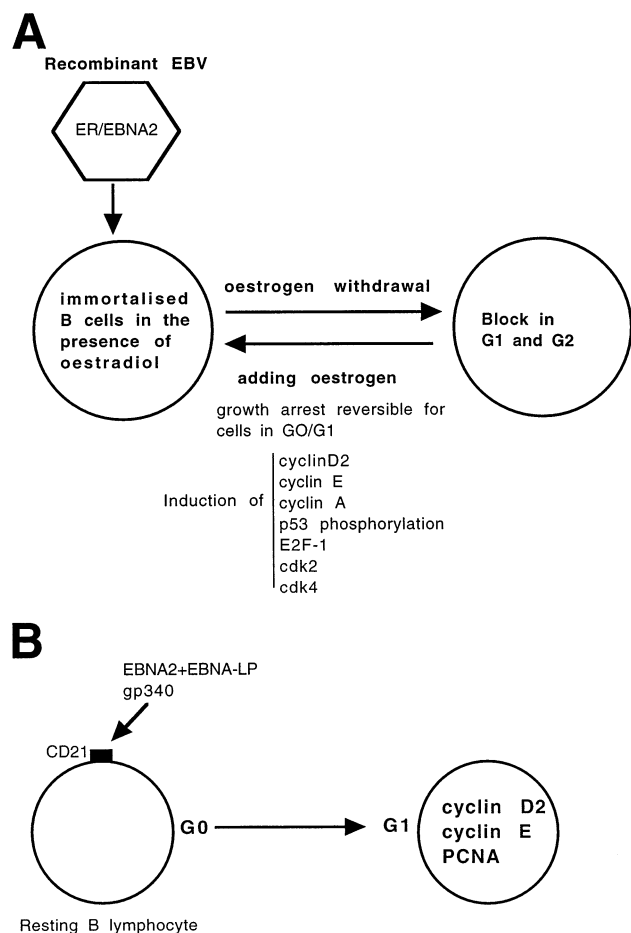


Fig. 3. EBNA2 is essential for both the initiation and the maintenance of B cell immortalisation in vitro. (A) An EBV conditional mutant in which EBNA2 was fused to the hormone-binding domain of the oestrogen receptor (ER/EBNA2), immortalises primary B cells in an oestrogen-dependent manner. Upon withdrawal of oestrogen from the culture medium, all B-cell clones stopped proliferating in G1 and in G2/M. Upon re-addition of oestrogen, only the cells arrested in G1 re-entered the cell cycle and the re-induction of cell growth was followed by the sequential activation of the expression of the early G1 regulating proteins cyclin D2 and cdk4, as well as cyclin E required for the G1/S transition. (B) By exposing resting human B lymphocytes to purified EBV glycoprotein gp340, which interacts with the EBV receptor CD21, it is possible to efficiently express transfected DNA in primary B cells without causing the cells to enter the cell cycle. Transfection of EBNA2 in such primary B cells induced cyclin D2 expression, suggesting that EBNA2 induced resting B cell to progress through the G1 phase, but only when acting in concert with EBNA-LP.

probably true for EBNA3B, but this has not yet been shown directly. There is therefore three proteins in EBV infected cells that can repress the EBNA2-activated transcription, and the mechanism of repression is identical. An obvious explanation for this puzzling observation would be that there is a loop of autoregulation of the EBV genes expressed in infected B cells, and only those cells in which latency III genes products are expressed in the correct relative amounts will survive and proliferate in vitro. However, this explanation leaves open the questions of why are three EBNA proteins required for such a negative loop of regulation, and are there other functions for the EBNA3s in immortalisation, and especially for EBNA3B which is not essential to this process.

2.2.2. EBNA3A is essential for initiation of B cell immortalisation

While producing the 71 kbp mini-EBV recombinant genome, one such mini-genome was found to have a point mutation in the EBNA3A gene. This mini-EBV was able to immortalise resting B cells, only when complemented by a helper virus which probably transprovided a functional EBNA3A protein. However, on the long term, the complementation by a functional EBNA3A protein was not necessary anymore, suggesting that EBNA3A was only essential for the initiation of B cell immortalisation, but was dispensable for the maintenance of B cell proliferation [21]. Since EBNA2 is the transcription factor that activates the expression of EBNA3A, it could well be that EBNA3A induced by EBNA2 is the key protein for the initiation of immortalisation of B cells.

2.2.3. EBNA3C as an oncoprotein co-operating with ras

Another function for EBNA3C, directly related to the cell cycle regulation has been reported by Parker et al. [42]. In this one report, it has been first shown by transient expression assays that EBNA3C activates the B-myb promoter in an E2F-dependent manner. The E2F-responsive promoter, B-myb promoter, is inactive in the G1 phase of the cell cycle, and only active when cells progress in the S phase. It was also shown that EBNA3C co-operates with (Ha)-ras to transform primary rat embryo fibroblasts (REFs), with an efficiency similar to the human papilloma virus E7 protein. As the Ras/E7 co-operation in transformation of REFs requires the binding of E7 to Rb and the displacement of E2F which then can exert its functions in the cell cycle progression, it was therefore tempting to speculate that EBNA3C interacts with pRb and induces the release of E2F. Such a possibility was indirectly documented in the following experiment: the cyclin-dependent kinase inhibitor p16^{INK4A}, by binding to the cyclin-dependent kinases 4 and 6, suppresses the pRb-

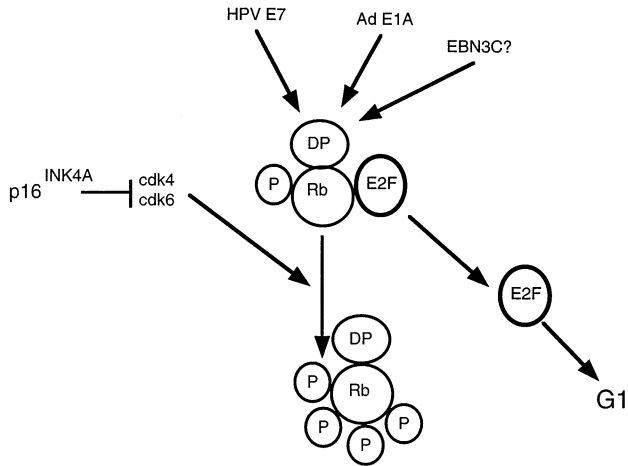


Fig. 4. EBNA3C is functionally equivalent to adenovirus E1A and HPV E7. p16^{INK4A} binds to and inhibits the activity of cdk4 and cdk6, which do not phosphorylate Rb and prevents both the release of E2F/(DP) and the proliferation of Rb-positive cells. In transformation assays of rat embryo fibroblasts by (Ha-)ras and E7 or (Ha-)ras and E1A or (Ha-)ras and EBNA3C, p16^{INK4A} has no effect, suggesting that EBNA3C contacts directly or indirectly Rb and induces the release of E2F/(DP).

phosphorylation, the release of E2F and thus progression through the G1 phase restriction point (Fig. 4). Therefore p16^{INK4A} prevents the proliferation of pRb-positive cells but not of pRb-negative cells. Consequently, it suppresses transformation of REFs by (Ha-)ras and Myc, but not by (Ha)ras and E7 or E1A. Similarly, if EBNA3C binds to Rb and releases E2F, then p16^{INK4A} should not suppress transformation of REFs by EBNA3C and (Ha-)ras, and that is what was observed. Although EBNA3C does not have the LX-CXE motif essential for interaction with the pRb 'pocket', pull down assays using GST-pRb fusion proteins suggested that EBNA3C and pRb may interact directly in vitro. However, no in vivo direct interaction could be shown. It is therefore possible that EBNA3C, in infected B cells co-operates with an unknown partner to neutralise the pRb/E2F induced growth repression, and allows the entry in S phase of the cell cycle. In EBV infected resting B cells, EBNA2/EBNA-LP-induced EBNA3A and EBNA3C might therefore respectively induce the progression from G0 to G1 and the entry in S phase (Fig. 6).

2.3. LMP1

LMP1 is an integral membrane protein, with a short cytoplasmic amino terminus, six membrane-spanning segments and a long cytoplasmic carboxy terminal (Fig. 5) [43]. This transmembrane protein has been recognised as a major EBV gene product essential for the

immortalisation of B cells [19], and due to its structure, LMP1 was proposed as a constitutively active receptor, influencing mitogenic signal transduction pathways in EBV infected cells. Indeed, LMP1 has been shown to trigger cellular NF- κ B activity through two C-terminal intracytoplasmic domains, CTAR1 and CTAR2 (CTAR stands for C-terminal Activating Region) [44,45], and recently, it has also been shown that CTAR2 triggers the activity of the N-terminal activation domain of c-jun through the Jun N-terminal kinase (JNK) pathway [46].

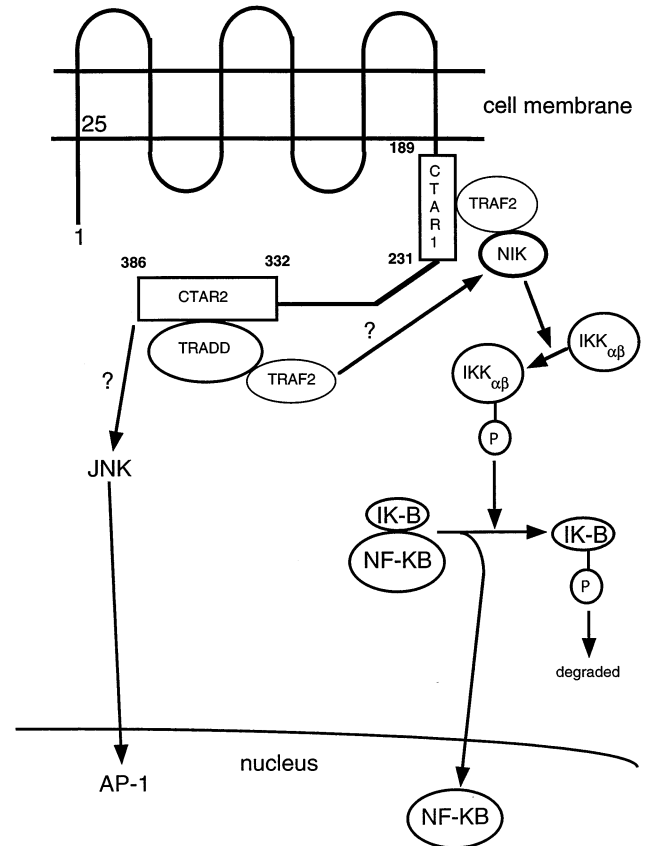


Fig. 5. LMP1 acts as a transmembrane receptor constitutively stimulating pathways leading to the activation of NF- κ B and AP-1. LMP1 is a transmembrane protein with no ligand binding domain identified. The activation of NF- κ B by LMP1 seems to operate through the C-terminal domain called CTAR1 (C-terminal domain activation 1). CTAR1 recruits TRAF2 (TNF receptor associated protein 2) which binds the NF- κ B activating kinase (NIK), that phosphorylates and activates the two IKK-kinase α and β (IKK). IKK- α is then phosphorylated by the IKK, ubiquitinated, degraded by the proteasome leading to the release of NF- κ B and its translocation in the nuclei. The CTAR2 domain induces the activity of both NF- κ B and Jun. For NF- κ B, one may speculate that since CTAR2 recruits TRADD (TNF receptor associated death domain) which contacts TRAF2, it might activate NF- κ B by a pathway similar to CTAR1. For the activation of AP-1, it seems to operate through the activation of the Jun N-terminal kinase (JNK), by mechanisms which are not yet elucidated.

2.3.1. LMP1 acts as a constitutively activated membrane receptor

It has long been suspected that LMP1 could act as a constitutively activated ligand-independent receptor. Indeed, LMP1 molecules aggregate and form patches at the cell membrane [47,48]. Like the activation of CD40 by its ligand, CD40L, LMP1 expression induces B cells surface activation markers and adhesion molecules [49–51]. Like growth factor receptors, LMP1 has a rapid turnover [52], and recently, it has been shown that LMP1 activates NF- κ B in part through a pathway that involves the recruitment by domain CTAR1 of TNF-RI receptor associated factors or TRAFs (Fig. 5) [45,53–55]. However, no direct experimental data were provided as a proof that LMP1 acts as a ligand-independent constitutive transmembrane protein. This is now done very elegantly by Gires et al. [56]: recombinant LMP1 proteins carrying the C-terminal domain of LMP1, linked either to one or several LMP1 transmembrane domains or to heterologous transmembrane domains were constructed. The induced-oligomerisation of the amino-transmembrane domains was sufficient to stimulate within minutes the activation pathway of NF- κ B, demonstrating directly that the carboxy-terminal region of LMP1 carries the signalling domain.

2.3.2. LMP1 induces NF- κ B and AP-1 activity

Although upon oligomerisation of wild type LMP1 or of chimeric LMP1 molecules, the NF- κ B activation pathway is induced, LMP1 transforms BALB/3T3 cells without inducing NF- κ B [57,58], indicating that LMP1 might activate other pathways. Indeed, it has been shown by Kieser et al. [46] that LMP1 also triggers AP-1 activity via the c-jun N-terminal kinase (JNK). The domain required for the AP-1 activation is contained in the LMP1 55 C-terminal amino-acids domain (CTAR2 domain), which is not contacting directly the TRAFs (Fig. 5). However, it has been shown that this CTAR2 domain, like the TNF-RI receptor, binds to TRADD (Fig. 5) (TNF receptor associated death domain) [59], which in fact can recruit TRAF2 and activate NF- κ B at least when recruited by TNF-RI. It is therefore possible that LMP1 activates the NF- κ B activity through TRAF2 via both CTAR1 and CTAR2, by recruiting the NIK (NF- κ B inducing kinase) which binds to and phosphorylates the I κ B kinases α and β (I κ B kinases α and β). The activated I κ B kinases would then recruit and phosphorylate I κ B and initiate its degradation pathway leading to the release and translocation of free NF- κ B to the nucleus where it will modulate the expression of NF- κ B-dependent gene expression (Fig. 5). Therefore, LMP1 seems to act as a constitutive transmembrane effector of different pathways that trigger the activation of NF- κ B and AP-1.

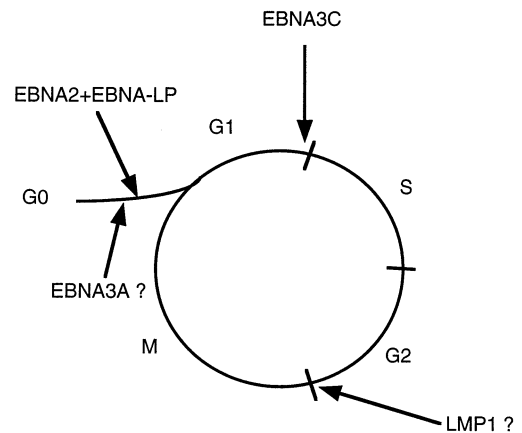


Fig. 6. Model on how the effect of EBV gene products expressed in latency III could affect the cell cycle. EBNA2 plus EBNA-LP are essential for the expression of EBNA3A, EBNA3C and LMP1. However, EBNA2 plus EBNA-LP as well as EBNA3A are essential for the initiation of immortalisation of quiescent B lymphocytes, although they might act on different targets. Once initiated, EBNA3C seems to be essential for the transition G1-S, and one could speculate that LMP1 is essential for the transition G2-M.

3. Conclusions and perspectives

A model could be proposed that partly explains how several EBV genes products are simultaneously required and essential for B lymphocytes permanent proliferation in vitro. It seems that they act at different points of the cell cycle, insuring that cells will continuously cycle, although some of these gene products might have redundant functions (Fig. 6). One open question is still to determine what will be the effect of a conditional expression of LMP1 on immortalised B cells in vitro. Knowing that inactivation of EBNA2 blocks the proliferation of B cells in vitro in G1 and G2, and since EBNA2 is a key regulator of LMP1 expression, it would not be surprising that a conditional inactivation of LMP1 would have the same effects as those of conditional inactivation of EBNA2. Many other questions stay open, like what are the molecular functions of EBNA3A and EBNA3C in immortalisation? Is EBNA-LP essential for B-cell immortalisation in vitro, and what are the exact mechanisms by which EBNA-LP activates transcription synergistically with EBNA2? At which level(s) are EBNA2, EBNA3A, EBNA3B and LMP1 acting on the cell cycle? Although EBNA3B and LMP2A/2B are not essential for B cell immortalisation, why are these genes conserved and expressed in immortalised B cells after many years of selective pressure by the immune system, and what are their functions? The answer to such questions is a challenge for the future, and might help understanding the molecular basis of the implication of the virus in the development of the diverse EBV-associated pathogenesis.

Acknowledgements

We apologise in advance for having not cited all the data which have been published on the topic discussed in the review. We also wish to thank Conrad B. Blunk for reading the manuscript.

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Biographies

Evelyne Manet, Ph.D., a graduate from Lyon I University, is a CNRS senior scientist working in the INSERM Unit U412 at the Ecole Normale Supérieure de Lyon in Dr Alain Sergeant's group. *Pierre-Yves Bourillot* is a Ph.D. student at the Ecole Normale Supérieure de Lyon working in Dr Alain Sergeant's group. *Lucas Waltzer*, Ph.D., a graduate from the Ecole Normale Supérieure de Lyon, is now working as a postdoctoral fellow in Dr Mariann Bienz's group at the Laboratory of Molecular Biology (MRC) in Cambridge, England. *Alain Sergeant*, Ph.D., a graduate from the University of Lille, received his virological education from Heschel Raskas (St. Louis, USA), Dominique Stehelin (Lille, France) and Walter Keller (Heidelberg, Germany). He currently is directing a group in the INSERM Unit U412 at the Ecole Normale Supérieure de Lyon.