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THE EPSTEIN BARR NUCLEAR ANTIGEN EBNA3C REGULATES TRANSCRIPTION, CELL TRANSFORMATION AND CELL MIGRATION

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1. ABSTRACT

The Epstein-Barr virus (EBV) infects most of the human population and persists in B-lymphocytes for the lifetime of the host. During the establishment of latent infection a unique repertoire of genes are expressed. The EBV nuclear antigen EBNA3C is essential for growth transformation of primary B-lymphocytes *in vitro*. EBNA3C regulates the transcription of a number of viral and cellular genes important for the immortalization process. Interaction of EBNA3C with the cellular transcription factor RBP-Jκ and HDAC1 modulates transcriptional activation. Additionally, EBNA3C disrupts the cyclin/cdk-Rb-E2F pathway that regulates cell cycle progression through the restriction point at G_1. Recent studies showed that the carboxy terminal region of EBNA3C from aa 366-992, essential for the immortalization of primary B-cells, interacts with Prothymosinα (ProTα) and Nm23-H1. The interaction of EBNA3C with ProTα as well as the histone acetylase p300 suggested a possible role in modulation of histone acetylation and chromatin remodeling. Cell migration assays geared towards determining the effect of EBNA3C on Nm23-H1 antimetastatic function suggests that EBNA3C suppresses the effects of NM23-H1 on the motility of breast carcinoma as well as Burkitt's lymphoma cells. This observation suggests that EBNA3C may be involved in driving the metastatic process in EBV associated human malignancies.

2. INTRODUCTION

Epstein-Barr Virus (EBV) is arguably the most intensely studied member of the gammaherpesvirus family. EBV predominantly infects two human cell types *in vivo*, classically establishing lytic infection in the oropharyngeal epithelium and latent infection in B-lymphocytes (1, 2). EBV is the etiologic agent of infectious mononucleosis and is also associated with a number of human malignancies, including Burkitt's lymphoma, nasopharyngeal carcinoma, post-transplant and AIDS-associated lymphomas, and Hodgkin's disease (2, 3). Preliminary studies have also demonstrated the presence of EBV in breast carcinomas (4, 5). *In vitro* infection of primary B-lymphocytes by EBV results in the formation of continuously proliferating lymphoblastoid cell lines (LCLs). This latent infection is usually established as so-called type III latency and is characterized by the expression of 11 latent viral gene products. These include six nuclear antigens, three latent membrane proteins, and two small non-polyadenylated RNA transcripts of uncertain function (2, 6-10). Of these 11 gene products, EBNA1 is essential for maintenance of the EBV episome in latently infected cells, and only a subset, namely EBNA-LP, EBNA2, EBNA3A, EBNA3C, and LMP1, are essential for the growth transformation of primary B-lymphocytes (1, 9, 11-13). The mechanisms underlying B-lymphocyte growth transformation mediated by EBV and the expression of a type III latency program have been under intensive investigation. These

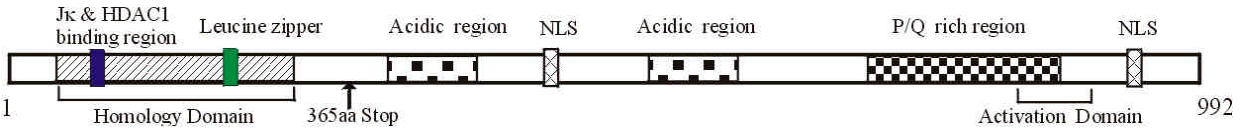

Figure 1. Schematic representation of Epstein Barr Nuclear Antigen EBNA3C (14). NLS – Nuclear Localization Signal. P/Q rich region – Proline and Glutamine rich region. Second site recombination studies (13) in which a stop codon was introduced at the SpeI site (amino acid 365) of EBNA3C showed that amino acids 366-992 are essential for the growth transformation of primary B-lymphocytes.

mechanisms are particularly relevant to the following: (1) the early stages of primary infection in humans, (2) lymphoproliferative disease that occurs in immunosuppressed patients, (3) the identification of genetically susceptible individuals, (4) the experimental infection of new world primates, and (5) other human malignancies in which a number of the essential latent gene products are expressed (1, 2, 15).

3. THE MOLECULAR GENETICS OF THE EBNA3 PROTEINS

The EBNA3 proteins share significant sequence homology and is tandemly arranged in the EBV genome suggesting gene duplication from a common ancestral gene (1, 16, 17). Each EBNA3 transcript is encoded by a short 5' exon and a long 3' exon. The predicted amino acid sequence of the type I and type II EBNA proteins are 944 and 925 for EBNA3A, 938 and 946 for EBNA3B, and 992 and 1069 for EBNA3C respectively (1, 16-18). The EBNA3 proteins encode a number of similar motifs, including a binding site for the transcriptional regulator RBP-Jκ, a putative leucine zipper motif, an acidic domain, proline and glutamine-rich repeats, and several arginine and lysine residues potentially important for nuclear localization (Figure 1) (14).

With advances in EBV recombinant molecular genetics in the early 1990s, it became possible to directly, and individually, evaluate the role of a latency gene product in EBV transformation of primary B cells. The effect of a single nonsense mutation in each of the EBNA3 genes was investigated by second site homologous recombination experiments. These experiments utilized a derivative of the Burkitt's lymphoma cell line Jijoye, referred to as P3HR-1, that harbors a transformation-deficient virus. This defective virus is deleted for the EBNA2 and EBNA-LP genes, important for B-lymphocyte transformation. By transfecting P3HR-1 with an EBV restriction fragment containing wild type EBNA2 and EBNA-LP genes, followed by induction of lytic replication, a population of transformation-competent viruses can be recovered by homologous recombination, which rescues the P3HR1 defect.

The EBNA3 second site recombination experiments involved co-transfecting a DNA fragment containing a null EBNA3 gene in addition to the EBNA2/EBNA-LP EBV rescue fragment. After inducing lytic replication, primary B-cells were infected with the virus pool and plated to allow outgrowth of LCLs. All LCLs established from the P3HR-1 virus will have wild type EBNA2 and EBNA-LP by recombination which rescues the deletion. Additionally, a sub-population of these LCLs can potentially undergo recombination for the null EBNA3 protein. When a nonsense codon was introduced at aa109 in the EBNA3B open-reading frame, the frequency with which LCLs carried virus recombinant for the null EBNA3B gene was about 12%. Of these, 50% were co-infected with P3HR-1 wild type for the EBNA3B gene. LCLs carrying EBNA3B null virus were indistinguishable from wild-type recombinant LCLs both in growth and in response to activation of lytic EBV infection. This clearly indicated that the last 829 codons of EBNA3B are not essential for growth transformation of primary B-lymphocytes *in vitro* (12). The fact that EBNA3B is dispensable for growth transformation *in vitro* suggests that this *in vitro* transformation assay may be limited under standard tissue culture conditions. EBNA3B, a potent stimulator of the T-cell response in humans, is unlikely to have persisted in the viral genome without an important role in viral infection *in vivo* (1, 2).

In contrast to EBNA3B, a nonsense codon introduced at aa309 in EBNA3A or aa365 in EBNA3C resulted in a small number of transformation-competent recombinants. Those that grew out were uniformly co-infected with P3HR-1 that provided the wild-type EBNA3 protein *in trans*. The frequency of recombinant-infected LCLs was less than 2% in both cases, and the recombinant EBV in co-infected LCLs lost their mutant genes through natural selection or via recombination with the co-infecting wild-type EBV genome (13). These data strongly suggest the importance of both the carboxy-terminal 640 amino acids of EBNA3A and 627 amino acids of EBNA3C in primary B-lymphocyte growth transformation. In another experiment, LCLs infected with P3HR-1 were transfected with an F-factor-derived plasmid containing the known transforming regions of the EBV genome and a frame shift mutation at codon 304 of EBNA3A (1, 14). All LCLs that arose from these experiments were co-infected with P3HR-1 helper virus or were EBNA-LP and EBNA2 rescued recombinant but wild type at the EBNA3A locus. However, two LCLs derived from the F plasmid based assay and lacked wild-type EBNA3A were isolated suggesting that the last 640 amino acids of EBNA3A may be dispensable for establishment and maintenance of LCLs (1, 14). More definitive experiments are required to confirm this result in terms of genetic analyses to determine the nature of the EBV DNA backbone containing these EBNA3A mutations.

A second approach for generating EBV recombinants utilized another transformation-deficient virus. This virus infects the naturally existing Raji cell line, one of the earliest derived EBV-infected Burkitt's lymphoma lines (19). The Raji EBV genome is defective in

both growth transformation and in replication due to two separate deletions in the viral genome (20). One of the deletions is approximately 3 KB in size and removes most of the EBNA3C coding region. The second deletion encompasses a portion of the BALF2 gene to the amino terminal region of the LMP2 open reading frame (1, 21). The BALF2 gene product is a single-stranded DNA binding protein essential for early lytic DNA replication (1, 19). Transfection of the Raji cell line with a BALF2 expression vector permits productive viral replication after treating stably expressing cells with chemical inducers of viral replication, phorbol ester and sodium butyrate (1, 19). Because the Raji virus is unable to growth transform primary B-lymphocytes due to EBNA3C deletion, this cell line provides an alternative tool for genetic analysis of the EBNA3 family of proteins. Inducing viral replication in this system and utilizing homologous recombination can rescue the EBNA3C deletion rescued using a wild-type fragment containing the EBNA3C gene and the flanking regions (19). The LCLs derived by these recombination events expressed wild-type EBNA3C similar to other wild-type LCLs but were unable to replicate or produce viral progeny, as they did not express BALF2. Additionally, over a period of six months the recombinant LCLs grew much slower and most of the cell lines were lost in culture. Nevertheless, by creating mutations linked to a DNA fragment, which overlaps the EBNA3C deletion, recombinant EBNA3C mutant viruses can be tested for their ability to growth transform primary B-lymphocytes. Similarly, the other EBNA3 genes as well as adjacent open reading frames can potentially be evaluated for their essential domains required for EBV mediated B-cell immortalization.

4. TRANSCRIPTIONAL REGULATION BY EBNA 3 FAMILY OF PROTEINS

EBNA3C is expressed in EBV-infected human B cells on activation of the major latent Cp promoter by the activity of another essential EBV antigen EBNA2. The level of EBNA3C remains remarkably steady in LCLs and is similar to other EBNA3 molecules (1). The highly hydrophilic EBNA3C protein contains a potential leucine zipper motif near the amino terminus, acidic domains, a carboxy-terminal glutamine-proline-rich domain that functions as a transactivation domain in gene fusion assays and several arginine and lysine residues that are potentially important for nuclear translocation (Figure 1) (1, 14, 22). Upregulation of CD21 in non-EBV-infected Burkitt's lymphoma cells (23) and down modulation of LMP1 expression in G_1-arrested Raji cells (24) was the first evidence showing transcriptional function of EBNA3C (1, 2). The EBNA3C protein does not bind to DNA specifically, although its amino acid sequence includes basic residues and a coiled coil domain reminiscent of c-Fos- and c-Jun-type transcription factors (22). Even though EBNA3C upregulates CD21, the level of activation is lower than that seen in studies with EBNA2 a well-studied acidic transactivator of viral and cellular gene transcription (23). In addition to CD21, EBNA2 upregulates the transcription of viral proteins including the EBNA3s, EBNA1 and LMPs and the cellular genes CD23 and c-fgr (23, 25-27). EBNA2 recruits cellular transcription factors to specific promoters through its interaction with sequence specific DNA binding proteins, including RBP-Jκ and PU.1 (20, 28-32). EBNA2 also activates transcription of the LMP1 promoter through its interaction with CBP, p300 and PCAF histone acetyl transferases (33).

Although genetic analysis clearly showed a critical role for EBNA3A and EBNA3C in EBV-induced growth transformation of primary B-lymphocytes, there is very little previous evidence to explain the biochemical function of these EBNA3 proteins in regulating transcription. Allday and colleagues showed that the EBNA3C protein might be involved in the regulation of LMP1 expression thereby playing an important role in influencing cellular and viral transcriptional events (24). EBNA3B was shown to be dispensable for growth transformation, however, upregulation of the cytoskeletal protein Vimentin and CD40 and down regulation of the Burkitt's lymphoma associated antigen CD77 by EBNA3B suggested that EBNA3B may function as a transcriptional regulator in EBV infected B-lymphocytes (34). Another line of experiments showed that the EBNA3 family of proteins upregulate the expression of Pleckstrin (an acronym for platelet and leukocyte C kinase substrate) thought to be involved in signaling and differentiation of hematopoietic cells (35).

Regulation of the LMP2 promoter by EBNA3 molecules was tested by cotransfecting EBNA3A, 3B or 3C either individually or together in combination with a vector containing the LMP2 promoter cloned upstream of a reporter gene. These experiments showed that the EBNA3 proteins could modulate EBNA2-mediated transactivation of the LMP2 promoter (36). However, individually the EBNA3 proteins stimulated neither the LMP2 promoter nor other heterologous promoters suggesting that the role of the EBNA3 molecules is intrinsically linked to that of EBNA2. Interestingly, the expression of stable EBNA3C in BL cell lines markedly diminishes the Jκ activity (19). It is now shown that EBNA3C and RBP-Jκ interact both *in vivo* and *in vitro*, and accumulation of EBNA3C in cells down modulates EBNA2 mediated transactivation of Cp and LMP1 promoters by disrupting the interactions of RBP-Jκ with its cognate sequence and competes with EBNA2. Thus, EBNA3C likely prevents runaway transcriptional activity from the Cp promoter and modulates the activating effects of EBNA2 on promoters like LMP1 and other RBP-Jκ responsive promoters.

The ability of EBNA3C to activate expression from the LMP1 promoter was examined using transient reporter gene assays. The results indicated that EBNA3C has two paradoxical functions on the LMP1 promoter when transfected alone or with EBNA2. Transcription activation greater than that seen with EBNA2 alone and inhibition of EBNA2-induced transactivation were shown to be due to binding to transcription factors that recognize and target distinct DNA sequences within the promoter. These results showed that the EBNA3 molecules are involved in the regulation of LMP1 gene expression (19, 24, 37, 38). The sequences involved in inhibition were identified as the

DNA binding cellular factor RBP-Jκ shown also to associate with EBNA3C and EBNA2 in EBV infected cells (39). Mutations either in the RBP-Jκ binding site of EBNA3C or in the RBP-Jκ binding site abolished EBNA3C-mediated repression. However, an adjacent DNA sequence was shown to be involved in activation. EBNA3C was able to activate an LMP1 promoter in which both the RBP-Jκ sites were mutated suggesting that this activation was independent of RBP-Jκ function (39). The transactivation domain was mapped to glutamine- and proline-rich domain of EBNA3C (aa 724-826) similar to a transactivation domain of another mammalian transcription factor Sp1 (38). In assays where EBNA3C was tethered to DNA using a fusion protein to the Gal4 DNA binding domain, two transcriptional repressor domains, aa 280-525 and aa 580-992, were identified (37). These studies therefore suggest that EBNA3C can function as an activator or repressor of transcription based on its interaction with specific cellular factors.

Studies carried out by Zhao and Sample showed that EBNA3C could activate the LMP1 promoter in conjunction with EBNA2 through a Spi-1/Spi-B binding site (40). A mutant EBNA3C protein incapable of binding to RBP-Jκ was able to activate expression from the LMP1 promoter, and a 41-bp LMP1 promoter fragment, containing the Spi-1/Spi-B but not the RBP-Jκ site, was sufficient for the EBNA3C-mediated activation (40). Like EBNA3C, EBNA2 also binds a Spi-1 site *in vitro* and this site in the LMP1 promoter is important for EBNA2-mediated transactivation (29, 41, 42). Of interest, it should be noted that although there is considerable homology between this element and the mouse immunoglobulin light chain promoter sequence to which the mouse homologue of Spi-1 binds with its dimerization partner IRF4, the IRF4-like binding sites in the LMP1 promoter are not involved in EBNA3C mediated activation (40). The *in vitro* and *in vivo* interaction of EBNA3C with Spi-1 and Spi-B occurs through the basic leucine zipper domain of EBNA3C and the ets domains of Spi-1 or Spi-B resulting in their targeting to DNA (40).

The repression of transcription associated with EBNA3C prompted the search for a role of histone deacetylases in EBNA3C mediated repression (43). In transient transfection assays using two different reporter plasmids, TSA (trichostatin A) relieved the repression induced by EBNA3C (43). A number of *in vitro* and *in vivo* assays showed that the histone deacetylase 1 (HDAC1) is recruited to the DNA through an association with EBNA3C, which is itself targeted to DNA by CBF1/RBP-Jκ. The region of EBNA3C (aa208-211) that is required for binding to CBF1/RBP-Jκ is also important for the association of EBNA3C with HDAC1 (43).

The ability of EBNA3C to target cellular transcription factors to affect transcription may be critical for EBV mediated oncogenesis. It is left to be determined through genetic analysis whether or not the functional associations with these cellular factors are critical for B cell transformation.

5. EBNA3C AS A REGULATOR OF THE CELL CYCLE

Studies in the last decade have resulted in the convergence of two independent fields; the cell cycle and oncogenesis – indeed, it now seems clear that deregulation of cell cycle machinery significantly contributes to the uncontrolled cell proliferation and the genetic instability typical of tumor cells (44). Leukemias and lymphomas are often associated with such genetic instability, the classic example being the 8:14 chromosomal translocation seen in EBV-positive Burkitt's lymphoma. This event translocates the *c-myc* proto-oncogene on chromosome 8 downstream of transcriptional enhancers of the immunoglobulin heavy chain locus on chromosome 14 (45). Current oncogenic theory suggests that an episode as dramatic as a chromosomal rearrangement is not likely to be the first "hit" in triggering malignant transformation. Instead, such an event is likely to be preceded by more insidious destabilizing events such as the inactivation of cell cycle regulators.

Classic work with polyomavirus SV40, adenovirus, and papillomavirus demonstrates that these "small" DNA tumor viruses prevent growth arrest and/or alter apoptosis in transformed cells (46-51). Specifically, the SV40 T antigen, the adenovirus E1A protein, and the papillomavirus E7 protein promote DNA replication and subsequently contribute to deregulation of the cell cycle by inactivating a common target – the retinoblastoma tumor suppressor protein (Rb) (52-54). Rb normally inhibits the progression of quiescent cells through the restriction point in G_1, roughly defined as the point beyond which cells are no longer dependent on stimulation by growth factors (55, 56). Rb and related family members p107 and p130 complex with, and inhibit the transcriptional activity of, the E2F family of transcription factors, a group of factors that activate the transcription of genes necessary for progression into S phase (57). Phosphorylation of Rb, first by the growth factor induced Cyclin D/cdk4, 6 complex and later by the Cyclin E/cdk2 complex, permits the release of E2F. This leads to the activation of genes for DNA synthesis and facilitates progression of the cell cycle (58). While at least this aspect of cell cycle deregulation by the "small" DNA tumor viruses has been well described, the likely connection between EBV antigens and the cell cycle continues to be elusive. However, recent work on EBV latent nuclear antigen EBNA3C has shown a link between EBNA3C and the cell cycle, most interestingly demonstrating an *in vitro* interaction between EBNA3C and the tumor suppressor Rb (59).

Initial lines of investigation linking EBNA3C and cell cycle regulation were noted during studies of LMP1 expression in the EBV-infected, EBNA3C-deficient Raji cell line (24). After infection of primary B-lymphocytes with EBV a number of viral and cellular events occur which lead to the expression of a specific set of viral and cellular genes. These genes are expressed in a controlled manner resulting first in activation, and ultimately in immortalization, of infected B-cells (1, 7). LMP1, the essential EBV oncogene is one of the last latent genes to be

expressed, the likely result of its apparent dependence on the expression of other latent EBNA genes, specifically EBNA2 (1). However, expression of EBNA2 does not uniformly induce expression of LMP1. In fact, during early infection of resting B cells no LMP1 is detected for many hours after EBNA2 is expressed (7). Similarly, the CD23-negative, EBV-positive population of primary cells does not express LMP1 but is consistent for EBNA2 expression (60). These results suggest that other cellular and/or viral factors contribute to the regulation of LMP1 gene expression.

Comparative analysis of Raji cells negative for EBNA3C expression with Raji cells strongly expressing EBNA3C indicates that LMP1 expression is dependent upon the proliferative state of the cell in the absence of EBNA3C expression. Shortly after feeding, LMP1 levels increase; however, 96 hours later the levels are almost undetectable when the majority of the cells are arrested in the G_1 phase of the cell cycle (24). The induction of LMP1 after feeding growth-arrested cultures occurred independent of the presence of serum in the diluting medium. This strongly suggests that LMP1 expression is not due to stimulation by serum factors. If EBNA3C is expressed *in trans* in Raji cells, the level of LMP1 closely mimics that seen in EBNA3C-expressing LCLs and is maintained through growth arrest of the cells in G_1 (24). This system was further characterized by determining the phosphorylation-state of Rb in relation to LMP1 repression and subsequent induction in Raji cells. In growth-arrested Raji cells Rb is hypophosphorylated and LMP1 expression is repressed (24). Expression of EBNA3C in this system derepresses and sustains LMP1 expression in spite of the presence of hypophosphorylated Rb (24). This suggests that EBNA3C is involved in regulation of LMP1 expression during the G_0/G_1 period of the cell cycle prior to cells passing the restriction point. These cumulative studies implicate EBNA3C as a potential player in early EBV infection prior to progression of infected cells through the restriction point and on into S phase. As mentioned previously, adenovirus and papillomavirus are known to target Rb through the E1A and E7 early antigens, respectively (53, 54). It is possible that an EBV essential antigen may function analogously to E1A and E7 in EBV-transformed cells.

B-*myb* is a cellular gene that is normally repressed in G_1 and is subsequently activated by the E2F family of transcription factors as Rb is phosphorylated and cells progress into S phase. Gene reporter assays with EBNA3C expression plasmid and either a wild type or mutant B-*myb* promoter element demonstrated that EBNA3C activates the human B-*myb* promoter in an E2F-dependent manner (61). This result is consistent with EBNA3C targeting the Rb-E2F pathway. Additionally, EBNA3C has transforming potential similar to papillomavirus E7 in a colony formation assay and cooperates with *H-ras* in the transformation of primary rat fibroblasts similar to E7 and *H-ras* in the same assay (61). To address whether the transforming potential of EBNA3C was related to the potential targeting of Rb pathways, transformation was assayed in the presence of the cyclin-dependent kinase inhibitor $p16^{INK4a}$ known to bind and inhibit the cyclin dependant kinases cdk4 and cdk6, potent phosphorylators of Rb (62). Additionally, $p16^{INK4a}$ inhibits the transformation of rat fibroblasts by *H-ras* in combination with potential oncogenes such as *c-myc* and a highly transforming p53 mutant that do not specifically target Rb (59). In contrast, viral oncoproteins such as E1A and E7 that antagonize Rb function are able to overcome $p16^{INK4a}$ suppression of transformation (63). EBNA3C has the ability to overcome this suppression similar to papillomavirus E7 on colony formation assay (61). This data provides an additional hint to the involvement of an Rb-related pathway in EBNA3C-induced transformation.
The most direct link between EBNA3C and Rb was provided by data obtained from an i*n vitro* binding experiment. *In vitro*-translated EBNA3C binds Rb in a manner that is at least partially dependent on the pocket domain of Rb (59). The pocket domain has been shown previously to interact with the consensus LxCxE binding motif present in Tag, E7, and E1A. Although this consensus motif is not present in EBNA3C, the EBNA3C protein showed several-fold lower affinity for an Rb pocket domain mutant, hinting that the Rb-EBNA3C interaction might display similar functionality to the interaction between Rb and the "small" tumor virus antigens (61). However, it must be noted that despite this apparently specific *in vitro* interaction, there was no evidence showing that EBNA3C and Rb associate *in vivo*. As such, it is left to be determined if these data can be substantiated with consistent *in vivo* experimental evidence.

Other experiments investigated the direct effect of EBNA3C on cell cycle progression. These experiments were performed with NIH3T3 cells transfected with EBNA3C cDNA and a neomycin resistance marker. Drug-resistant colonies were then pooled and analyzed by flow cytometry. EBNA3C-positive and negative cells showed little difference for cell cycle markers when grown in 15% serum. However, serum-starved cells showed increased DNA content in the EBNA3C-positive compared to the EBNA3C-negative populations. After 48 hours in 0.5% serum, 32 percent of EBNA3C-positive cells showed 4N DNA content, in contrast to 10 percent of EBNA3C-negative cells (64). Additionally, re-gating of the same experiment demonstrated that a significant percentage of EBNA3C-positive cells had greater than 4N DNA content suggesting that DNA replication was proceeding in the absence of cell division. Indeed, cells containing from 1-7 nuclei were easily visualized by microscopic examination (64).

To eliminate the possibility that long-term neomycin selection influenced the cell profile, further experiments were performed with U2OS cells that had been manipulated to express EBNA3C upon Muristerone A induction. Serum-deprived, EBNA3C-expressing U2OS cells showed a similar DNA profile to that observed previously, displaying a higher DNA content relative to EBNA3C-minus cells in 0.1% serum (64). Interestingly, immunoblot analysis demonstrated that U20S cells showed a striking decline in the levels of the cyclin-dependent kinase inhibitor (CDKI) $p27^{Kip1}$ upon EBNA3C induction

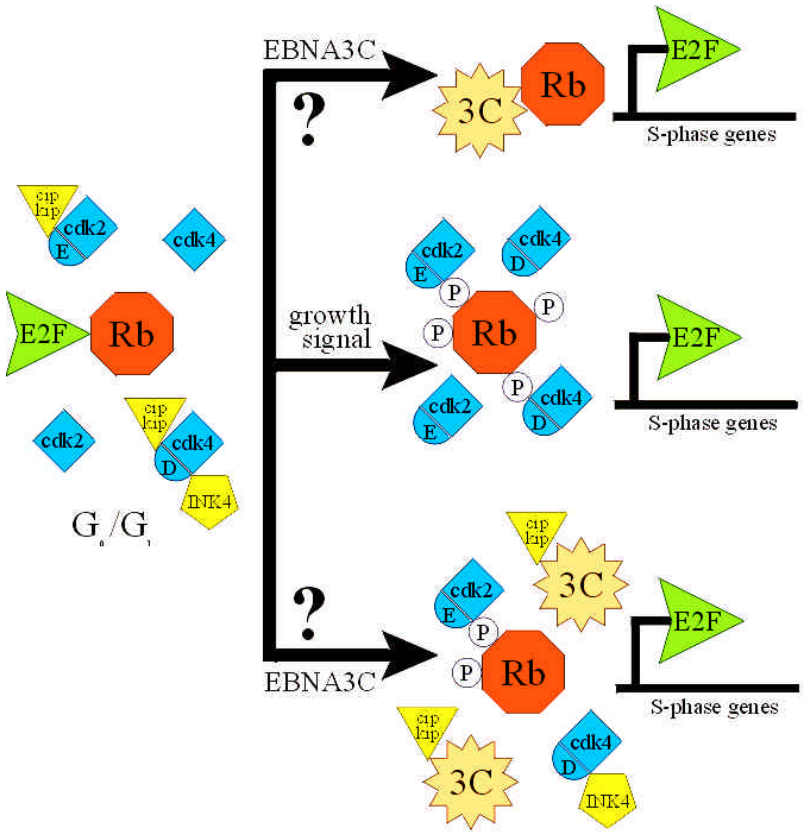

Figure 2. Potential targets of EBNA3C at the G_1/S transition. Upon growth factor stimulation, phosphorylation of Rb increases. Increased kinase activity is a result of increased transcription of the Cyclin D and Cyclin E genes and degradation of cyclin-dependent kinase inhibitors (cip/kip and INK4 families). Phosphorylation of Rb frees E2F family members to activate the transcription of S-phase genes. EBNA3C promotes S phase entry by targeting the Rb pathway at the restriction point. 3C – EBNA3C. cdk – cyclin dependent kinase. D – Cyclin D. E – Cyclin E.

(64). $p27^{Kip1}$ is a member of the Cip1/Kip1 family of CDKIs, a class of potent inhibitors that bind G_1 and S-phase cyclin/cdk complexes including cyclin E/Cdk2 and cyclin A/Cdk2. In general, levels of $p27^{Kip1}$ are high in senescent cells and dramatically decrease upon entry into the cell cycle as $p27^{Kip1}$ is marked for degradation by cyclin E/Cdk2 phosphorylation (65). Therefore, $p27^{Kip1}$ is an attractive target for EBNA3C as a deregulator of the cell cycle, potentially explaining, at least in part, the previously discussed phenotypic data (Figure 2). However, it is also possible that the decrease in $p27^{Kip1}$ levels is an indirect consequence of EBNA3C promoting entry into the cell cycle by an unrelated mechanism. Surprisingly, in these studies Rb does not show a shift to the hyperphosphorylated state as would be expected in cycling cells, although total levels of the hypophosphorylated species do appear to decrease (64). This suggests that EBNA3C potentially targets and disables active, hypophosphorylated Rb repression complexes (Figure 2). However, it should be reiterated that a reproducible *in vivo* link between Rb and EBNA3C has yet to be demonstrated despite considerable interest in such an interaction by the EBV research community.

The data described above suggests that EBNA3C may target checkpoints later in the cell cycle as EBNA3C expression results in increased DNA content. A mitotic spindle checkpoint exists to monitor proper assembly of the spindle at metaphase before separation of sister chromatids at anaphase (66, 67). Experimentally, microtubule-poisoning drugs such as nocodazole arrest the cell cycle in metaphase (68). In a series of experiments, EBNA3C-negative U2OS cells demonstrated a high mitotic index (80-90%) following treatment with nocodazole suggesting that the majority of cells had been arrested by the mitotic spindle checkpoint. In contrast, EBNA3C-expressing cells had a dramatically lower mitotic index (10%) as these cells potentially ignored the aforementioned checkpoint and proceeded through mitosis. This suggests that EBNA3C may regulate later checkpoints, including mitosis, in EBV-transformed cells.

As a whole these cell cycle data, from the early work with LMP1 expression in the Raji cell line to more recent cell-sorting studies, strongly suggest that EBNA3C plays a role in deregulation of the G_1/S transition, specifically the restriction point. Several lines of evidence suggest targeting of the Rb regulator or other molecules

functionally associated with the Rb pathway. This evidence includes rat fibroblast transformation studies suggesting that EBNA3C can overcome $p16^{INK4a}$ suppression, activation of an E2F-responsive promoter by EBNA3C in an E2F-dependent manner, and a robust decrease in $p27^{Kip1}$ levels with EBNA3C induction. While data exists to support a specific *in vitro* interaction between EBNA3C and Rb, the inability to similarly demonstrate this association *in vivo* suggests that EBV has developed a more complex symbiotic interaction with the restriction point than the relatively "simple" LxCxE-Rb interaction seen with the "small" DNA tumor viruses. Nevertheless, the preliminary evidence is thought provoking and should provide a framework to more completely understand the underlying mechanism by which the essential EBV antigen EBNA3C functions to deregulate the cell cycle.

6. INTERACTION OF EBNA3C WITH PROTHYMOSINα MODULATES THE HISTONE ACETYL TRANSFERASE ACTIVITY OF P300

Recent lines of investigation has shown that EBNA3C may be involved in the regulation of chromatin remodeling by targeting acetylases, deacetylases or other factors associated with these complexes. These lines of investigation identified a cellular molecule Prothymosinα (ProTα) as a strong interactor of EBNA3C. ProTα interacts with the carboxy terminal region of EBNA3C with implications for the growth of normal cells as well as in the proliferation of mammalian cells undergoing malignant transformation (69-72). Although, this protein was initially characterized as the precursor of thymosin α_1 more than 15 years ago its physiological role remains unclear (71). It is a nuclear acidic protein of 109-111 amino acid residues with an acidic-rich region of aspartic acid and glutamic acid residues located within the center of the molecule and a nuclear localization signal KKQK at the carboxy terminus (73). ProTα is highly conserved and ubiquitously expressed in a wide variety of cells, tissues and organisms suggesting that it plays an essential role in cell function (69-72, 74). Several lines of evidence suggest a link between ProTα and cell proliferation. It is induced upon expression of the known oncogene c-myc as well as the HPV E6 protein and its promoter element is activated by E2F (75, 76). Further, studies investigating the role of ProTα when over-expressed in Rat-1 cells by phenotypic transformation assays demonstrated that ProTα is capable of inducing significant cell proliferation and transformation by the classical transformation assays (77).

Another recent and interesting finding showed modulation of histone acetyl transferase activity through interaction of EBNA3C and ProTα (78). Previous studies showing the interaction of ProTα with histones *in vitro* suggested a possible role for this molecule in chromatin remodeling in mammalian cells (79-81). Cellular gene expression is regulated at the level of the chromatin structure through histone acetylation/deacetylation by the recruitment of histone acetyl transferases (HATs) like p300 and CREB-binding protein (CBP) and deacetylases (82-84). The chromatin and nucleosomal structure dissociates due to the addition of the acetyl groups to core histones, thereby rendering transcriptional regulatory sites accessible to the transcriptional machinery (85). EBNA3C associates with the acetyltransferases p300 potentially through the recruitment of EBNA3C to the chromatin by ProTα which functions to stabilize complexes involved in chromatin remodeling (78).

A number of *in vivo* and *in vitro* assays demonstrated that ProTα interacts with the carboxy terminal region of EBNA3C essential for the growth transformation of B-lymphocytes (78). More detailed interaction studies showed that EBNA3C binds with ProTα between aa 366 and 393, a region that is conserved in Type I and Type II EBV strains (78). This region lies immediately downstream of aa 365. A stop codon inserted after position 365 rendered the recombinant EBV incapable of B-lymphocyte transformation. This suggested that the region downstream of 365 is critical for EBV mediated transformation and that molecules that directly interact with the region of EBNA3C are functionally important in terms of EBV mediated B-lymphocyte transformation. This *in vitro* data mapping the binding of ProTα to the region 366-393aa of EBNA3C would therefore indicate that this interaction might have critical implications as to EBV biology and the role of EBNA3C as an essential latent antigen.

Co-immunoprecipitation experiments indicated that EBNA3C, p300, and histone H1 associate with ProTα in EBV-infected cells. The association of ProTα with the carboxy terminus of p300 was about 70 fold greater than that seen with the amino terminus although there was a clear indication of binding to both the amino and the carboxy terminus of p300 (78). The fact that EBNA3C and ProTα may compete for similar regions on the 3' terminus of p300 may have critical implications in terms of regulation p300 function as other viral oncoproteins, including E1A, large T antigen, bind to the 3' region of p300. P53 binds to both the amino and the carboxy terminus also suggesting regulation of p300 HAT activity (86, 87). Although the mechanism by which ProTα and EBNA3C modulate HAT activity in EBV-transformed cells is not fully understood, a model can be proposed based on the E1A protein and the transcription factor Twist, both well-described inhibitors of p300 and pCAF HAT activity (88, 89). It is possible that either ProTα or EBNA3C independently binds to the HAT/CH3 domain of p300 directly and consequently regulates the activity of the HAT domain. Alternatively, they may interact with the CHI amino terminal domain and so regulate the activity of p300 at the HAT domain in EBV-infected cells. It has been suggested that the viral transactivator EBNA2 acts as a functional homologue of intracellular activated NOTCH1 (78, 90). EBNA2 can potentially displace repression complexes including corepressors and deacetylases, while simultaneously recruiting transcriptional activators such as p300. In such a scenario, transcriptional activation would be a consequence of histone acetylation by an activation complex including other essential EBNA proteins, ProTα and p300. However, this increased acetylation would not go

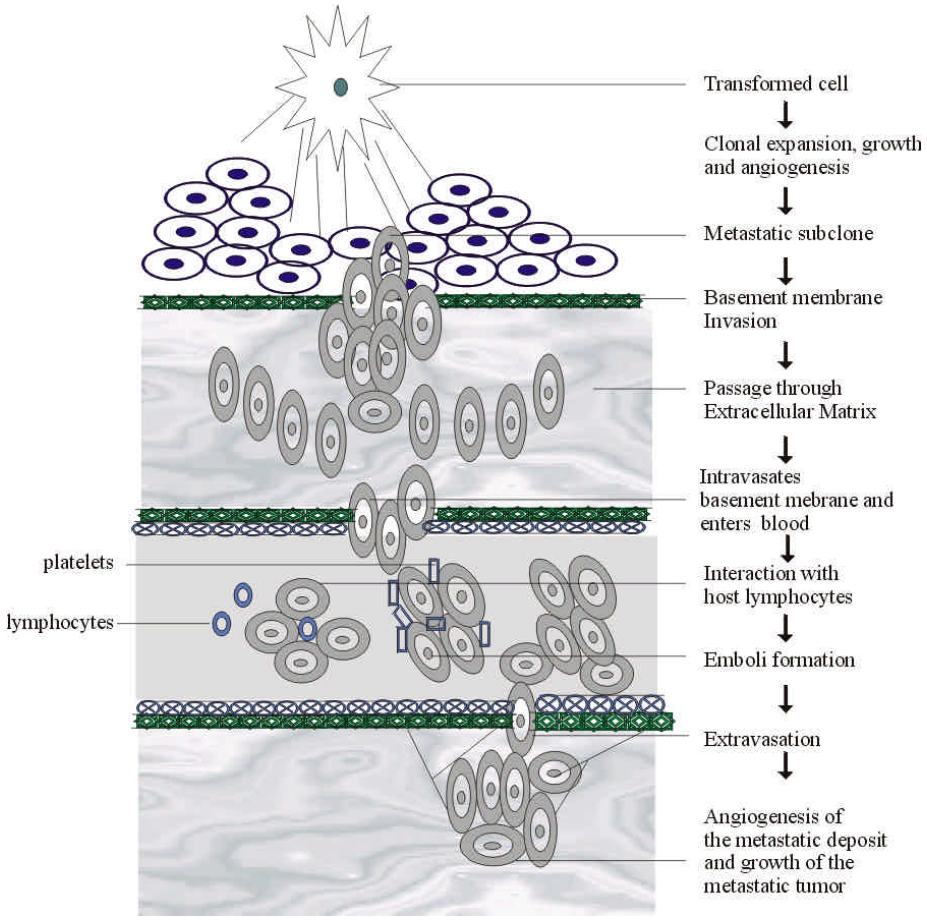

Figure 3. Schematic illustration of the sequential steps involved in the metastatic cascade (91). The tumor cells leave the primary mass, enter blood vessels or lymphatics and develop overt metastasis at the secondary site.

unchecked but would be stringently regulated by competition between EBNA3C and ProTα for binding to p300. This competition would result in down modulation of the p300 acetylation activity.

In summary, ProTα is potentially an important cellular molecule involved in the recruitment of p300 and other acetylases to the nucleosome in EBV proliferating cells. ProTα is upregulated in proliferating lymphocytes and may play a role in increasing the acetylation of core histones through recruitment of acetylases. The above studies suggest a role for the essential EBV protein EBNA3C in modulating the acetylation of cellular factors, including histones, during immortalization of primary human B-lymphocytes.

7. EBNA3C ASSOCIATES WITH NM23-H1 IN EBV LYMPHOBLASTOID CELL LINES

To identify cellular proteins interacting with the region of EBNA3C downstream of aa365 shown to be critical for B-lymphocyte transformation, a yeast two-hybrid screen using an EBV positive LCL derived cDNA library was employed. Control target RBP-Jκ was used as it maps to the amino terminus of EBNA3C (Figure 1)(78). One cellular target known to be associated with the carboxy terminus of EBNA3C was Nm23-H1 (92). This interaction was also shown by *in vitro* binding studies confirming that Nm23-H1 binds to the carboxy terminus of EBNA3C. Co-immunoprecipitation experiments also demonstrated the association of these proteins *in vivo* in EBV-transformed human primary lymphocytes. As Nm23 has a number of family members with similar identity its possible association with Nm23-H2 was also tested in this assay. However, little or no specific binding was seen with Nm23-H2 and EBNA3C suggesting that the interaction with Nm23-H1 was a specific functional interaction. Immunofluorescence data showed that Nm23-H1 was predominantly localized in the cytoplasm in B-lymphocytes. However, in EBV transformed LCLs and in B cells transfected with EBNA3C, Nm23-H1 predominantly localized to the nucleus in the same compartments to EBNA3C. This observation suggested that EBV, probably through EBNA3C and possibly through other essential EBNA proteins, may influence directly or indirectly the cellular localization and hence the function of Nm23-H1 in EBV-infected cells.

The metastatic suppressor gene Nm23-H1 was identified by differential colony hybridization of murine

melanoma cell lines (93). The Nm23 gene family is highly conserved among a wide variety of eukaryotic species. Eight distinctly different genes have now been identified in humans (94). Expression of Nm23 genes has been associated with suppression of tumor metastasis, cellular differentiation, apoptosis, proliferation, and DNA mutation rate (95). In carcinomas of the breast, ovaries, cervix, and gastric epithelium, in hepatocellular carcinoma, and in melanoma an inverse relationship is observed between the expression of Nm23-H1 and tumor metastasis (95, 96). However, in other neoplasms like neuroblastoma, in which a mutation was observed in the putative leucine zipper motif of Nm23-H1, there was a direct correlation between metastatic potential and Nm23-H1 expression (97, 98). Although the exact role of Nm23-H1 in metastasis is not fully understood, experimental data accumulated thus far strongly suggests that Nm23-H1 plays a significant role in the regulation of metastasis in diverse types of human cancer (99).

Recent studies showed an association of EBV with invasive breast carcinoma despite previous contradictory results that question the role of EBV in breast cancer (4). Further findings demonstrated that EBV is capable of infecting a number of invasive breast carcinoma cell lines (100). Taken together the interaction between EBNA3C and Nm23-H1and the potential role of Nm23-H1 in metastasis suggest that EBV may be associated with carcinomas and may influence the ability of these neoplasms to metastasize in humans.

8. POTENTIAL ROLE OF EBNA3C IN MODULATING CELL MIGRATION

Metastatic spread of tumor cells is the major naturally occurring complication of most cancers. The presence and treatment of metastasis greatly influences the mortality of cancer patients. Metastasis is a multi-step process that involves angiogenesis, invasion, and changes in adhesion characteristics (101). During metastasis, cells detach from the tumor mass, migrate toward lymph and/or blood vessels, disrupt the vascular basement membrane, penetrate the vascular lumen and evade the immune surveillance system. This is followed by adhesion to endothelium of organs distant to the primary site as well as growth, extravasation, and survival in the invaded microenvironment (Figure 3).

In a graded series of related tumor cell lines that differed in metastatic ability, the metastatic potential directly correlated with increased cell motility (102). Cell motility plays a role in the metastatic cascade at a number of points, including during intravasation of tumor cells into the tumor vasculature or nearby lymphatic channels and during extravasation of tumor cells from the vasculature into the tissue parenchyma at selected secondary sites. As would be predicted, transfection of a wild-type Nm23-H1 expression plasmid inhibits the migration of human breast carcinoma and murine melanomas cell lines in response to growth factors, including serum, platelet derived growth factor (PDGF), and insulin-like growth factor (IGF) (103). In another study MDA-MB-435 breast carcinoma cells were transfected with wild type or mutated forms of Nm23-H1 (104). These were then examined for the motility phenotype using the *in vitro* Boyden chamber assay. Tumor cell motility to either serum or partially purified autotoxin (ATX) was reduced by transfection with either wild type or a serine 44-mutated form of Nm23-H1, but not by transfecting either a serine 120 or a proline 96-mutated Nm23H-1. Further, biochemical studies carried out using mutant forms of Nm23-H1 demonstrated that mutations lacking motility suppressive activity are also deficient in histidine-dependent protein phosphotransferase pathways *in vitro* (105). These studies strongly suggest a correlation between metastasis and motility.

Tumor cells that exhibit increased motility *in vitro* show increased propensity to metastasize *in vivo*. As such, understanding the molecular mechanisms by which a cell generates locomotion will undoubtedly be of significance in the development of treatment strategies to inhibit the metastatic process. Studies using the breast carcinoma cell line MDA-MB435 and the Burkitt's lymphoma cell line BJAB demonstrated that EBNA3C could reverse the anti-metastatic potential of Nm23-H1 in an *in vitro* motility assay (92). This data builds upon previous studies in which increased expression of Nm23-H1 resulted in a decrease in motility of breast carcinoma cells when chemoattractants like serum, insulin growth factor (IGF) and platelet derived growth factor (PDGF) were added to the medium (103). When EBNA3C was expressed with Nm23-H1, the ability of Nm23-H1 to suppress cell motility was clearly inhibited with EGF and fibronectin as chemoattractants. This is likely due to the direct interaction of Nm23-H1 with EBNA3C in the transfected cells. Importantly, EBNA3C expression had little effect on cell migration when overexpressed in the absence of exogenous expression of Nm23-H1. In summary, the suppression of migration by Nm23-H1 in breast carcinoma and Burkitt's lymphoma cell lines is reversed by the expression of EBNA3C in an *in vitro* migration assay (92). This has clear implications as to the potential role of this essential EBV latent antigen in driving tumor metastasis in EBV associated cancers.

9. CONCLUDING REMARKS

EBNA3C is an essential EBV latent antigen for EBV mediated B-lymphocyte transformation (44). Numerous studies by a number of investigators strongly suggest that EBNA3C plays a critical role in EBV biology, specifically in B-lymphocyte transformation, cell cycle regulation, proliferation and metastasis (Table 1). EBNA3C targets the RBP-Jκ transcription repressor and is capable of modulating transcription from the RBP-Jκ?responsive promoters (19) ?Additionally, other studies demonstrate that EBNA3C interacts p300 and ProTα and modulates the activity of the histone acetyl transferase p300 (78). Clearly, specific domains of EBNA3C can mediate regulation of the function of specific cellular target genes particularly those responsive to RBP-Jκ and EBNA2.

Table 1. Proteins that interact with Epstein Barr Nuclear Antigen EBNA3C and their related functions

Target Protein	Related Function	References
RBP-Jκ	DNA-binding cellular protein. Binding of Jκ with EBNA3C modulates EBNA2-mediated transactivation.	19
HDAC1	Chromatin modifying enzyme. Recruitment of HDAC1 activity by EBNA3C enhances repression of transcription from EBV Cp.	43
ProTα	Cellular protein important for cell division and proliferation. It is involved in transcriptional regulation at the level of histone acetylation through interaction with EBNA3C and p300.	78
Nm23-H1	Human metastatic suppressor protein. EBNA3C reverses the ability of Nm23-H1 to suppress the migration of Burkitt's lymphoma and breast carcinoma cell lines.	92
CtBP	E1A C-terminal binding protein. Binding of this corepressor to PLDLS motif of EBNA3C (aa728-732) affects the capacity of EBNA3C to cooperate with H-ras in transforming primary rodent fibroblasts.	106

Ongoing studies into the role of EBNA3C in cell proliferation and metastasis will certainly shed light on these interactions and their potential effects in EBV associated human cancers.

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